1

DEVELOPMENT OF GLUCAN SULFATES WITH BETTER INHIBITORY ACTIVITY ON THE SELECTIN-MEDIATED CELL ADHESION THAN HEPARIN

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The adhesion of cells to the endothelium plays an important role in leukocytes recruitment into tissues during inflammation as well as in tumor cell metastasis. The initial adhesive event is mediated by selectins which bind to oligosaccharide structures of the corresponding receptors. Heparin is known to interfere with this process. This could contribute to its moderate antiinflammatory activity and its survival time prolonging effect in tumor patients. However, compared to its high anticoagulant activity, the inhibition by heparin of the selectin-mediated cell adhesion is relatively weak.
We developed a new class of partial synthetic glucan sulfates, which exhibit antithrombotic activity similar to heparin, but in vivo antiinflammatory activity better than heparin. The aim of the presented study was to compare their inhibitory influence on the selectin-mediated cell adhesion with that of heparin and to establish structure-activity relationships. In adhesion assays, the glucan sulfates inhibit the L- and P-, but not the E-selectin-mediated cell adhesion. Their activity depends not only on the degree of sulfation and the molecular weight but also on the sulfation pattern. Further, the basic polysaccharide structure was shown to play an important role, e.g. the glucan sulfates are considerably more active than heparin.
These results obtained under static conditions correlate well with the effects observed in a flow chamber model. The latter examines the influence of the test compounds on the interactions of selectin expressing cells with a vascular surface imitate containing Sialyl Lewis x under shear flow. Whereas heparin is inactive in this dynamic test system, the glucan sulfates structure-dependently reduce the number of adhering cells and prolong the rolling velocity of the cells.
In conclusion, the cell adhesion inhibitory potency of glucan sulfates is suggested to contribute to their in vivo antiinflammatory activity. They may be interesting alternatives to heparin for thrombosis prophylaxis in clinical situations accompanied by inflammation as well as in tumor patients.

2

DOES LAMININ REPRESENT A TARGET FOR THE ANTIMETASTATIC ACTIVITY OF HEPARIN AND SULFATED POLYSACCHARIDES?

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Tumor cell metastasis requires the interaction of tumor cells with components of the basement membrane. One of these is the glycoprotein laminin, which is involved in a number of steps of tumor cell invasion and metastasis. Cancer cell lines, which are highly metastatic in mice, were shown to exhibit an enhanced attachment to laminin, spreading on it and migration toward it. Antibodies to laminin inhibited tumor cell metastasis. Heparin is known to prolong the survival time of tumor patients. According to manifold in vitro- and animal experiments, heparin influences manifold processes involved in tumor metastasis. The aim of the presented study was to examine whether heparin also affects the laminin-dependent steps of metastasis. To establish structure-activity relationships, structurally defined partial synthetic sulfated polysaccharides were included in the study. To investigate the effects of the test compounds on the tumor cell adhesion, a microplate coated with laminin was incubated with the metastatic breast cancer cell line MDA-MB231 in presence or absence of increasing concentrations of the test compounds. After washing, the adhering cells were lysed and quantified by means of their lactate dehydrogenase activity. Whereas heparin turned out to be unable to inhibit adhesion of MDA-MB231 cancer cells to laminin, partial synthetic sulfated polysaccharides show a dose dependent inhibition. The inhibitory activity of the sulfated polysaccharides can be neutralized by protamin sulfate. Mechanistic studies suggest that the sulfated polysaccharides interact with laminin and not with the laminin receptor structures. Essential to any effect are sulfate groups covalently bound to the polysaccharide. The inhibitory activity increases with increasing molecular weight and degree of sulfation of the test compounds. In addition beta-1,3-glucan sulfates are significantly more active than alpha-1,4/1,6 glucan sulfates. Since these sulfated polysaccharides are also superior to heparin in other test systems recording interference with tumor metastasis, they may represent interesting candidates for the development of new antimetastatic drugs for the adjuvant tumor therapy.

3

MODIFICATION OF THE ACTION PROFILE OF HEPARINS AND SULFATED CARBOHYDRATES WITH ELASTASE INHIBITING AND ANTICOAGULANT ACTIVITIES

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The extravasation of tumor cells is an important prerequisite for the development of metastasis. Proteolytic enzymes such as elastase and matrix metalloproteinases contribute to this process by degrading extracellular matrix. As shown by clinical studies, breast and lung cancer patients with high levels of elastase activity have a significantly shorter survival rate and poor prognosis compared to patients with low levels of elastase activity. Heparin proved to prolong the survival time of tumor patients. One of the manifold effects of heparin which may contribute to this clinical outcome, is its elastase inhibiting activity (EIA). However, the high anticoagulant activity (ACA) of heparin generally limits the therapeutic use of all these antitumor effects. Therefore, the question arises whether the action profile of heparin/heparinoids can be modified, i.e. whether the EIA can be increased and the ACA can be reduced. In the presented study different heparins and heparin derivatives as well as commercial heparinoids and partial synthetic sulfated polysaccharides were tested for their anticoagulant and elastase inhibiting activity. By reducing the chain length of heparin, both the EIA and the ACA are reduced. Similar results were obtained by specific desulfation of heparin. This dependency on the chain length and the degree of sulfation was also shown for the commercial heparinoids and partial synthetic sulfated polysaccharides. However, in most cases their action profile is changed in favour of the EIA. For example, a short chain laminarin sulfate and pentosan polysulfate has an EIA similar to heparin, but a 4 times lower ACA in the APTT and a curdlan sulfate is as active as unfractionated heparin in the APTT, but has a 2fold higher EIA. These EIA are based on a chromogenic substrate assay. However, by using a more physiological elastinolyse assay, heparin turned out to be even much less active than these partial synthetic sulfated polysaccharides. These results demonstrate that specific carbohydrate design results in new sulfated carbohydrates with action profiles different from that of heparin, which may be advantageous for the use in tumor patients.

4

USE OF A LOW MOLECULAR WEIGHT HEPARIN FOR CARDIOPULMONARY BYPASS: A DOSE-FINDING STUDY IN YUCATAN MINI PIGS

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Unfractionated heparin (UFH) is still the anticoagulant of choice for standard cardiopulmonary bypass (CPB), although it has several disadvantages. For example, it has to be neutralized by protamine, its monitoring is not satisfactory and especially cardiac surgery patients have a high risk to develop heparin-induced thrombocytopenia (HIT). Since low molecular weight heparins (LMWH) are known to induce less HIT, they might be a an interesting anticoagulant for CPB.
In the present study, the LMWH dalteparin was used as anticoagulant for extracorporeal circulation (120 min) during open heart surgery in Yucatan mini pigs. To determine the effective dose, different dosage regimens were used for the 12 pigs. They ranged from a 1500 aXa-U priming solution dose followed by a an i.v. bolus of 200 aXa-U/kg b.w. 30 min before ECC and a continuous infusion of 10 (aXa-U/kg)/h during the first 90 min of ECC down to 750 aXa-U priming solution dose followed by a an i.v. bolus of 12.5 aXa-U/ kg b.w. 30 min before ECC and a continuous infusion of 5 (aXa-U/kg)/h during the first 90 min of ECC. Only at the highest dosage regimen, dalteparin was neutralized with protamine after the ECC. In addition to further laboratory parameters, the anticoagulation was monitored by determination of ACT, Heptest, aXa- and aIIa-activities and TAT-complexes from pre-ECC to 6 h after ECC.
Only at the two lowest dosage regimens clot formation was observed. Compared to the use of UFH, the drainage volume was generally decreased by a factor of 3-4 with dalteparin. The Heptest, aXa- and aIIa-activities showed a clear linear dose-response. Due the longer elimination half live of aXa- versus aIIa-activity, the aXa/aIIa-ratio increased from about 2.2 (pre-ECC) to about 5.0 (1 h after ECC). Therefore, the successful anticoagulation is supposed to be mainly based on the aXa-activity.
According to this dose-finding study in Yucatan mini pigs, a middle dalteparin dosage regimen represents a promising alternative for anticoagulation during open heart surgery without any risk of bleeding complications or thrombosis. Further, the application of dalteparin does not re-

quire neutralisation with protamine. Finally, there are several options for reliable monitoring the adequacy of its anticoagulant effect of this LMWH during ECC.

5

COMPARISON OF METHODS FOR MONITORING THE ANTICOAGULATION BY A LOW MOLECULAR WEIGHT HEPARIN DURING CARDIOPULMONARY BYPASS

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To determine the effective dose of the LMWH dalteparin for anticoagulation during cardiopulmonary bypass (CPB), a dose finding study was carried out in Yucatan mini pigs. The activated clotting time (ACT) is the test routinely used for monitoring anticoagulation during open heart surgery. However, it does not correlate with heparin levels because of its lack of specificity for heparin and its high variability. To examine whether there are more reliable methods for monitoring the LMWH used in this study, we compared the ACT with the Heptest (HT) and a chromogenic aXa-assay. In addition, the aIIa-activities and TAT-complexes were measured.
Twelve Yucatan mini pigs received 5 different LMWH dosage regiments for anticoagulation during the extracorporeal circulation (ECC). Twenty-two samples of citrated blood were taken beginning before ECC, during ECC (2 h) until 6 h after ECC. In addition, baseline pig citrated plasma (PP) was supplemented with LMWH for comparison with corresponding standard curves with human citrated pool plasma (HP).
In the aXa- and aIIa-assay, the PP- does not significantly differ from the HP-standard curve. Thus, these tests specifically measure the conc. of heparin molecules with aXa- and aIIa-activity, resp.. In contrast to this, the HT turned out to be more sensitive to PP than to HP, i.e. lower heparin conc. result in longer coagulation times. This coagulation assay does not exclusively record the aXa-activity of heparin, but is influenced by several other plasma components. Nevertheless, in contrast to the ACT, these three assays proved to be suitable for LMWH determination during ECC. There was a linear dose-response with r=0.904±0.09 for aXa-activity, r=0.927±0.08 for aIIa-activity and r=0.906±0.144 for HT. In addition, the HT activities showed a linear correlation with the aXa-activities (r=0.976±0.027). The pharmacokinetics of the LMWH-induced aXa- and aIIa-activities considerably differed. The HL(el) of the aIIa-activity was much shorter than that of the aXa-activity resulting in a time-dependent increase of the aXa/aIIa-ratio.
In conclusion, the aXa-assay is most reliable to monitor anticoagulation by LMWH during CPB. Since this assay is generally not well practicable in the OR, the HT performed with citrated blood might be an useful alternative.

6

RAPID TITRATION OF ANTI-HEPARIN/PF4 ANTIBODIES FOR "BEDSIDE" CONFIRMATION OF HEPARIN-INDUCED THROMBOCYTOPENIA

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HIT is a prothrombotic syndrome of antibody-mediated thrombocytopenia, which requires prompt recognition in order to avoid severe thromboembolic complications. Its diagnosis is based on clinical criteria and should be confirmed by demonstration of heparin-dependent antibodies. However, therapeutic decisions must often be made before reliable laboratory results become available. In fact, functional assays are not suited for emergency testing and immunologic assays detect clinically irrelevant antibodies as well. The aim of our study was to investigate whether rapid titration of anti-HPF4 antibodies could improve HIT recognition.
We examined data of 148 patients with suspicion of HIT in whom a test for heparin-dependent platelet aggregation had been performed in our laboratory between 1.1995 and 6.2001. In 139 patients clinical data allowed assessment of HIT likelihood. Frozen plasma samples were available for all 148 patients in order to perform a recently described particle gel immunoassay for antibodies directed against the HPF4 complex, PaGIA-HPF4 (Lancet 1999;354:1525), which allows antibody detection within 20 minutes. In addition, the titer of anti-HPF4 antibodies was determined by the same assay.
Among the 148 patients, 69 (47%) had detectable anti-HPF4 antibodies. Titers varied between 1 and 256 and were reproducible. Clinically likely or very likely HIT were significantly more frequent among patients with titers of 4 or higher (39/41) compared to those with no detectable antibodies (9/70), a titer of 1 (4/22), or a titer of 2 (2/6). All 19 samples with positive platelet aggregation test showed anti-HPF4 antibodies, with titers ranging from 4 (n=1) to 256 (n=4). Thromboembolic complications under heparin were found to be significantly more prevalent among patients with antibody titers of 4 or higher (26/41) compared to those with undetectable antibodies (6/79) or a titer of 1(2/22). Eleven out of the 22 patients with a titer of 1 were maintained on heparin and in none of them did thrombocytopenia worsen or thromboembolic complications develop.
We show that the PaGIA-HPF4 allows rapid and reproducible titration of anti-HPF4 antibodies, and that the antibody titer is of clinical relevance. We suggest that the PaGIA-HPF4 can be used as a rapid semi-quantitative confirmatory test to complement a clinical likelihood score for patients with suspected heparin-induced thrombocytopenia.

7

TISSUE FACTOR AND ITS DUAL ROLE IN ANGIOGENESIS: INTERACTION OF TISSUE FACTOR WITH PLASMINOGEN AND PLASMINOGEN FRAGMENTS

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Background: The plasminogen/plasmin system is involved in a series of biological processes such as basement membrane degradation and cell migration required for angiogenesis including protease induction, migration, proliferation and differentiation. Moreover, proteolytic fragments of plasminogen (plg) such as K1-3, K1-4 and K1-5 are known to be potent antagonists of angiogenesis and inhibitors of endothelial cell migration and proliferation.
Methods and Results: In a solid phase binding assay, we found that TF binds specifically to plg, to plg kringle domains K1-3, K1-5, K4, and to mini-plg (K5m). Inhibition of binding of plg and its kringle domains to TF by aminohexanoic acid suggests that lysine-binding sites are involved in plg interaction with TF. In addition, the effects of cellular or soluble TF on adhesion of human vascular endothelial cells (HUVEC) to immobilized plg were analyzed. HUVEC bound to plg, however, there were no differences between unstimulated HUVEC with low TF expression (0.33 ± 0.31ng/10^6 cells) and TNF-stimulated HUVEC with high TF expression (1.10±0.63 ng/10^6 cells). Although these results indicate that plg binding was not dependent on cell surface TF expression, addition of soluble TF significantly reduced adhesion of HUVEC to immobilized plg. In the presence of soluble TF, the inhibitory effect of K1-5 on bFGF-mediated HUVEC proliferation, however, was dose-dependently and saturably abolished. This suggests that TF can interfere with the antagonistic effect of angiostatin on endothelial cell proliferation. TF by itself had no effect on the endothelial cell proliferation.
Conclusions: In conclusion, the competetive effect of TF on the interaction of HUVEC with plg may represent a possible mechanism to negatively influence angiogenic processes by reducing cell bound plasmin formation. On the other hand, the binding of TF to K1-5 may antagonize the antiangiogenic effects of plg fragments.

8

STUDIES INTO THE MECHANISMS OF INHIBITION OF PLATELET AGGREGATION BY SPHINGOSYLPHOSPHORYLCHOLINE

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It could be observed that sphingosylphosphorylcholine (SPPC) inhibits human platelet activation and aggregation in response to different agonists. The present experiments were designed to obtain further insight into the underlying mechanisms.
Platelets were isolated from blood of healthy donors, washed and resuspended in a HEPES-buffer (200.000/µl). Fibrinogen and calcium were added as indicated. Platelet activation and aggregation was induced by the thrombin receptor activating peptide TRAP-6, the Ca^{2+}-ATPase inhibitor thapsigargin, and the ionophore calcimycin. SPPC, prostaglandin E1 (PGE_1) or vehicle were added 2 or 5 minutes prior to stimulation. In some experiments, cells were pre-incubated with the protein kinase A (PKA) inhibitor H-89. Platelet aggregation response was quantified by turbidimetry. Surface expression of P-selectin, fibrinogen binding and phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) were analysed by flow cytometry. Changes of intracellular Ca^{2+} [Ca^{2+}]i were analysed in fura-2-AM-loaded platelets. Data are means±SEM of 4-6 experiments. Pre-incubation with SPPC (10/20 µM) inhibited thapsigargin-induced (0.5 µM) [Ca^{2+}]i elevation by 68±1% and 71±4% ($p<0.01$)

in the presence of extracellular Ca^{2+}, but did not significantly affect $[Ca^{2+}]i$ increases in response to calcimycin (0.5 µM). Inhibition of thapsigargin-induced $[Ca^{2+}]i$ was less pronounced if extracellular Ca^{2+} was absent. Nevertheless, SPPC (10/20 µM) inhibited platelet aggregation in response to thapsigargin (44±7 and 84±8%, $p<0.01$) and calcimycin (25±8 and 57±6%, $p<0.01$). SPPC (20 µM) induced phosphorylation of VASP, an indicator of PKA activation, but to a smaller extent than the known adenylyl cyclase-stimulating PGE1. PGE1 (1 µM) also inhibited TRAP-induced $[Ca^{2+}]i$ elevation, expression of P-selectin, fibrinogen binding and aggregation. The PGE_1 effects could be antagonised by the PKA inhibitor H89 (20 µM). H89 also partly antagonised inhibition of TRAP-induced $[Ca^{2+}]i$ elevation by SPPC (20µM) (9±1 vs. 23±3% of control, $p<0.05$). In contrast, H89 did not significantly antagonise inhibitory effects of SPPC on TRAP-induced (10 µM) expression of P-selectin, fibrinogen binding and aggregation.
We conclude that SPPC inhibits agonist-induced $[Ca^{2+}]i$ elevation and the associated aggregation in human platelets. While inhibition of $[Ca^{2+}]i$ elevation may involve PKA activation, this can only partially explain the inhibitory effects of SPPC on platelet aggregation.

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INHIBITION OF PLATELET ACTIVATION AND AGGREGATION BY SPHINGOSYLPHOSPHORYLCHOLINE; AN ENDOGENOUS LYSOSPHINGOLIPID

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Lysosphingolipids such as sphingosine-1-phosphate (SPP) are stored in human platelets from which they can be released upon activation. Since SPP was reported to induce platelet activation, we have investigated the effects of the related lysosphingolipid sphingosylphosphorylcholine (SPPC) on human platelet function.
Isolated platelets from citrate-anticoagulated blood of healthy donors were washed and resuspended in a HEPES-buffer at 200.000 cells/µl. Fibrinogen (0.5 g/l) and calcium (2 mM)were added as indicated. Platelet activation was induced by adenosin diphosphate (ADP), the thrombin receptor activating peptide SFLLRN (TRAP-6), collagen, and the thromboxane A2 mimetic U-46619. SPPC (0.1-20 µM) or vehicle were added 2 minutes prior to stimulation. Analyses of platelet aggregation, expression of activation-dependent platelet surface receptors P-selectin, GP 53, GP Ib, the activated GP IIb/IIIa receptor (PAC-1), fibrinogen binding and changes of intracellular calcium concentrations ([Ca2+]i) were were used to quantify platelet activation. Data are means±SEM of 4-6 experiments.
SPPC alone (0.1-20 µM) caused only very small elevations of $[Ca^{2+}]i$, did neither significantly change surface receptor expression nor induced platelet aggregation. Pre-incubation with SPPC concentration-dependently and almost completely inhibited platelet aggregation in response to different agonists [pEC50 ADP (5/20 µM) 5.80±0.3/5.41±0.3, TRAP (5/20 µM) 5.78±0.3/4.77±0.6, collagen (20/50 µg/ml) 6.19±0.3/5.53±0.3, U-46619 (1 µM) 5.27±0.3]. Agonist-induced $[Ca^{2+}]i$-increase [pEC50 ADP (20 µM) 6.01±0.1, TRAP-6 (20 µM) 5.80±0.1, U-46619 (1 µM) 5.50±0.2] as well as surface expression of P-selectin, GP 53, PAC-1, internalisation of GP Ib, and fibrinogen binding were also inhibited by SPPC in a concentration dependent manner with an almost complete inhibition at 20 µM SPPC.
We conclude that the endogenous lysosphingolipid SPPC is able to almost completely inhibit activation and aggregation of human platelets in response to different agonists.

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HYPERHOMOCYSTEINEMIA, DVT AND PULMONARY EMBOLISM IN A PATIENT WITH CROHNS DISEASE

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Patients with inflammatory bowel are at increased risk of venous thrombosis. We present the case of a 38 yr old female patient who was admitted because of bilateral thrombosis of the popliteal and superficial femoral veins and multiple pulmonary embolism. The patient had had Crohn's disease for six years and took mesalazine and, intermittently, loperamide for diarrhea. At first diagnosis of Crohn's disease, borderline low levels of vitamin B12 and folate as were noted, and substitution recommended. This recommendation, however, was ignored by the patients GP. The patient had started a third generation contraceptive one month before admission. On hospitalization, absent ankle reflexes, sever ataxia, hair loss and a depressed mood with affect instability was noted. The patient complained of marked general weakness and irregular periods Crohn's disease was quiescent reflected in a CDAI of 80 points. Mean corpuscular volume was 130 fl, and a diagnosis of severe B12 deficiency with neurological manifestations was made. Thrombophilia screening was negative for Factor V Leiden mutation, prothrombin mutation, Lupus anticoagulant and anti-Phospholipidantibodies. However, a homocysteine value of 75 µmol/l (normal value less than 15) was found. Vitamin B12 level was low (175 pg/l, normal >225), folate was normal (4,0 ng/ml (>3,0)). Upper endoscopy and serologic testing (parietal cell antibodies) ruled out atrophic gastritis.
The extensive thromboembolic disease in this patient highlights the importance of elevated homocysteine in the development of thromboses in patients with inflammatory bowel disease. Several studies examined homocysteine levels in patients with ulcerative colitis and Crohn's disease. Elevated plasma levels of homocysteine associated with low folate levels were found in patients, however, the elevation was mild compared to controls and ranged from 5 to 15% higher values in IBD patients than in healthy populations with a mean value of 15.6 µmol/l. Extremely high homocysteine values are associated with long-standing vitamin deficiency, and for the prevention of thrombotic disease in IBD patients, vitamin status and homocysteine should be determined.

11

ACTIVATED PLATELETS INCREASE SYNTHESIS OF PLASMINOGEN ACTIVATOR INHIBITOR-1 IN CULTURED HUMAN ENDOTHELIAL CELLS BY HUMAN TRANSFORMING GROWTH FACTOR BETA-1

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Hepatic veno-occlusive disease (VOD) is a life threatening complication following allogeneic bone marrow transplantation (BMT). High dose cytoreductive therapy seems to cause endothelial injury leading to a hypercoagulable state and to the obliteration of terminal hepatic venules. Thrombocytopenia and refractoriness to platelet transfusion in these patients suggest that an interaction between platelets and the sinusoidal endothelium might play an additive role in the pathogenesis of VOD.
We investigated the influence of activated platelets on the synthesis of plasminogen activator inhibitor-1 (PAI-1) in human endothelial cells isolated from umbilical cords using collagenase (HUVEC). Platelet rich plasma was activated with thrombin and brought into close contact with confluent HUVEC by centrifugation. HUVEC (1×10^6) and activated platelets (5×10^6) were coincubated for 24 h at 37°C (5% CO_2). The release of PAI-1 into the cell culture supernatant was investigated by ELISA technique.
1) Human endothelial cells conincubated with activated platelets show a significantly increased release of PAI-1 into the supernatant. 2) HUVEC incubated with activated platelets and an antibody against CD154 still show an increased synthesis of PAI-1. Incubation with an antibody against hTGF beta-1, however, blocks this effect. 3) HUVEC stimulated with hTGF beta-1 for 24 h also show a significantly increased synthesis of PAI-1.
Our studies demonstrate that thrombin-activated platelets induce an increased synthesis of PAI-1 in cultured human endothelial cells by hTGF beta-1, a mediator known to be released by activated platelets. By releasing PAI-1, this novel mechanism might contribute to the hypercoagulable state of the sinusoidal endothelium in VOD.

12

A SAFETY SURVEY OF INHIBITOR DEVELOPMENT AND VIRUS TRANSMISSION IN 6 HEMOPHILIA B – PATIENTS TREATED WITH A PLASMA-DERIVED FACTOR IX-CONCENTRATE

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Hemophilia B affects about 1 in 25000 males. We study 6 patients with hemophilia B. 3 have a severe (F IX<1%) and 3 patients a less severe form (F IX 1,8-2,5%). While the 3 patients with F IX <1% were exclusively treated according to clinical need with one pd F IX-concentrate (Octanyne) the other 3 patients were treated first with other pd F IX-concentrates. The molecular defect was investigated in all 6 patients. According to the recommendations of the GTH-Inhibitor-Incidence Study Group we tested the inhibitor formation. All patients were vaccinated for hepatitis A and B and developed antibodies. 3 patients are on regular prophylaxis and 3 are treated on demand. 4 patients had surgical interventions, 2 of them with continuous infusion.Until now all patients have no incidence of inhibitor formation. The exposure days are in 2 patients >250 ED, in 1 patient >50 ED, in 2 patients >25 ED and in 1 patient 4 ED. All 6 patients remained HIV and HCV negative at repeated control tests and have as well normal ALT values. The clinical efficacy of the treatment with this pd F IX-concentrate is good, it is well tolerated and no serios adverse events are reported.

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ENHANCED COAGULABILITY IN THROMBELASTOGRAPHY (TEG) IN HAEMODILUTION IS AN ARTIFICIAL RESULT

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Recent publications reported on enhanced coagulability in haemodilution determined by TEG. In contrast, prolongation of in-vivo-bleeding time in anaemia and increased microvascular bleeding in haemodiluted patients during cardiac surgery have been observed. In order to throw some light on this discrepancy diluted blood samples were investigated by TEG as well as by in-vitro-bleeding tests (IVBT) which simulate primary haemostasis ex vivo.
Material and methods: Citrated blood of 10 healthy blood donors. Dilution by 20 % with either saline, HES 6% or autologous platelet poor plasma (PPP). Parallel examination of undiluted and diluted blood samples: Rotation thrombelastography (roTEG, Pentapharm, Munich) with (PTT reagent, InTEG-LS Aktivator, Nobis, Endingen; InTEG) and without specific activators of coagulation ($CaCl_2$; NaTEG). Determination of coagulation time (CT), clot formation time (CFT), alpha angle (alpha), maximal amplitude (MA). IVBT with PFA-100 (Dade-Behring, Marburg) with collagen-epinephrine-coated cartridges (PFA-EPI). Additionally, a modified IVBT method for research purposes (VCP2) was used ensuring constant shear forces and controlled flow during the test cycle independently from haematocrit.
Preliminary results: Only dilution with PPP showed consistent changes of the various parameters in NaTEG as defined for enhanced coagulability (CT shortened 5/5, CFT shortened 4/5, CT+CFT shortened 5/5, MA increased 5/5). Samples diluted with saline gave inconsistent results (CFT shortend 4/5, MA reduced 3/5). With HES, parameters mainly changed as defined for reduced coagulability (CFT prolonged 4/5, MA reduced 5/5), but CT was shortened in 4 of 5 samples. In InTEG the changes described were even more obvious. However, with HES all parameters now showed reduced coagulability (CT prolonged 5/5, CFT prolonged 5/5, alpha reduced 5/5, MA reduced 5/5). In the two IVBT methods dilution always prolonged the closure time (PFA-EPI: saline +51±18.3%, PAP +46±26.6%, HES +76±30.5%; VCP-EPI: saline 44±16.3 %, PPP 36±28.4%, HES (n=2) 66±30.7%).
Conclusions: Haemodilution, particularly with HES, clearly disturbs primary haemostasis. Changes of the TEG parameters as defining enhanced coagulability are artificial and seem to be due to the unphysiological flowing in the test tube.

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PROTEASE ACTIVATED RECEPTORS IN BRAIN CAPILLARY ENDOTHELIAL CELLS

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Brain microvascular endothelium with astrocytes constitute the border between blood circulation and the brain interstitium being assigned as the blood-brain barrier (BBB).Thrombogenic and fibrinolytic enzymes acting through PAR-s (proteolytically activated receptors) may play a role in the opening of the BBB. PCR analysis detected the mRNA of both thrombin receptors, PAR-1 and PAR-3 in primary cultures of rat brain capillary endothelial (RBCE) cells and in astrocytes. To determine the contribution of PAR-1 and PAR3/PAR4 to thrombin signalling, the changes in intracellular Ca2+ level ([Ca2+]i) in response to thrombin, the PAR-1 agonist peptid SFRLRN and the PAR-4 agonist peptid GYPGKF was examined.The peak $[Ca^{2+}]$i rise attributable to SFRLRN was less than the increase induced by thrombin. Complete blockade of the thrombin response by maximal doses of SFRLRN did not occure, although almost no remaining $[Ca^{2+}]$i signal was measured after repeated stimulation with thrombin. The PAR-4 agonist peptid GYPGKF elicited a transient increase in $[Ca^{2+}]$i in RBCE cells, maximal increase being similar in magnitude to the SFRLRN response. Desensitisation with GYPGKF decreased significantly the rise in $[Ca^{2+}]$i, induced subsequently by thrombin, but did not influence the subsequent SFRLRN response, indicating that in RBCE cells PAR-1 and PAR-4 mediate thrombin signalling independently. RBCE cells also expressed PAR-2 mRNA and functional PAR-2 was proved by measuring $[Ca^{2+}]$i after activation with trypsin and the PAR-2 agonist peptide SLIGRL. Trypsin activated both PAR-1 and PAR-2, the relative agonist activity of trypsine and thrombin on PAR-s of RBCE cells compared to that of SLIGRL were 112% and 48%, respectively. Both plasmin and elastase reduced the trypsin-induced [Ca2+]i signal dose-dependently, whereas the thrombin-induced $[Ca^{2+}]$i signal was completely eliminated by plasmin.The results suggest the presence of distinct PAR-s in the cells of the BBB, contributing to the thrombin or trypsin induced increase in $[Ca^{2+}]$i and the susceptibility of the receptors to inactivation with plasmin and elastase.

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***MUTATION ANALYSIS OF FACTOR VII DEFICIENCY IN SLOVAKIA**

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FVII (FVII) is a vitamin K-depended serine protease. The FVII gene is 12.8 kb long and was localised on chromosome 13q34-qter. The clinical features are quite variable with rather poor correlation between reported coagulant activity and clinical bleeding tendency.
We have analysed the factor VII gene of 24 unrelated subjects with FVII deficiency from Slovakia. All exons, the exon-intron-boundaries and the promotor region of the FVII gene were analysed by sequencing.
The molecular defects of FVII deficiency in 24 unrelated patients from Slovakia were identified. 7 different mutations: *-62C>T, Gln100 Arg, Ala294 Val, *Val362 Phe, *Gly365 Ala, Cys310 Phe and Ala294 Val; 404 del C were analysed in the FVII gene. Three (*) of these lesions are novel FVII mutations (MRC database FVII 2001). The most frequent lesion was the double mutation Ala294 Val; 404 delC in exon 8. This mutation was analysed in 25 unrelated FVII alleles in patients from Slovakia.
The FVII mutations were found in patients in different genetic conditions. The results of the mutations and haplotype analysis of families with inherited factor VII deficiency will be presented.

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***NEW THERAPY POSSIBILITIES TO PREVENT DISASTROUS COMPLICATION IN PATIENTS WITH NEONATAL ALLOIMMUNE THROMBOCYTOPENIA**

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Neonatal alloimmune thrombocytopenia (NAIT) is a fetomaternal incompatibility. The NAIT is usually induced by platelet-specific antibodies against HPA-1a (Zwa) or HPA-5b (Bra) and other most low-frequency alloantigens on the platelet glycoproteins. The frequency is given 1-1,5/1000 liveborn neonates. The clinical picture of these antibodies is different. The children with a NAIT are born have a isolated mild thrombocytopenia to life threatening intracranial bleeding. Around these severe complications should to be able very much prematurely the diagnosis are protected. The detection of the genetic difference between the parents covered on known thrombocyte alloantigens, the antibody detection in the maternal serum and the positive crossmatch (maternal serum against paternal test cell) are a presupposition for the sure diagnosis. Currently to pregnant women takes place no routine screenings for an immune mechanism in her blood. Around intracranial bleeding in the fetus should to bend forward therapeutically everything are tried to hold the titer of the antibody in the maternal blood so low as possible. Because it is here about the formation of an IgG-antibody, the illness can already appear in the first pregnancy. Treatment with high dosis from iv-IgG are expensive and have led to no continuous reduce of the titer. The treatment of intrauterine platelet transfusions for the prevention of severe intracranial bleedings had many side effects for the mother. A new much promising therapy possibility is the immunoadsorption. With a continoues decrease of the antibody titer in the maternal blood on 1:8 it is prevented that antibodies with a high titre lead to severe fetal thrombocytopenia. A 29-old women with a history of five habitual abortions and a high titer of Anti-HPA (1:32), Anti-HLA (>1:2000 non-complementfixed) could be treated successfully with 42 immunoadsorptions. The woman delivered a healthy boy with birth weight of 2360g in the 34 week of pregnancy by cesarean section. At birth the child had 199 GPt/l thrombocytes (lowed 49 GPt/l in 24 hours) and the Anti-HPA titer was 1:2.

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***CRITICAL ESTIMATION OF ABSOLUTE RISK FOR TRAVELLERS THROMBOSIS**

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Estimate of the absolute risk for the general population: From several case-control studies an absolute incidence of 6 to 24 deep venous thromboses (DVT) per 100.000 travellers can be extrapolated for a time interval of three weeks after travelling. Employing prophylactic measures with a 100% efficacy, this would translate into 4.000 to 7.000 persons who would need to undergo prophylatic measures in order to avoid one DVT (number-needed-to-treat (NNT). The incidence of pulmonary embolism (PE) without prophylaxis would range from 6 to 24 per million passengers, assuming a 10% rate of symptomatic PE in patients with symptomatic DVT. This incidence is about 30-fold higher than the one recently reported for severe symptomatic PE diagnosed after arrival. In this study,

however, the incidence may have been underestimated, as only severe clinical signs, such as syncope, resulted in a call for emergency medical services. Also, thromboembolic events evolving over the ensuing weeks were not included.
Estimate of the absolute risk for risk populations: Extrapolation based on an odd's ratio of 1.0 (1): For patients who previously had had DVT, 461 thromboses per 100.000 travellers would occur during a three weeks period. For an odd's ratio of 2.2 (2) this would translate into 1.084 DVTs, equivalent to 6 fatal pulmonary embolism per 100.000. For an odd's ratio of 4.0 (3) a number of 1.844 DVT per 100.000 would be expected. The results reported by Scurr et al. (4) with 10% asymptomatic DVT per 100.000 travellers would result in approximately 1.000 symptomatic DVT and 6 fatal pulmonary emboli. For patients with a history of DVT (odd's ratio 15.0) this would result in 15.000 symptomatic DVT and 90 fatal pulmonary emboli per 100.000. These numbers appear – with respect to the observed incidence in the vicinity of large airports – not plausible. The results for risk populations are summarized in the table.

Risk estimate for DVT associated with long distance travel for risk populations (preceeding thromboembolic event)

Risk of long history of TE	distance travel	DVT per **100.000** fatal PE travellers (≤3 wks.)	
OR: 1.0 (1)	OR: 15.0	461	**1**
OR: 2.35 (2)	OR: 15.0	1.084	6
OR: 4.0 (3)	OR: 15.0	1.844	10
OR: 33.0 (4)	OR: 15.0	15.000	90
(assuming 10% asymptomatic DVT)			

1) Kraaijenhagen, 2000; 2) Samama, 1999; 3) Ferrari, 1999; 4) Scurr, 2001

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***IN VIVO AND IN VITRO RESPONSE OF FIBROBLASTS TO PLASMATRANSGLUTAMINASE (F XIII) IN PARAPLEGIC PATIENTS**

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The treatment of chronic pressure sores in para- and quadriplegic patients remains challenging. Surgical treatment is still limited to available tissue demanding safe wound/flap healing. We performed an in vivo study with F XIII on patients with chronic pressure sores and an in vitro study regarding stimulation response of fibroblast to plasmatransglutaminase (F XIII/Fibrogammin®).
Material and methods: Patients with long standing pressure sores (>3 months) and deep defect (>type 3 A after Daniel/Seiler) were treated preoperatively during flap surgery with 1250 IU F XIII iv. daily for 8 days. Follow-up was performed clinically and with photo documentation. Cases with contraindications to surgery were treated with topic application of 250 IU F XIII/d.
Tissue harvesting of infected pressure sores, in vitro stimulation after growth in DMEM with F XIII in different dilutions, analysis of cellular growth (MTT, ABS).
Results: 17 Patient involved (14 Pat. systemic, 3 Pat. topic). Systemic group treated surgically with 21 flaps (8 TFL, 2 biceps femoris, 1 gracilis,1 fasciocutaneous, 3 vastus lateralis, 6 gluteal – flaps). 9 patients showed additional risk factors for impaired wound healing due to 4 local infections with MRSA, 3 plasmocytoma and 2 diabetic patients. Uneventful healing of 17 flaps. Four revisions were performed, but no flap necrosis was seen. All flaps with underlying chronic osteomyelitis survived. No systemic side – effects of F XIII application were seen. Topic treatment had to be stopped after allergic reaction/local necrosis in all cases.
Cell survival was mainly seen after enhancing the DMEM media with antibiotics after sensitivity testing. In comparison to normal tissue, in vitro stimulation of fibroblasts occurred only after high dilution.
Conclusion: We found a safe and rapid healing of flaps after intravenous application, however no effects in topic application were seen. The overall local response of fibroblasts to F XIII in paraplegic patients is different from normal tissue. This effect may be due to changed cellular patterns in paraplegic patients and has to be investigated further.

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DISTRIBUTION OF SUBTYPES OF VON WILLEBRAND DISEASE IN A SECONDARY LABORATORY. IN THE GENETICALLY DETERMINED VON WILLEBRAND DISEASE TYPE 1 FREQUENT OR RARE?

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Von Willebrand disease (VWD) is the most common inherited disorder of primary hemostasis with a prevalence of 1:3000. Usually 70% of the patients are classified as mild, type 1 VWD.
The distribution of different subtypes of preselected patients in a our laboratory is in contrast to published data.
In 1999 over a 12 months period, n=2438 plasma samples of symptomatic patients were investigated, either to confirm VWD or to rule out a bleeding tendency. In all samples determination of VWF:Ag, VWF:CB and multimers in a high and low resolution gel system (in house methods) were performed.
Abnormal results were found in 489 (20%) of the samples. The diagnosis of inherited VWD was made in 303 (62%) cases, all with a typical bleeding history (and positive family history).
Further subtyping of our cohort revealed the following distribution: VWD type 1 40,3%, type 2 56,4% und type 3 in 3,3%. In 10% of the type 2 patients the ratio VWF:CBA/Ag was normal; however, multimeric analysis detected the abnormal pattern. 32% of the patients were diagnosed with acquired VWD, which will be not subject of this presentation.
VWD type 2 was more heterogeneus than has been reported in the literature. Interestingly we found a high proportion of type 2M Vicenza. In 90% of the type 2 patients we had an excellent phenotype – genotype correlation. Therefore, correct phenotyping results in a cost efficient and faster genotyping (price per exon 300-400 Euro, total gene sequence up to 8500 Euro).
It remains open, if due to the preselection of our patients the proportion of VWD type 1 is unrealistic low or if type 1 is less frequent than previously published; the latter would explain the problems in recruiting patients with type 1 in all participating centers for an international study.
A reliable diagnosis and proper classification of VWD can be achieved only by the combination of different methods - together with clinical information on the patient.

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TWO YEARS EXPERIANCE WITH THE COLLAGEN-BINDING ASSAY FOR QUANTIFICATION OF VON WILEBRAND-FACTOR-CLEAVING PROTEASE (VWF-CP)

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In patients with chronic thrombocytopenia the introduction of the VWF-CP assay is very helpful in classification of microangiopathic thrombocytopenia. However, recent reports doubt the specificity of the assay.
The mayor advantage of the collagen binding assay is the fast turn-over time of only one day and it is less technical demanding than the originally described vWF-multimer analysis method by Furlan et al (NEJM 1998). Instead of a plasma preparation we use recombinant von Willebrand factor as the uncleaved substrate.
Over a period of 2 years we analysed approximately 500 samples of patients with thrombocytopenia, including patients with TTP, HUS, HELLP-syndrome and patients after bone marrow transplantation (BMT). Additionally, we investigated 100 patients with metastazised solid tumors.
In our opinion patients with TTP are characterized specifically by a complete absence of the VWF-CP due to an inhibitor in most of the non-familiar cases.
Patients with HUS or women with HELLP-syndrome never showed a relevant decrease of the VWF-CP activity. Some patients after BMT had a considerably decreased activity but were always distinguishable from non-BMT-TTP-patients. In cancer patients we observed a decrease of the VWF-CP, but only in 2 of 100 patients the VWF-CP was absent completely. These data are in contrast to published data by Oleksowicz et al (CANCER RESEARCH 1999).
In our experiance the assay is a fast and reliable tool in the differential diagnosis of microangiopathic thrombocytopenia.

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SPECIFICITY OF SEVERE VON WILLEBRAND FACTOR-CLEAVING PROTEASE DEFICIENCY FOR THROMBOTIC THROMBOCYTOPENIC PURPURA

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Background. vWF-cleaving protease (vWF-cp) deficiency has been found in most patients with a clinical diagnosis of TTP (NEJM 1998;339:1578-84; NEJM 1998;339:1585-94). It may be hypothesized that the defective processing of vWF leads to the presence of unusually large and extremely adhesive vWF multimers (ULvWF), which might be responsible for the microvascular platelet clumping process underlying the acute TTP episodes. N-terminal aminoacid sequencing of purified vWF-cp and genetic linkage analysis of families with congenital TTP led to the identification of vWF-cp as a new member of the ADAMTS family of metalloproteinases (ADAMTS13). Twelve different mutations of the ADAMTS13 gene have been identified in seven families, affected patients being homozygous or doubly heterozygous for ADAMTS13 mutations (Nature 2001;413:488-94). Besides these rare congenital vWF-cp deficiencies, most patients with acute TTP show an acquired deficiency of vWF-cp due to autoantibodies inhibiting its activity. vWF-cp deficiency has been recently claimed not to be specific for TTP, being found in various thrombocytopenic conditions different from TTP(Blood 2001;98:1842-46) as well as other conditions (Blood 2001;98:2730-35). Our own experience is contradictory to these data, severe vWF-cp deficiency (<5%) having been found exclusively in patients with a clinical picture compatible with TTP, even though no formal study has been performed. Our aim was to investigate, by a formal study, the vWF-cp activity in patients with thrombocytopenia related to various disorders, in order to clarify whether a severe deficiency of vWF-cp is a specific diagnostic finding for TTP.
Methods. We have recruited 17 thrombocytopenic patients with severe sepsis/septic shock ± DIC from a previous study, 16 cases of heparin-induced-thrombocytopenia (HIT) collected from a parallel study and 30 prospectively enrolled patients with thrombocytopenia of various etiology (ITP, osteomyelofibrosis, myelodysplastic syndrome, leukaemia, aplastic anemia, etc.). vWF-cp activity was assayed by an immunoblotting method previously described. Results. The results are expressed by median [range] (see table).
Conclusions. This study demonstrates that a low vWF-cp activity can be found in several thrombocytopenic patients, but a severe deficiency of vWF-cp (<5% of normal plasma) is specific for classic TTP. Furthermore, there was no correlation between platelet count and vWF-cp activity.

	Age (mean±SD)	**PLT** (×10⁹/L)	**vWV-cp** (%)
HIT (n=16)	68±13	69 [19–108]	50 [15–100]
Severe sepsis/ septic shock (n=17)	62±13	69 [7–129]	32 [18–46]
Osteomyelofibrosis (n=3)	68±13	25 [7–69]	72 [49–100]
Myelodysplastic syndrome (n=3)	65±6	34 [12–52]	98 [95–100]
ITP (n=10)	44±15	56 [9–132]	98 [25–100]
Leukaemia (n=5)	60±10	60 [23–83]	100 [88–100]
Aplastic anemia (n=2)	58±13	39 [4–74]	100 [100–100]
Miscellaneous (n=7)	48±20	33 [7111]	100 [72–100]

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VWF-CLEAVING PROTEASE IN SEVERE SEPSIS AND SEPTIC SHOCK

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Background. Severe sepsis and septic shock are characterized by a disturbed endothelial function, main cause of the derangement of the haemostasis system leading to disseminated intravascular coagulation (DIC) and contributing to multiple organ failure (MOF). Due to its physiological role in haemostasis, vWF might be involved in haemostasis activation and platelet consumption in sepsis, contributing to the development of MOF and leading to death. From previous studies, it is known that a severe hereditary or acquired deficiency of the vWF-cp leads to a massively enhanced microvascular platelet clumping in thrombotic thrombocytopenic purpura (TTP), due to the fact that unusually large vWF multimers, released by the activated endothelium, cannot be cleaved. At present hardly any data is available about the role of vWF-cp in severe sepsis and septic shock.
Methods. We studied 40 consecutive patients admitted to ICU, enrolled at the moment of diagnosis of severe sepsis and septic shock. The patients have been matched by gender and age with 40 healthy controls (CTRLs). vWF:Ag was measured by ELISA, vWF-cp by immunoblotting assay and vWF:RCof by aggregation of normal platelets by patient plasma in the presence of ristocetin in an optical aggregometer.
Results. Among the 40 patients, 32 (80%) were affected by severe sepsis and 8 (20%) by septic shock. Septic patients had a decreased vWF-cp activity if compared to CTRLs (median [range]: 34% [18-100%] vs. 81% [35-162%]; p<0.0001), while both vWF:Ag and vWF:RCof were significantly elevated (vWF:Ag: 372% [133-816%] vs. 89% [48-209%]; p<0.0001 and vWF:Rcof: 372% [127-653%] vs. 118% [55-253%]; p<0.001). No difference has been highlighted in these parameters between the severe sepsis and the septic shock groups.
Conclusion. In severe sepsis and septic shock vWF has a pivotal role. In this study it has been confirmed that vWF:Ag and vWF:Rcof are highly elevated in sepsis and, moreover, a decreased activity of vWF-cp in septic patients has been demonstrated. A vWF-cp <5%, characteristic of TTP, does not occur in severe sepsis and septic shock. vWF-cp may behave as a negative acute phase protein being already decreased at the time of the diagnosis of severe sepsis and septic shock.

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MENTAL STRESS INDUCES ACTIVATION OF THE TRANSCRIPTION FACTOR NF-KB IN MONONUCLEAR CELLS

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Mental stress is supposed to contribute to the development of atherosclerosis, however little is known about the cellular mechanisms that convert psychosocial stress into cellular dysfunction. The transcription factor NF-kB is rapidly induced in response to a variety of stimuli. Since several NF-kB controlled genes are known to be upregulated in atherosclerosis, NF-kB might be a good candidate to transduce psychosocial stress into atherosclerosis relevant pathophysiological changes. When healthy volunteers were subjected to a brief laboratory stress test, mononuclear cells from 17 of 19 volunteers demonstrated rapidly induced NF-kB binding activity during stress exposure, that returned to basal levels after a 60 min recovery period. When cultured THP-1 cells were induced with physiological amounts of adrenaline or noradrenaline for 10 min, only noradrenaline resulted in a dose and time dependent induction of NF-kB binding activity and NF-kB dependent gene expression. The effect of noradrenaline was blocked in the presence of the alpha1-inhibitor prazosin and the beta-blocker butoxamine (beta2 > beta1), suggesting the involvement of both alpha- and beta adrenergic receptors. Exposing transgenic mice, carrying an NF-kB driven beta-globin reporter gene, to mental stress resulted in increased beta-globin expression, which could be reduced in the presence of alpha1- and beta2-inhibitors. These data indicate that noradrenaline dependent adrenergic stimulation results in activation of NF-kB and NF-kB dependent gene expression in vitro and in vivo. Activation of NF-kB might therefore represent a downstream effector for the neuroendocrine response to stressfull psychological events and link changes in plasmatic activity of the neuroendocrine axis to the cellular response important for development of vascular disease.

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UROKINASE RECEPTOR (UPAR) DEPENDENT PROTEOLYTIC ACTIVITY CAN BE REGULATED BY VEGF OR BFGF VIA NON TRANSCRIPTIONAL MECHANISM

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Vascular endothelial growth factor (VEGF) and the basic fibroblast growth factor (bFGF) play an important role in the angiogenic process. Urokinase type plasminogen activator receptor (uPAR) is tightly regulated by these cytokines in ECs and itself plays an important role for the angiogenic process that is dependent on local proteolytic activity. Using flow cytometry we could confirm previous data that VEGF or bFGF upregulate expression of uPAR in a time and dose dependent manner via the MEK/ERK pathway, starting 8 hours after addition of growth factors. For the first time we can show here an additional immediate short term effect of these growth factors on the distribution of uPAR within the cell.

When VEGF (50 ng/ml) or bFGF (10 ng/ml) was added to the cells, uPAR was partly internalized within 30 min via a LRP dependent mechanism, as we could proof by using the Receptor Associated Protein (RAP), a specific inhibitor of LDL- receptor family mediated internalization. To further delineate the initial events by growth factors, we analyzed cell surface bound urokinase by fluorometric cell assay and ELISA, using antibodies recognizing specifically the inactive pro-uPA as well as the antibodies specific for both the active and inactive forms of uPA, respectively. We found a loss of pro-uPA on the cell surface upon VEGF stimulation, while total uPA antigen followed the changes in uPAR. Internalization of uPAR and activation of uPA in response to VEGF was absent in the presence of PI3-kinase inhibitors, but could be restored adding exogenous active uPA to the PI3-kinase inhibited cells. Because a specific inhibitor of gelatinases (MMP-2, MMP-9) could inhibit the redistribution of uPAR and the loss of pro-uPA induced by growth factors in ECs, MMP-protease dependent pro-uPA activation by VEGF can be assumed to be involved in the PI3-kinase dependent mechanism. By using an antibody, which only binds to the active form of beta-1-integrins, we found that VEGF down regulates the binding activity of beta-1-integrins. The soluble tripeptide RGD, known to inhibit integrin activity, was added to the ECs. By this measure, cell bound pro-uPA was acivated, leading to a redistribution of urokinase receptor in a similar pattern as VEGF does. We conclude that down regulation of beta-1-integrin activity by RGD or VEGF leads to immediate metalloprotease activation. This in turn results in urokinase activation, followed by RAP dependent internalization of its receptor.

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EUROPEAN COLLABORATIVE STUDY OF THE ANTENATAL MANAGEMENT OF FETAL ALLOIMMUNE THROMBOCYTOPENIA

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The antenatal management of pregnancies complicated by fetal alloimmune thrombocytopenia (FAIT) has been a subject of controversy. The aims of this study were to determine the success of different types of antenatal intervention and to assess if the severity of FAIT in the current pregnancy could be predicted from the history. We studied 56 fetuses who all had a sibling affected by FAIT due to HPA-1a alloimmunisation. Three of the study cases suffered intracranial hemorrhage (ICH) and 3 others died. These results compared favorably as 15 study cases had siblings with ICH and in 8 of these cases the sibling died. 92% who had a sibling with an antenatal ICH and 66% with siblings known to have had platelet counts of $<20\times10^9$/l had severe thrombocytopenia. Maternal therapy, mostly with intravenous immunoglobulin (ivIgG), resulted in a platelet count of $>50\times10^9$/l in 67% of cases. None of the fetuses managed by serial platelet intrauterine transfusions suffered ICH after treatment started. However, the most serious complications encountered by the study cases were associated with fetal blood sampling and were highest in the group receiving serial platelet transfusions. We recommend maternal therapy as first line treatment for the antenatal management of FAIT, and that serial platelet transfusions be reserved for those not responding to maternal therapy.

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***STRUCTURES OF COAGULATION COMPLEXES**

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The processes of coagulation, anticoagulation and fibrinolysis are governed by activation cascades involving trypsin-like serine proteinases. Despite their highly similar architecture, these proteinases display major differences in their specificities and activities. Specificity is achieved by subtle differences in subsites and exosites, but frequently also via formation of complexes with cofactors. Examples for such proteinase-cofactor complexes are the intrinsic and the extrinsic Xase, the prothrombinase, the thrombin-thrombomodulin complex, and the plasmin complexes formed with the bacterial non-enzymatic activators streptokinase and staphylokinase. We have determined the crystal structures i) of a ternary complex formed by two µ-plasmin molecules and staphylokinase [1], and ii) of a binary complex formed by α-thrombin and the active EGF-like 4-5-6-fragment of the transmembrane receptor thrombomodulin [2]. Both structures revealed that these cofactors upon binding to their target proteinase, besides blocking some surface regions, provide novel exosites, which allow to bind their wanted protein substrate (plasminogen and protein C, respectively) such that the cleavage site of the substrate is presented to the active center of the cognate proteinase with precise geometry optimal for proteolytic cleavage. The structure of the membrane-binding C2 domain of cofactor Va [3], besides explaining the hydrophobic and simultaneously hydrophilic membrane interactions, suggested a mechanism for stereospecific binding to phospatidylserine-rich membranes.

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FIRST CONTACTS OF AGGREGATING PLATELETS: αIIbβ3-FIBRINOGEN-CONTACTS OR CONTACTS CONTAINING TIGHT JUNCTIONS PROTEINS?

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Platelets aggregate by formation of focal contacts mediated by fibrinogen molecules that connect αIIbβ3 integrins on the opposite plasma membranes. Within these contacts, so-called tight contacts are present. Tight contacts (TC) are focal contacts with some characteristics of tight junctions (TJ) of epi- or endothelial cells, i.e. TCs contain the TJ molecules occludin and ZO1 (1). The functional role of these contacts remained to be elucidated. To investigate if the TCs play a role at the beginning of aggregation, we studied the very early phases of aggregation using transmission electron microscopy.
The platelet aggregation in citrated PRP of human donors was induced using ADP (5mM) under stirring. The experiments were stopped as fast as possible by addition of buffered glutardialdehyde under stirring. It was possible to prepare samples from activated platelets after 1.5 and 2.5 sec. Pellets of the samples were post fixed with OsO4 and processed for ultra thin serial sectioning.
1.5 sec after ADP stimulation the platelets showed a discoid shape and had formed aggregates. Fibrinogen was bridging the focal contact spaces as shown earlier with immunocytochemistry(2). TCs were not found in such aggregates. After 2.5 sec - and longer lasting - ADP stimulation aggregates were formed by shape-changed platelets. Fibrinogen was bridging focal contact spaces, too, but TCs were present within the fibrinogen contacts in such aggregates. In the immediate neighboring of the TCs, membrane invaginations are observable, sometime coated with clathrin. The internalization of fibrinogen is demonstrated at these places.
1. Fibrinogen molecules bound to αIIbβ3 contacts and obviously these molecules initiate the contact of platelets that are firstly aggregated discocytes. 2. TCs are formed only between aggregated platelets that have changed their shape. 3. Occludin as well as ZO-1 play rather regulatory or functional than structural roles in the formation of tight junctions in cells (3). In activated platelets such molecules might be involved in the internalization of fibrinogen as shown here or of fibrin fibers during clot formation (4).
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CORRELATION OF VWF-CLEAVING PROTEASE ACTIVITY, VWF:AG AND FREQUENCY OF RELAPSES IN 17 TTP-PATIENTS OBSERVED BETWEEN 1982 AND 2001

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Background: The pathogenesis of thrombotic thrombocytopenic purpura (TTP) has been enlightened by identifying the deficiency of VWF cleaving protease (cp) activity in acute TTP. Congenital cp-deficiency is caused by mutations in the ADAMTS13 gene, whereas IgG auto-antibodies against the protease have been characterised in various cases of acquired TTP. However, the aetiology for onset and relapse remains unclear. We report the retrospective analysis for 17 TTP-patients observed between 1982 and 2001 in respect of vWF-cleaving protease activity, vWF:Ag and frequency of relapses. The age of the 17 pts. at initial episode was 13-52 years (mean=29) for females (n=10) and 36-54 years (mean=39) for males (n=7).
Methods: Cp-activity was tested using a recently developed method in our laboratory, which is based on the positive correlation between multimeric size and RistocetinCofactor activity of the vWfactor.

Results: 8/17 pts. (M:F=3:5) experienced a total number of 21 relapses, 8/15 (M:F=3:5) pts. are without relapse until now. 1 patient died during initial episode. Cp-activity (expressed in % of normal) was severely deficient (<12,5%) in all initial plasma samples of the acute episode tested (n=20). The cp-activity in remission was normal in 6/16 pts. (>58%), mildly reduced (35 to 47%) in 6/16 pts. and severely reduced (<12,5%) in 4/16 pts. Patients without relapse showed a higher mean of cp-activity and a lower mean of vWF:Ag in remission than pts. with relapse (55% vs. 31% and 135% vs. 163%, respectively). To further evaluate the influence of cp-activity and vWF:Ag on relapse we investigated the patients with an observation period longer than 23 months after first episode (n=13). We found a significant correlation between cp-activity and number of relapses (r=-0685, p=0,009), whereas vWF:Ag level and observation time was not significantly correlated to the number of relapses (p>0,1). If cp-activity combined with vWF:Ag is correlated with the number of relapses in multiple analysis we obtained a slightly higher correlation (r=0,730, p=0,022).
Conclusions: An isolated deficiency of vWF-cleaving protease activity does not necessarily lead to TTP episodes as it has been also reported by others. Our data indicate though, that low cp-activity in remission, especially if combined with high vWF:Ag, might be a significant risk factor for a future relapse.

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THE SIGNIFICANCE OF THE VWF-CLEAVING PROTEASE IN THE PATHOGENESIS OF TTP

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Background: The severe deficiency of vWF-cleaving protease (cp) activity in acute thrombotic thrombocytopenic purpura (TTP) has been a consistent finding since the enzyme was characterised in 1996. A wide number of pts. in remission are partial deficient or normal in cp-activity. The decrease in cp-activity during acute episodes in those patients is due to auto-antibodies or hitherto unknown factors. We describe results from serial monitoring of cp-activity from 3 TTP-pts. with mild reduction of cp-activity in remission, where decrease of cp-activity to an undetectable limit without severe thrombocytopenia, rise in LDH-level and clinical symptoms of acute TTP was observed.
Methods: Cp-activity was tested using a novel in house method based on the correlation between multimeric size and RistocetinCofactor activity (RCo). After digestion of a protease-free vWF concentrate (gift from LFB, France) in the presence of barium ions and urea the residual RCo is measured and used to assess the cp-activity in the samples (expressed in % of normal).
Results: In Patient 1 and Patient 2 the decrease of cp-activity from 20% and 60% to <1% was followed by decrease in platelet count, increase of LDH-level and fragmented erythrocytes in the peripheral blood smear thus diagnosing an acute TTP episode. Plasmapheresis against FFP lead to an immediate increase in cp-activity to 32% and 23%. Patient 3 with a decrease in cp-activity from 44% to <1% stayed without further evidence of relapse and cp activity increased within 40 days to 10% without any treatment.
Conclusions: We demonstrate for the first time that decrease of cp-activity can indicate a relapse before the occurrence of severe thrombocytopenia, increased LDH-level, fragmented erythrocytes and clinical symptoms. The decrease of cp-activity might be an early marker for acute TTP thus secure early diagnosis and rapid treatment. However, the severe decrease of cp-activity in patient 3 without clinical or biochemical evidence of an acute episode demonstrate, that further parameters are needed to diagnose acute TTP. It might be speculated, that the cp decrease in patient 3 verifies the occurrence of undiagnosed acute episodes. It might also be, that decrease of cp-activity is not a specific beacon of acute TTP, but a hint for a hitherto unknown disease (e.g. liver dysfunction). This would go in line with findings of low cp-activity in other diseases.

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VARIABLE PLATELET RESPONSE TO THERAPY WITH ASA/ CLOPIDOGREL IN PATIENTS WITH CARDIOVASCULAR DISEASE – IS THERE A SUBGROUP OF CLOPIDOGREL NON RESPONDERS?

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Background: Antiplatelet therapy is an important adjunctive treatment that reduces ischaemic complications in patients with percutaneous coronary intervention (PCI). Clopidogrel is an oral antiplatelet agent, which inhibits the platelet ADP receptor. When clopidogrel is given with acetylsalicylic acid (ASA), the antiplatelet effect is synergistic. The combination of clopidogrel with ASA has now emerged as standard therapy in PCI.
Methods: In 99 patients undergoing PCI and pretreated with 100 mg ASA/day we investigated platelet function before, and 3, 6 and 24 hours after a loading dose of 450 mg clopidogrel. The effect of ASA on platelets was measured in anticoagulated whole blood as closure time (CT) with the platelet function analyzer PFA-100® (Dade Behring) using test cartridges coated with collagen/epinephrine. ASA prolongs CT (cutoff value=<170s). Inhibitory effect of clopidogrel on platelet aggregation was tested with a Chrono-log® whole blood aggregometer (Nobis) using 10 µM ADP as aggregation agent. Clopidogrel reduces ADP-induced aggregation, and an impedance value of >=7 ohm was set as the cutoff defining clopidogrel non response.
Results: Dependent on pretreatment with 100 mg ASA as a single dose (long-term dosage), and dependent on time intervals of 3, 6 and 24 hours after the intake of 450 mg clopidogrel aggregometric analysis showed the expected inhibition of platelet aggregation by impedance values of <7 ohm in 59 (76), 76 (94) and 86 (94) percent of the patients with PCI. No adequate platelet response (impedance value >=7 ohm) to clopidogrel exhibited 8 of the 99 PCT patients, and 6 of whom additionally had an ASA resistance, which was documented by CT values =<170 s in the PFA-100® analysis.
Conclusion: Our results supply the evidence of clopidogrel non responders in 8 percent of patients with cardiovascular disease. In addition, in some of these patients non response to clopidogrel was associated with ASA resistance. Therefore, diagnostic monitoring of the efficacy of an antiplatelet treatment with clopidogrel and ASA in patients undergoing PCI should be taken into account.

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***CURRENT DEVELOPMENTS IN NEW ANTITHROMBOTIC DRUGS, LIMITATIONS AND SCOPE**

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Many new antithrombotic agents are under development. These include oral heparins, new inhibitors of F. IIa, VIIa and Xa, new glycosaminoglycans, orally active thrombin inhibitors, thienopyridine compounds, orally active GP IIb/IIIa receptor antagonists and other platelet function inhibitors. New antithrombotic agents have chances if they improve current treatment regimens. A special need for new drugs exists in high risk surgery where a reduction of the thrombosis incidence below 3% might be achieved and in acute coronary syndromes, where the occlusion rate and rethromboses after stent implantation might be reduced. For long-term antithrombotic treatment vitamin K-antagonists, which need monitoring and have a relatively high risk of bleeding, could be replaced by safer drugs which need less monitoring. There still is a hope that antithrombotic drugs could be effective in stroke or in peripheral arterial occlusive disease (PAOD). New antithrombotic agents should have advantages at least in some limited indications. During the development of new agents extensive monitoring is strongly advised. It may not be necessary later but monitored patient groups will reveal whether under- or overdosing are responsible for reduced efficacy or bleeding complications.
Sufficiently large and well designed dose finding studies are essential to avoid negative experiences like those with hirudin in acute coronary syndromes. It is likely that in the future drug combinations will be used in many indications. The growing combined use of aspirin and clopidogrel is a good example. Other combinations, especially of existing drugs with new agents, may increase efficacy and safety in many indications. It is conceivable that new antithrombotic agents will only be used in combinations. Such combinations could lead to a more effective prevention of arterial occlusions in PAOD and coronary heart disease, where aspirin at present is still used worldwide. Interaction studies will be increasingly important in the future. They are essential for drug combinations but they should also cover frequently expected overlapping treatments.

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***TREATMENT OF ACUTE DEEP VEIN THROMBOSIS WITH THE LOW-MOLECULAR-WEIGHT HEPARIN, REVIPARIN – RESULTS OF THE CORTES STUDY**

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In the CORTES trial, 1137 patients with an acute deep vein thrombosis (DVT) were randomised to three different regimens: intravenous unfractionated heparin (UFH) (group A), twice daily low molecular weight heparin (reviparin) for one week (group B) or once daily reviparin for four weeks (group C). All patients received vitamin K-antagonists, but patients in group C started oral anticoagulation at Day 21. Primary end-

point was thrombosis regression assessed by phlebography. Secondary endpoints were recurrent venous thromboembolism, mortality and major bleeding. Blood samples withdrawn at base line, weeks 1 and 3 were analysed using markers of in vivo thrombin generation, TFPI release and standard coagulation parameters. During the first three weeks symptomatic recurrent DVT/PE occurred in 17 of 375 patients (4.5%) in group A compared to 4 of 388 patients (1.0%) in group B and 9 of 374 patients (2.4%) in group C. After 21 days 40.2% of patients in group A, 53.4% in group B and 53.5% in group C showed a 30% or greater reduction in thrombus size. Group B patients had significantly greater reduction in D-dimer, prothrombin F1+2, ETP levels and TAT complexes compared to groups A and C. TFPI release and reduction of fibrinogen were significantly more pronounced in group C. Non-responders at baseline had significantly higher fibrinogen, TAT and F1+2 values. At weeks 1 and 3 thrombin generation was less inhibited in group C. The inhibition of thrombin generation was strongest in group B. If all patients independent of the treatment regimens are considered significant differences were found: At base line but also after 1 and 3 weeks non-responders had significantly higher values of fibrinogen, TAT complexes and F1+2 than responders. In addition in group C also significant differences of responders and non-responders were observed at weeks 1 and 3: Non-responders had higher values of fibrinogen, TAT, F1+2, ETP and D-dimers. Reviparin administered twice daily plus vitamin K-antagonist is more effective in inhibiting in vivo thrombin generation compared to i.v. UFH plus vitamin K-antagonist. But reviparin once daily without initial oral anticoagulation strongly affects fibrinogen and increases TFPI. Whether the observed changes in coagulation parameters are causally related with clinical or phlebographic effects remains an interesting question.

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***MITOGENIC EFFECTS OF THROMBIN AND FACTOR XA ON HUMAN VASCULAR SMOOTH MUSCLE CELLS**

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At sites of vascular injury the coagulation cascade is activated resulting in the generation of factor Xa (FXa) and thrombin. Besides their key function in thrombus formation both coagulation enzymes also elicit cellular responses. In the present study FXa- and thrombin-induced intracellular signaling and mitogenic effects in vascular smooth muscle cells of the human saphenous vein (SMC) were investigated. mRNA of protease-activated receptors (PARs) was detected by RT-PCR. Increase in intracellular calcium was measured by confocal laser scanning microscopy. Activation of ERK-1/2 was detected by Western blotting and increase in DNA-synthesis was determined by measuring [^{3}H]-thymidine incorporation. Stimulation of the cells by FXa (10-100 nM) elicited a concentration-dependent activation of ERK-1/2 and an increase in [^{3}H]-thymidine incorporation. Both effects were inhibited by the FXa inhibitor DX-9065a and were found to be independent of endogenous release of PDGF from the SMC. Investigations on thrombin-induced cellular effects revealed that in addition to the well known thrombin receptor PAR-1, the recently cloned PAR-4 is also present in SMC. Treatment of the cells with the PAR-4-activating peptide GYPGQV (200 µM) induced a transient increase in intracellular calcium. Low concentrations of thrombin (10 nM) and the PAR-1-activating peptide SFLLRN (200 µM) activated ERK-1/2 with maximum effect after 5 min. Higher thrombin concentrations (100 nM) as well as GYPGQV (200 µM) phosphorylated ERK-1/2 with a more prolonged time course with a maximum at 60 min. Thus, it is suggested that PAR-1 and PAR-4 are activated by thrombin at distinct concentrations and with distinct kinetics. Similar to thrombin and SFLLRN, GYPGQV also induced a mitogenic effect. Incubation of cultured SMC with recalcified platelet poor plasma resulted in a significant thrombin generation in the cell supernatant. SMC-mediated thrombin generation was inhibited by anti-tissue factor antibody, tissue factor pathway inhibitor, inactivated factor VIIa and DX-9065a. These data indicate that the generation of FXa and thrombin by SMC may contribute to thrombus formation and intimal hyperplasia.

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***SAFETY ASPECTS OF THE USE OF LOW-MOLECULAR WEIGHT HEPARINS IN PATIENTS UNDERGOING MAJOR ORTHOPAEDIC SURGERY**

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The use of anticoagulants to reduce thromboembolic events is limited by potential bleeding effects. In major orthopaedic surgery using UFH the still high rate of thromboembolic events, which is about 30 to 35 per cent, can be reduced by application of higher doses of LWMH. Half of the DVT are found in proximal veins, which are responsible for pulmonary embolism. Reduction of these thromboses therefore is of more importance than the reduction of the total DVT. The long-term consequences of distal DVT are not finally clarified.
Therefore it seems to be more important to reduce the clinically important thromboembolic events which are proximal DVT, fatal and non-fatal thromboembolism.
In former studies it has been demonstrated that the LWMH are individual substances. Raising the dose of the drug may be limited by the occurrence of major bleeding during and after operation. Therefore, it is necessary to regard in clinical trials not only efficacy, but also the side effects of an anticoagulant. Every severe adverse event has to be analyzed by an independent board whether it is potentially drug related and a study has to be stopped when there is an important indication that there are influences of the drug. As recent studies have shown that major bleeding is relative rare using LMWH a higher bleeding rate under a modified dose of a LMWH can only be detected by a suffient number of patients. In the recent studies this safety aspect has been more considered, although it is difficult to find an objective definition of “severe bleeding”, which can be accepted for all anticoagulant studies. Proposals will be made basing on the results of some newer trials.

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RELEASE OF MONOCYTE CHEMOTACTIC PROTEIN-1 FROM ENDOTHELIAL CELLS IS STIMULATED BY HUMAN ACTIVATED PROTEIN C – AN IN VITRO SEPSIS MODEL

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Background: Monocyte chemotactic protein-1 (MCP-1) plays a critical role in recruiting monocytes to stressed endothelium as an early response to vascular injury. The activated protein C (APC) pathway has been suggested to be a common link between coagulation and inflammation. In addition to its anticoagulant properties, APC may also function to restore hemostasis via cytokine/chemokine induction, thereby amplifying the local inflammatory reaction. This has been supported by reports showing that APC could induce the production of interleukin-6 (IL-6) and IL-8 in endothelial cells. The aim of this in vitro study was to investigate the effect of APC on the release of chemokine MCP-1 in human umbilical vein endothelial cells (HUVEC).
Methods: HUVEC were treated with human APC (2.5-10 µg/ml; from two different commercial sources) either alone or in combination with tumor necrosis factor-alpha (TNF-alpha 0.1-1 ng/ml). After an incubation period of 2-24 h, MCP-1 was analyzed in supernatants by commercially available ELISA-kits and MCP-1-mRNA was determined in total cell lysates by a colorimetric mRNA quantitation assay (Quantikine-assay®). Statistical analysis was performed by ANOVA.
Results: APC stimulated MCP-1-gene transcription and MCP-1-protein-synthesis in a time and dose dependent manner: MCP-1-gene transcription was up-regulated compared to controls after 2 up to 8 hours of incubation with APC. MCP-1 was detected in HUVEC-supernatants as early as 4h after the addition of APC and continued to increase through the 24 h incubation period ($p<0.01$ for APC 2.5ug/ml). TNF-alpha stimulated mRNA transcription and release of MCP-1 after 2 hours of incubation ($p<0.001$ for TNF-alpha 0.1 ng/ml). HUVEC pretreated with APC for one hour (2.5-10 µg/ml) strongly enhanced the TNF-alpha-stimulated MCP-1-release ($p<0.01$). Recombinant hirudin (100 µg/ml) and Polymyxin B (10 µg/ml) had no effect on the APC-stimulated MCP-1-release indicating that the effect of APC was not due to LPS- or thrombin-contamination of the APC preparation.
Conclusion: By up-regulation the production of MCP-1 in endothelial cells - at both the transcriptional and protein level - APC contributes to leukocyte trafficking and adherence at the site of vascular injury. Thereby APC may aid in the local inflammatory reaction at the endothelium, initiate wound repair and may contribute to the host`s response to infection.

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MODULATION OF THE ANTICOAGULANT EFFECTS OF UNFRACTIONATED AND LOW MOLECULAR WEIGHT HEPARIN BY HYDROXYETHYL STARCH

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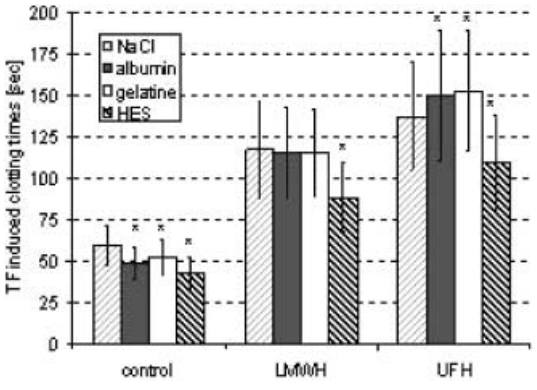
While intravascular volume replacement solutions are often used simultaneously with various anticoagulants, there has been no evaluation concerning their interaction in literature. As several effects of hydroxyethyl



starch (HES) on various coagulation proteins have been described, interactions of hemodilution and anticoagulation are possible. We examined the interaction of different volume replacement solutions with unfractionated heparin (UFH) and low molecular weight heparin (LMWH) in vitro. Citrated plasma from 36 patients was diluted in vitro (by 40%) using NaCl 0.9%, 5% albumin, 3.5% gelatin or 6% HES solution (molecular weight 200.000). UFH (0.5 U/ml) or LMWH (Dalteparin, Pharmacia, 1 anti-factor Xa U/ml) or NaCl 0.9% (control) were added. Coagulation was triggered using 5 ng recombinant tissue factor/ml. Clotting was detected optically. Hemodilution with HES, albumin or gelatin significantly accelerated coagulation activation when compared to NaCl. Hemodilution with HES shortened the clotting times of the UFH and LMWH samples (versus NaCl, albumin and gelatin). We conclude that a decreased anticoagulant effect of UFH, LMWH and endogenous antithrombin occurs during hemodilution with HES, when compared to NaCl, gelatin and albumin. This effect could compensate for the anticoagulant effects of HES and thus contribute to its clinical safety.

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AUTOSOMAL DOMINANT INHERITED GIANT PLATELET SYNDROMES; GENOTYPING OF GENETIC DEFECTS ON NONMUSCLE MYOSIN HEAVY CHAIN 9

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Background: Giant platelet syndromes (GPS) consist of a heterogenous group of autosomal dominant inherited syndromes with haematological manifestations and minor bleeding tendency. In Sebastian platelet syndrome (SPS), May-Hegglin anomaly (MHA), and Fechtner syndrome (FS) the triad of macrothrombocytes, thrombocytopenia, and granulocyte inclusion bodies occurs. In addition, FS patients suffer from nephritis, cataract and neurosensorial deafness. Recently chromosome 22 could be associated with GPS and the heavy chain of nonmuscle myosin 9 (MYH9) was identified as the disease causing gene.
Methods: Sequence-specific primer (SSP)-PCRs were developed for 12 point mutations on MYH9 associated with macrothrombocytopenia. Patients were diagnosed for SPS, MHA and FS after assessing the family history of bleeding tendency, macrothrombocytopenia, granulocyte inclusion bodies (May-Grünwald-Giemsa staining, electron microscopy), nephritis, cataract, and neurosensorial deafness.
Results: 12 unrelated families were investigated using SSP-PCR. 6 different mutations were found in 8 of the investigated families as follows; SPS(4): D1424N(3), no mutation(1); MHA(2): D1424H(1), no mutation (1); FS(2): R702C(1), R702H(1); MHA/SPS(4): K371N(1), R1933X(1), no mutation(2). The mutations were only found in heterozygote form. In 5 families relatives of the index patient were assessed (16 affected and 7 unaffected individuals), and in all cases only the affected individuals carried mutations of MYH9.
Discussion: The genetic defect in inherited macrothrombocytopenia was localised on MYH9 in 19 individuals of 8 unrelated families. In 4 families none of the known mutations was found, indicating the presence of further point mutations on MYH9. Importantly, all index cases were initially wrongly diagnosed and treated as chronic autoimmune-thrombocytopenia, several of the index patients underwent splenectomy unnecessarily. The presented SSP-PCR methods enable rapid identification of these missense mutations and will further improve differential diagnosis of chronic thrombocytopenia.

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INHIBITION OF PLATELET ADHESION BY THE SEQUENCE H483-K502 OF DOMAIN 5 OF HIGH MOLECULAR WEIGHT KININOGEN

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Proteolytic cleavage of single chain high molecular weight kininogen (HK) by kallikrein releases the short-lived vasodilator bradykinin and leaves behind two-chain high molecular weight kininogen (HKa). HKa and particularly its His-Gly-Lys-rich domain 5 have been previously reported to exert anti-adhesive properties by binding to the extracellular matrix protein vitronectin (VN). In this study the ability of HKa and domain 5 to interfere with platelet adhesion was investigated. In a purified system HKa but not HK and particularly domain 5 inhibited the binding of VN to the alpha(IIb)beta(3)-integrin, whereas the binding of fibrinogen (FBG) to this integrin was not affected. Moreover, the sequence H483-K502 from HK domain 5 was identified as responsible for inhibition of the VN/alpha(IIb)beta(3)-integrin interaction, as this portion was also found to mediate kininogen´s binding to VN. Through these interactions, HKa, the isolated domain 5, and the sequence H483-K502 abrogated the alpha(IIb)beta(3)-integrin-dependent adhesion of human platelets as well as the alpha(v)beta(3)-integrin-dependent adhesion of different megakaryoblastic cell lines to VN but not to FBG. Codistribution of VN and HK at sites of fibrin polymers within platelet thrombi was demonstrated by transmission electron microscopy, indicating that the described interactions are likely to take place in vivo. Taken together, our data emphasize the regulatory role of HKa in platelet adhesion to a provisional wound matrix and new strategies for platelet antiadhesion therapy could be based on this antithrombotic action of HKa.

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PROTEIN S ACTIVITY VS. FREE PROTEIN S ANTIGEN DETERMINATION: CORRELATION TO FACTOR V LEIDEN STATUS AND FACTOR VIII:C ACTIVITY

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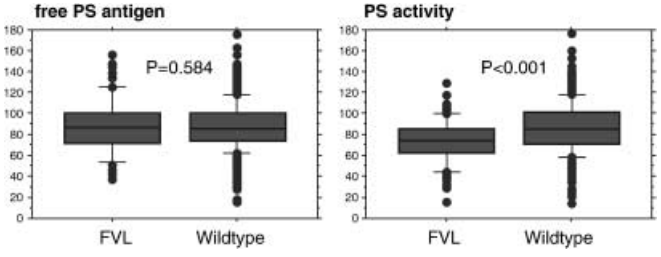
Protein S (PS) deficiency is an inherited risk factor for thromboembolic events. Different methods are available for the laboratory determination of PS. PS activity tests are known to have several analytical problems and a limited specificity. We evaluated the influence of the factor V Leiden (FVL) mutation and the FVIII:C activity (FVIII:C) on PS activity, free PS antigen and their ratio.
Methods: Citrated plasma and EDTA blood from 735 thrombophilia patients (no oral anticoagulant therapy) was analyzed. PS activity was determined with the Staclot PS assay (Stago). Free PS antigen was determined with the Asserachrom free PS ELISA (Stago). The FVL status was determined using the Coatest APC Resistance test (screening) and PCR analysis (confirmation). FVIII:C was determined using an aPTT-based method (Instrumentation Laboratory).
Results: 556 patients had a wild type FV, 164 a heterozygous FVL mutation and 6 patients had a homozygous FVL mutation. The correlation coefficient of PS activity and free PS antigen was 0,613. While there was no significant difference of free PS antigen between FV wildtype and FVL carriers (p=0,5836, ANOVA), significantly higher PS activity levels were determined in the FV wildtype patients vs. the FVL carriers (p <0.001, ANOVA). Correlation coefficient of FVIII:C vs. free PS antigen=0.089, FVIII:C vs. PS activity= -0.157, FVIII:C vs. PS activity/free PS antigen= -0.325.
Conclusion: While FVL and FVIII:C had no significant influence on free PS antigen, there was a significant correlation vs. PS activity and the ratio of PS activity/free PS antigen. For the determination of the PS status both the PS activity and free PS antigen should be assessed.



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RADIOACTIVE PLATELET LABELLING: 111IN-OXINATE OR 99MTC-HMPAO

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The kinetic and in vivo-behaviour of 111In-labelled platelets are well documented. 99mTc-HMPAO-labelling of platelets was described in clinical studies of thrombodiagnosis. But there are only few data about kinetics of 99mTc-labelled human autologous platelets in clinical investigations.
Methods: We analysed the results of kinetic investigations of 70 patients. The platelets were isolated and labelled with 370-550 MBq 99mTc-HMPAO. The labelling procedure was according to the method described for oxinate. We determined the labelling efficiency (LE) in vitro, the recovery over 24 hrs and kinetic data after reinjection by dynamic anterior and posterior images including heart, liver and spleen (double-head camera Bodyscan, Siemens). Blood samples were obtained at 5, 10, 20, 60, 120 minutes and twice daily for two days. The results were compared with those of 15 patients, investigated with 111In-oxinate to diagnose active venous thrombi.
Results: The LE of 99mTc-HMPAO labelled platelets was 70+/-7%, in case of 111In-oxinate 92+/-4%. There was no difference in the early in vivo platelet kinetic in spleen, liver and blood, but there was significant difference in the disappearance-rate of labelled platelets: 26+/-7 hrs (99mTc-HMPAO); 60+/-30 hrs (111In-oxine).
Conclusions: Because of the high activity (MBq per thrombocyte) the 99mTc-HMPAO procedure is ideal for kinetic measurements over two days, one example is the thrombodiagnosis. Investigations longer than 3 days need the low-activity 111In-labelling procedure. Further investigations are necessary to compare the 99mTc-HMPAO labelling of isolated autologous cells with a procedure without cell isolation (receptor-labelling with help of P 280 - ACUTECT(TM)).

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EFFECTS OF TISSUE FACTOR PATHWAY INHIBITOR AND ANTITHROMBIN ON THROMBIN GENERATION IN CORD AND ADULT PLASMA

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Background: Despite of low concentrations of clotting factors, newborns have an excellent haemostasis. Thus, it was the aim of our study to investigate weather low concentrations of the anticoagulatory proteins tissue factor pathway inhibitor (TFPI), and antithrombin (AT) compensate for low levels of procoagulatory proteins. In contrast to the routine determinations of clotting parameter, clotting was initiated by applying low concentrations of tissue factor (TF).
Material and Methods: Concentrations of inhibitors were increased by administration of purified concentrates or decreased by addition of the respective antibodies. TFPI levels were determined by means of the ImubindTM Total TFPI ELISA kit. Factor Xa- and thrombin generation were measured by means of subsampling techniques. Results: Determination of the physiologic TFPI level in cord plasma: The cord plasma level is 42% of adult value. Effect of different amounts of TF on clotting time: When high amounts of TF (more than 30 pM) are applied as a trigger for coagulation, adults have shorter clotting times than newborns. Under low procoagulant challenge (TF below 30 pM), to the contrary, neonates have shorter clotting times. Effect of TFPI and AT on FXa- and F IIa-generation: Augmentation of TFPI- and AT-levels in cord plasma to adult values results in retarted and decreased FXa- and FIIa-generation. This effect increases when the amount of TF used to initiate coagulation is successively lowered.
Conclusions: Under a mild stimulus, cord plasma clots earlier than adult plasma, and, correspondingly, FXa- and FIIa-generation starts earlier. Since we have shown in the present study that the levels of TFPI and AT have a marked influence on FXa- and FIIa-generation, we conclude that the physiologic low levels of this two inhibitors allow sufficent thrombin generation in cord plasma despite of low procoagulants to provide good hemostasis in the newborn.
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ANALYSIS OF THE ANTITHROMBIN GENE OF 22 PATIENTS WITH SUSPICION OF HEREDITARY ANTITHROMBIN DEFIENCY

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Antithrombin (AT) plays a major role in regulating haemostasis by inhibiting procoagulant serine proteases such as thrombin and factor Xa. Its (functional) deficiency is a strong risk factor for venous thrombosis. We investigated the molecular and genetic basis of 22 patients with moderate AT deficiency. The Coamatic LR Antithrombin assay (Chromogenix-Instrumentation Laboratory SpA) was used for the quantitative determination of the heparin cofactor activity of AT in human citrated plasma. Gene analysis comprises the whole translated region including all exon-intron boundaries of the AT gene. DNA sequencing was performed on both strands using nested primer for the amplified products, dye-deoxy terminator method and an automated 310 DNA sequencer (Applied Biosystems). We detected two new and two known mutations in our patient group. New mutations: The first single amino acid substitution (Ala54Val, 3159G>A) is identified in exon 2 of the AT gene (H. sapiens gene for antithrombin III, GenBank Accession X68793). The base pair exchange was detected in heterozygous form in two patients with an AT activity of 64% and 71%. The second single amino acid substitution (Cys21Arg, 3059T>A) is located in exon 2 of the AT gene in heterozygous form. The measured AT activity of the patient was 53%. Known mutations: In a patient with an AT activity of 35% we detected only one homozygous base pair exchange in exon 3a (Arg129Gln, 5916G>A, AT Geneva). The amino acid substitution Arg129Gln (AT Geneva) is part of the AT region which contains the highest portion of basic residues, and is known from chemical modification studies to be involved in heparin binding. The second known mutation in heterozygous form was found in the AT gene of a patient with an AT activity of 63% (Pro407Thr, 13871C>A). Point mutations in and immediately adjacent to strand 1C have pleiotropic effects on AT, leading ultimately to failure of its regulatory function.
Besides two known mutations we could identify two novel mutations in regions near the heparin binding domains and in conformational important domains. The N-terminale binding site of heparin is located five amino acids before the Ala54Val mutation. It might have an impact of the heparin binding activity of AT protein. The Cys21Arg mutation disables one (Cys-21-Cys-95) of three disulphide linkages in the AT protein and could change the conformation of the AT protein and influence the domains of biological activities.

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A DIRECT AUTUMATED ASSAY FOR FREE PROTEIN S ON HITACHI 911: IL TEST FREE PROTEIN S

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Protein S is a vitamin K-dependent, potent natural anticoagulant plasma glycoprotein. Protein S has a molecular weight of approximately 70 kDa and circulates in plasma in two forms. About 40% of Protein S in plasma is in a free form, whereas 60% is complexed with C4b-binding protein. Only the free Protein S functions as a cofactor to APC in the APC-dependent degradation of FVa and FVIIIa. Protein S defiency is associated with an increased risk of thrombosis.
We have adapted the IL Test Free Protein S on Hitachi 911 (Boehringer Mannheim) and have compared the results of plasmas from patients with COALIZA PROTEIN S – FREE (Chromogenix). Both methods base on a procedure described by Dahlbäck and colleagues: Free protein S in plasma is captured by C4b-protein that is immobilized on latex beads (IL) or in microtiter plates (Chromogenix) and then recognized by a monoclonal antibody immobilized on latex beads (IL) or enzyme labeled (Chromogenix). Latex agglutination correlated to the concentration of free protein S (IL) or the amount of colour in the well after addition of substrate-chromogen (Chromogenix).
We found stable calibration from day to day (done for three days) for the IL Test Free Protein S on HITACHI 911. The standard deviation and variation coefficient for three control plasmas in precision (n=10) was 1.29 and 1.48 (median free Protein S:87.4%), 3.33 and 5.84 (median free Protein S: 57.0%), 2.18 and 11.28 (median free Protein S: 19.3%). We investigated 29 plasmas from patients with IL TestTM Free Protein S on Hitachi 911 (variable y) and COALIZA PROTEIN S – FREE (Chromogenix, variable x) in comparison. The Passing Bablok regression calculates the following constantes in the comparision with the two methods: y=1.458× – 15.667, n=29. The IL TestTM Free Protein S on Hitachi 911 leads to slightly increased concentrations in comparision with the COALIZA PROTEIN S – FREE.

In conclusion the IL Test Free Protein S on Hitachi 911 represents a simple, faster and totally automated method. It correlates reliably with established methods measuring a wide range of clinical samples with different free Protein S levels.

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***PREVENTION OF STROKE AND SYSTEMIC EMBOLISM IN PATIENTS WITH ATRIAL FIBRILLATION USING XIMELAGATRAN A NOVEL ORAL DIRECT THROMBIN INHIBITOR**

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Ximelagatran is a novel oral direct thrombin inhibitor that shows a highly reproducible pharmacokinetic and no significant food/drug or drug/drug interactions. Hitherto anticoagulants such as warfarin are used to lower the risk of stroke and systemic embolic events in patients with nonvalvular atrial fibrillation (NVAF).
In the SPORTIF-study programme the tolerability and safety of Ximelagatran was tested. This programme consists of five clinical studies including one pharmacokinetic and one dose guiding study, two identical large-scale efficacy studies (one in Europe and one in US) and a long-term safety trial.
In the SPORTIF II study, the tolerability and safety of three doses of Ximelagatran were compared with warfarin in NVAF patients with medium or high risk for stroke. A total of 257 patients were randomised to treatment and 254 patients received study drug ranging from 20 mg to 60 mg.
Results: all fixed doses of Ximelagatran were well tolerated with a most favourable benefit/risk ratio for 36 mg bid. There was no need for dose adjustment or routine coagulation monitoring, during a 3-month treatment period in NVAF patients with medium or high risk for stroke.
SPORTIF IV is an open-label continuation of SPORTIF II. Patients with NVAF and at least one additional stroke risk factor received either a fixed dose of Ximelagatran (36 mg bid) or warfarin (dose-adjusted to an INR of 2-3). Patients have been treated up to 24 months and the study is still ongoing. No coagulation monitoring was performed or required with Ximelagatran, and no intracerebral or fatal bleeds occurred, yet.
The large scale efficacy studies SPORTIF III and SPORTIF V are ongoing and results are expected at the end of 2002.
These data suggest that Ximelagatran seems to be promising as an effective and well-tolerated agent for long-term anticoagulation in order to prevent stroke and systemic embolism in NVAF patients. Further clinical studies are ongoing.

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APPLICATION OF A SOLUBLE TNF RECEPTOR AMELIORATES CARDIAC AND RENAL END-ORGAN DAMAGE IN ANG II-DEPENDENT HYPERTENSION INDEPENDENT OF BLOOD PRESSURE: AN IMPORTANT INTERACTION BETWEEN ANG II AND TNFA SIGNAL TRANSDUCTION PATHWAYS

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We recently reported that rats harboring both human renin and angiotensinogen genes (dTGR) feature moderate hypertension, severe renal and cardiac damage, and a 50% mortality at 7 weeks. We also showed that TNFα is increased in both hearts and kidney by western blot and immunohistochemisty in this model of angiotensin II (Ang II) induced end-organ failure. Rats over expressing TNFα develop chronic heart failure associated with severe leukocyte infiltration in both the atria and the ventricles. Moreover chronic treatment with a soluble TNFα receptor (sTNF-R) in patients with heart failure resulted in a significant dose-dependent improvement in LV structure and function and a trend toward improvement in patient functional status. Thus, we tested the hypothesis that TNF signaling is involved in Ang II-induced cardiac and renal damage. We treated rats chronically with sTNF-R for 3 weeks. sTNF-R had no effect on blood pressure (211±8 vs. 194±7 mm Hg, Sprague Dawley rats (SD): 110±1 mm Hg), however cardiac hypertrophy index was reduced (5.64±0.13 vs. 4.75±0.09 mg/g, $p<0.05$, SD: 3.5±0.05 mg/g) compared to dTGR. sTNF-R reduced 24 h albuminuria by 50 % (37.3±8.1 vs. 16.9±3.2 mg/d, $p<0.05$, SD: 0.2±0.02 mg/d). Vasculopathy and perivascular cardiac fibrosis were markedly ameliorated by sTNF-R. Immunohistochemical analysis showed increased infiltration of monocytes and T-cells in dTGR which was significantly reduced by sTNF-R. sTNF-R was only partially effective in the kidney. Electrophoretic mobility shift assay showed increased NF-kB and AP-1 DNA binding activity in heart and kidney of dTGR. The DNA-binding activity of both transcription factors was reduced by sTNF-R in heart and kidney. Immunohistochemical analysis shows increased expression of the p65 NF-kB subunit in dTGR that was reduced by sTNF-R treatment. Similarly, immunohistochemistry showed that the TNF-regulated adhesion molecule ICAM-1 was reduced in heart in the sTNF-R treated compared to untreated dTGR. These results demonstrate that TNF signaling is involved in Ang II-mediated end-organ damage in vivo. Blocking TNFa signaling does not reduce Ang II induced hypertension in vivo. sTNF-R reduced NF-kB and AP-1 DNA binding activity and reduced cardiac damage independent of blood pressure.

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COMPARISON ON THE PERFORMANCE OF DIFFERENT COMMERCIAL FIBRIN SEALANT PREPARATIONS IN EXPERIMENTAL SURGERY AND BY IN VITRO SPAY METHODS

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Background: The main elements of fibrin sealants (FS) fibrinogen and thrombin are present in all products, however, they vary significantly concerning presentation, additives, concentration of the active components, addition of antifibrinolytic agents or factor XIII and the device used for application. The aim of this investigation was to compare the properties of FS from 8 different companies with their appropriate devices in vitro and in vivo.
Methods: FS were investigated 1. for their in-vitro spray patterns (homogeneity of mixture and droplet size), 2. for their efficacy to induce hemostasis after partial liver resection, a rabbit model for oozing bleeding 3. in a partial kidney resection model in rabbits where hemostatic as well as sealing properties of a FS are requested. The individual sealants were coded with a capital letter from A–H (company) and a number (different FS from the same company) followed by the device abbreviation (s=manual spray tip, a=air assisted spray, n=needle).
Results: It has been found that device construction is crucial for achieving good spray patterns and differed significantly between the individual devices. Especially manual devices with two separate spray nozzles appeared to be unfavourable. In the partial liver resection model immediate cessation of oozing bleeding in 100% of animals was achieved with FS A1(s), A1(a), A2(s), B4(a), B3(s) and H1(s). With the remaining FS the following data were obtained: B1(a), A2(a2), E1(a): 90%; A2(a1): 80%; B2(a), G1(n): 70%; E1(s): 60%; D1(a): 40%; F1(a): 30%; and C1(a): 20%. Good mixing properties by the application system and a sufficient fibrinogen concentration in the FS was shown to be prerequisite to stop oozing bleeding. A study in the kidney resection model was performed with a limited amount of FS, A1(s), B1(a), B2(a), B3(s), D1(a), F1(a) demonstrated that the presence of factor XIII is essential to prevent the occurrence of rebleeding.
Conclusions: Although all commercial FS share the same basic principle it was concluded that they vary considerably in their device performance and their efficacy to achieve hemostasis. Crucial for a successful FS is a device with excellent mixing properties and the composition of the FS, especially the presence of factor XIII.

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DETECTION OF COAGULATION ABNORMALITIES IN A RAT SEPSIS MODEL BY THROMBELASTOGRAPHY AND INFLUENCE OF FIBRINOGEN CONCENTRATE SUBSTITUTION

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Background: Sepsis is a disorder frequently associated with disseminated intravascular coagulation (DIC), one of its lead symptoms is the decrease in plasma fibrinogen. In this study we used the thromboelastography to define the coagulation abnormalities in rat sepsis model. To improve coagulation abnormalities, we substituted with a fibrinogen concentrate (Haemocomplettan®P).
Methods: A sepsis associated with a severe DIC was induced in female rats by the i.v. injection of 40 mg/kg bacterial lipopolysaccharide (LPS). Fibrinogen plasma levels were determined by CTS Fibrinogen (Dade Behring) and abnormalities in the coagulation system by thrombelastography (Haemascope Thrombelastograph). Haemocomplettan®P was administered in doses at 25, 50, 100 and 200 U/kg i.v.
Results: The administration at LPS induced a drop of plasma fibrinogen from 2.1±0.2 to 0.2±0.1 g/l. The DIC was associated with severe alternation in coagulation: Maximum amplitude (MA) of the thrombelastography dropped from 74.1±22 to 9.3±7.9 mm, reaction time (RT) increased from 205±62 to 648±274 seconds. Substitution with Haemocomplettan®P normalised fibrinogen plasma levels and the coagulation abnormalities (see table below).
Conclusion: Thromboelastography is a useful tool to detect a coagulopathy associated with sepsis. The administration of a fibrinogen concentrate could normalise fibrinogen plasma levels and improve the severe DIC.

Haemocomplettan (U/kg i.v.)	Fibrinogen plasma level (g/l)	MA (mm)	RT (sec)
Non-septic control	2.1±22	74.1±22	205±62
Sepsis control	0.2±0.1	9.3±7.9	648±274
25	0.37±0.06**	33.8±7.2**	325±104.7*
50	0.61±0.08**	33.4±6.0**	326.5±42.3*
100	1.18±0.31**	55.9±10.6**	234±35.6*
200	1.97±0.37**	55.1±11.8**	270±59.5*

* p<0.001, ** p<0.0001 (two-sided t-test)

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PREOPERATIVE PROLONGATION OF APTT CAUSED BY PREKALLIKREIN (FLETCHER FACTOR) DEFICIENCY

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Background: The prolongation of aPTT indicates a hemostatic dysfunction and has to clarify before elective surgery. An accurate differential diagnosis is necessary for therapeutic decisions. Mostly deficiencies of coagulation factors or pathological inhibitors are responsible for aPTT-prolongation, but prekallikrein deficiency has to be considered as a possible cause in rare causes too. Following we report about a patient with preoperative aPTT - prolongation which was explained as lupus anticoagulant-inhibitor in an outward laboratory.
Methods: We examined a 24-year old male patient of croation nationality who had to be sub-jected horseshoe-kidney surgery. APTT was performed by using an assay obtained from Roche-Diagnostics, Mannheim, Germany. Prekallikrein-activity was determined using one-stage assay (Sigma-Aldrich, Taufkirchen, Germany). The quantitative determination of prekallikrein was performed by enzyme-linked immunosorbent assay (ELISA) using antibodies obtained from Affinity Biologicals, Ontario, Canada. Measurement of high molecular weight kininogen was performed employing a Fitzgerald trait plasma (Progen, Heidelberg, Germany).
Results: In the preoperative state the patients aPTT was prolonged to 46.0 seconds. Other coagulation assays including fitzgerald factor activity were in the normal range. Prekallikrein-activity was strong decreased to less than 1 percent. The measurement of prekallikrein-concentration resulted in 40 mg/l comparable with amount of normal plasma suggesting an underlaying prekallikrein-deficiency type 2. Surgery was performed without remarkable bloodloss and without any complications.
Conclusions: With the evaluation and interpretation of clotting analysis, in case of aPTT -prolongation an prekallikrein deficiency, an even rare event, has to be taken into consideration. In this anomaly there is no increased risk of bleeding. After exclusion of all other possible causes of aPTT-prolongation the patient is operable without transfusion requirement.

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SYNDECAN-4 AS ANTITHROMBIN RECEPTOR OF HUMAN NEUTROPHILS

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Antithrombin inhibits chemokine-induced migration of neutrophils by activating heparan sulfate proteoglycan-dependent signaling. Mechanisms of antithrombin's effects on neutrophils were, therefore, studied by testing function and expression of heparan sulfate proteoglycans in RT-PCR or flow cytometry and cell migration assays, respectively. In vitro effects of antithrombin on human neutrophil migration in modified Boyden chambers were abolished by pretreating cells with heparinase-1, chondroitinase, sodium chlorate and anti-syndecan-4 antibodies. Expression of syndecan-4 mRNA and protein in neutrophils was demonstrated in RT-PCR and anti-syndecan-4 immunoreactivity assay, respectively. In the presence of pentasaccharide, antithrombin lost its activity on the cells. Data suggest that antithrombin regulates neutrophil migration via effects of its heparin-binding site on cell surface syndecan-4.

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***ROLE OF ENDOTHELIAL FUNCTION IN THE CORONARY CIRCULATION IN HUMANS**

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The endothelium provides a variety of important functions involved in the cardiovascular homeostasis. The assessment of endothelial function in humans has focused primarily on endothelial dependent vasomotion in response to the release of NO. In particular, clinical studies have evaluated vasomotor tone following changes in flow or stimuli for the release of NO from the endothelium, such as acetylcholine, substance P or serotonin (1). However, NO not only acts as a vasodilating substance but also affects other functions of the endothelium such as the adhesive properties of the endothelium with respect to the interaction with leucocytes and platelets (2). To critically review the role of NO in coronary endothelial dysfunction in humans the present review focuses on observations made by the *in vivo* assessment of endothelial dependent vascular responses. There is clear evidence that endothelial-dependent vasodilation is impaired in coronary artery disease and this functional alteration is associated with impaired myocardial perfusion and ischemia. Thus, improvement of endothelial vasodilator capacity is a clincially relevant target for therapy and may prevent clinical symptoms of ischemia. Moreover, there is a growing body of evidence that endothelial function has prognostic implications in terms of future cardiovascular events. Several interventions have been effective in restoring endothelial vasodilator responses such as lipid lowering, antioxidants or ACE-inhibitors. Further studies should be able to elucidate whether the overall clincially benefical effects of these interventions are related to improvement of endothial function which incudes much more than vasodilator capacity, i.e. attenuation of leucocyte adhesion, prevention of platelet aggregation or favoring profibrinolytic activity.

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FVIII DETERMINATION IN PLASMA AND CONCENTRATES: AN APPROACH TO ESTABLISH A TEST EQUALLY SUITED FOR BOTH MATRICES

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Background: The FVIII level in patients is determined by the one stage clotting test while concentrates must be tested by the chromogenic test according to EurPharm. Under certain circumstances these test systems lead to conflicting results. We have addressed this question by establishing an assay which may overcome this problem.
Materials and methods: Coagulation factors were purified from human plasma and activated where needed according to published procedures. The FVIII assay was applied to commercial FVIII concentrates as well as to plasmas from healthy donors and FVIII deficient plasmas reconstituted with FVIII. FVIII was determined in a FXa generation system using a FXa specific fluorogenic peptide substrate.
Results: We obtained evidence that FVIIIa functions optimally only for a very restricted time (less than normally needed to perform chromogenic assays). Therefore, similar to the situation in vivo, no preactivation of FVIII is applied. Rather FVIII- activation, FX- activation and FXa mediated substrate conversion are started simultaneously. Once a certain predilution is achieved, the assay gives comparable results with plasma and FVIII concentrates as the FVIII source. Interestingly, our assay shows dramatic differences when applied to the analysis of different FVIII deficient plasmas, e.g. immunologically and chemically prepared FVIII deficient plasmas. When reconstituted with FVIII, only the immunologically prepared deficient plasma exhibits a performance similar to normal plasma.
Conclusion: The assay described appears to be a suitable compromise between the two current tests applied to the analysis of the two different matrices. In particular, the described assay appears to be the first one considering that (and how much) FVIII must not only be present, but also how it is activated under physiological conditions.

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A PROSPECTIVE STUDY ON THE IMPACT OF HEPARIN-INDUCED THROMBOCYTOPENIA ON FATAL IN HOSPITAL PULMONARY EMBOLISM

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Background: Heparin-induced-thrombocytopenia (HIT) is a hypercoagulable syndrome associated with thrombosis and thrombocytopenia. This prospective study addressed the issue of whether unrecognized HIT might be a common explanation for fatal pulmonary embolism (PE).
Patients: Over a time period of 30 months, all consecutive patients at our university hospital in whom PE was found during autopsy were studied. Blood samples were taken from the cardiac chamber for further testing. Clinical details were taken from the patients' records.
Methods: The heparin-induced platelet activation test (HIPA) was performed as published. A positive result was defined as specifically positive, if platelet activation could be inhibited by the monoclonal antibody IV.3. An in-house ELISA with surface bound antigen consisting of PF4/heparin complexes was used as antigen test. Conjugate detecting IgG/A/M as well as monospecific conjugates were used.
Results: During the study period 80439 patients had been treated at our university hospital, 1262 deaths occurred of which 292 were autopsied. In 23 patients PE was found, of whom 20 were evaluable (15 female, 5 male, age 33-82 years (median 69)). This results in an incidence of 7.8% of lethal PE in the 292 autopsied patients or an adjusted incidence rate of 0.12%. Ten patients were from the surgical (8) or neurosurgical (2) departments, 1 from neurology and 9 from medical wards. All received heparin (UFH: 15, LMWH: 5) for an average of 13 days (3-32) before they died. 12/20 patients had a decrease in platelet count of >50% or to <100,000/µl. In the HIPA test one serum tested positive, whereas in the ELISA in none of the sera antibodies against PF4/heparin could be detected. The one patient, who was positive in the HIPA test, had a decrease in platelet counts >50%. The underlying disease was biliary duct carcinoma which may explain the thromboembolic complication.
Discussion: None of the 20 consecutive patients who died of PE while receiving heparin in our hospital, had evidence that HIT was the underlying cause. We thus assume, that screening for HIT-antibodies would not have prevented any of these deaths.

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DEVELOPMENT OF AN IN-HOUSE PF4/HEPARIN ELISA AND COMPARISON WITH THE ID-H/PF4 ANTIBODY TEST (DIAMED) AND FURTHER ANTIGENIC AND FUNCTIONAL ASSAYS FOR DETECTION OF HIT-ANTIBODIES

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Background: Heparin-induced thrombocytopenia (HIT) is an immune mediated complication of heparin treatment. Several in vitro assays are available to detect HIT-antibodies: functional assays require freshly prepared platelets, are time consuming and need trained laboratories; commercial immunologic tests based on ELISA technique are expensive. We developed a method for purification of platelet factor 4 (PF4) and an in-house ELISA for detection of HIT-antibodies.
Methods: PF4 was obtained from platelet concentrates on heparin-sepharose and was eluted using a sodium chloride gradient. Recombinant PF4 was produced by expression in E.coli. The purified PF4 was complexed with heparin in stochiometric amounts. The PF4/heparin-antigen was coated on microtiter plates for use in a sandwich-ELISA. The in-house ELISA was compared with 1) the ID-H/PF4 antibody test (DiaMed), 2) 14C-serotonin release assay, 3) HIPA-test, and 4) two commercial ELISAs.
Results: The in-house ELISA using human PF4 for the PF4/heparin-antigen showed a comparable sensitivity but higher specificity than commercial ELISAs in 100 HIT-antibody positive sera and in sera of 40 normal donors. No differences in specificity and sensitivity were observed between the in-house-ELISA, the ID-H/PF4 antibody test and the functional assays. The in-house-ELISA allowed the detection of HIT-IgG, -IgM, -IgA antibodies by using of monospecific conjugates. Recombinant PF4 was unsuitable for detection of HIT-antibodies because of high background signals.
Discussion: Our method allows a cost-effective extraction of human PF4 from outdated platelet concentrates, which is suitable for application in a diagnostic ELISA. The observed dependency of quality of PF4 from the extraction method could be a cause for described test differences in the commercial ELISAs in comparison to the reference methods. Furthermore, our in-house ELISA allows the differentiation of HIT-immunoglobulin classes which could resolve in prospective studies the role of these immunoglobulin classes in HIT.

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GLANZMANN THROMBASTHENIA AND ARTHROPATHY

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Glanzmann thrombasthenia is typically associated with mucocutaneous bleeding such as epistaxis, gingival bleeding or menorrhagia. We report here two patients with Glanzmann thrombasthenia showing an atypical pattern of bleeding: They both suffer from recurrent hemarthroses and arthropathy. The first patient, a 13 year old girl, had a fracture of the distal humerus at the age of 11 years. She wore a cast for three weeks. The patient subsequently presented at our hospital showing a severe elbow bleeding. Attempts to treat this joint bleeding by desmopressin and a single infusion of recombinant F.VIIa failed. As the joint function was severely impaired, an arthroscopic chondrolysis was inevitable to restore the joint function necessitating the infusion of platelet concentrates. Following this first hemarthosis recurrent bleeding episodes affecting the same joint occurred after minor trauma. These episodes could partially be treated by infusions of NovoSeven. The second patient is a 18 year old young woman who experienced a hemarthrosis of the ankle after minor trauma at the age of 8 years. The joint improved under therapy with desmopressin infusions but hemarthroses recurred in the following years. Both patients have developed considerable arthropathy. They show clinical symptoms such as a chronically swollen joint, impaired joint function and pain. Moreover, radiographic abnormalities can be demonstrated by X-ray and MRI scan. Apart from hemarthroses, both patients experienced significant mucocutaneous bleeding episodes. The diagnosis of Glanzmann thrombasthenia was confirmed in both patients by flow cytometry showing a nearby complete absence of the GPIIb/IIIa receptor (CD41 and CD 61). No additional bleeding disorder such as von Willebrand's disease could be found. Analysis of the underlying genetic defect is underway. Recurrent hemarthroses and chronic arthropathy causing considerable morbidity might complicate the clinical course of Glanzmann thrombasthenia at least in a subset of patients. A possible link between atypical phenotype and genotype remains to be established. New treatment strategies have to be sought to improve patient's outcome.

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METHODS AND RESULTS OF THE IMAGING OF VENOUS THROMBI BY LABELLED PLATELETS

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The current discussion about receptor imaging of active venous thrombi (Taillefer et al.; J.Nucl.Med.: 1999, 2029-2035) was the cause to analyse our data to diagnose fresh venous thrombi by labelled platelets. This could give some data to compare the two methods. At present P 280 is not available in Germany.
Methods: The autologous platelets were labelled with 10-15 mCi 99m Tc-HMPAO (70 patients) or with 0,5 mCi 111In-Oxinate (15 patients). After reinjection we performed dynamic studies (60 min.) over liver, spleen and heart to document the biological behaviour of the labelled cells. Whole-body pictures were done by a double-head camera (Bodyscan/Icon/Siemens) after 3 hrs and 24 hrs, in the case of 111 In-platelets additionally after 48 hrs. The reference methods for thrombodiagnosis were contrast venography and radionuclide venography as well as ultrasound.
Results: 35 patients were without any pathological platelet deposition, but 6 of them showed signs of an old venous thrombosis. From the 50 patients with local accumulation of the labelled platelets 43 investigations were done with 99m Tc-platelets and 7 with 111 In-platelets. Single hot spots were documented in 11 patients, multiple spots in 35 patients, linear deposition patterns in 4 patients. The "sensitivity" in respect to the diagnosis of a thrombosis was 71%.
Conclusions: This study confirms the problem to find a "Gold Standard" for thrombodiagnosis (Editorial; J.Nucl.Med.; 1999, 2036-2037). We had to compare functional results with morphological methods, trying to find out functional activities. Therefore the combination of methods seems to be an acceptable diagnostic way at present: morphological methods to document the localisation of a thrombus and methods of nuclear medicine to give important functional information in research and clinical work about thombus activity and therapy effect.

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SOLUBLE FIBRIN IS THE MAIN MEDIATOR OF STAPHYLOCOCCUS AUREUS ADHESION TO PLATELETS

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Background: S. aureus is an important pathogen in intravascular infections. Bacterial adherence to platelets on the cardiac valve surface is believed to be critical in the induction of infective endocarditis. To enlighten the molecular pathomechanism behind this process we examined platelet dependent factors that are potentially involved in S. aureus adhesion to platelets.
Methods: Association of Syto-13 labelled S. aureus and with PE-conj. mAb against CD42a labelled platelets was measured using flow-cytometry. To pinpoint the binding sites of the platelets involved in the interaction, platelets from knock out mice, platelets from patients with rare selective inherited deficiencies of membrane proteins or granules, and platelets activated with thrombin, ADP, collagen in the presence or absence of ancrod, an enzyme from malayan pit viper venom, that induces soluble fibrin formation, without activating platelets by itself, were used.
Results: Platelets bound S. aureus in a specific, dose dependent manner. The number of S. aureus-platelet associates increased up to 5-10 fold using thrombin activated platelets, while ADP or collagen activation in the presence of hirudin had no effect. GPIIb/IIIa, CD36 and P-selectin could be excluded as direct receptors for S. aureus. In contrast to controls binding of S. aureus to thrombospondin-1 deficient platelets from patients with Gray platelet syndrome or alpha-delta storage pool deficiencies could not be increased by thrombin activation. Activation of platelets with ADP, addition of fibrinogen (200 µg/ml) and ancrod (0,4 U/ml) together with thrombospondin-1 (15 µg/ml) increased the amount of platelet-S. aureus associates to at least the same extend as thrombin activation.
Discussion: Platelets may facilitate endovascular infection, especially catheter-associated infections, by supplying a highly attractive substrate for microorganisms to adhere. In an animal model of endocarditis the treatment with aspirin and/or ticlopidine together with antimicrobial therapy improved the rate of sterilisation and reduced vegetation weight. On the basis of our results thrombin inhibitors might be superior as supplemental treatment. This hypothesis is under investigation.

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TREATMENT OF PREGNANT WOMEN WITH MECHANICAL HEART VALVE PROSTHESIS WITH LOW MOLECULAR WEIGHT HEPARIN

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Introduction: Patients with mechanical heart valve prothesis (MHVP) are treated with oral anticoagulants (OAC) to prevent thromboembolic complications. The treatment of women with MHVP of childbearing age, who wish to become pregnant, is complex and often associated with complications. OAC may cause embryopathy during the first trimester, while neurologic complications, stillbirth and abortion may appear during the second and third trimester. We decided to treat this population with body weight adjusted therapeutic dosages of low molecular weight heparin (LMWH) during the whole pregnancy at the university hospitals in Frankfurt and Mainz.
Patients and methods: From 1/1997 to 12/2000 eight female patients, seven with MHVP, one with atrial fibrillation, were started on LMWH before or at the beginning of pregnancy. The dose was 150-200 U/kg/day aiming at a target range of 0.4-0.8 anti Xa units. LMWH was applied twice daily and was monitored every 4 weeks by measurement of anti Xa-levels.
Results: We observed neither valve thrombosis, embolic complications nor malformations or serious bleeding complications during these pregnancies. No complications occurred in the fetus. Cesarean section was necessary in three of the patients, while the others delivered spontaneously. Three patients developed heart failure during the third trimester, that resolved after treatment of the myocardial insufficiency.
Conclusions: Treatment with body weight adapted LMWH in pregnant women with MHVP seems to be safe and efficient. It is necessary to perform a continuous interdisciplinary monitoring because of the underlying heart disease, which in kontrast to current opinion represents a serious comorbidity.

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LONG-TERM EXPERIENCES IN PATIENTS UNDER SELFMANAGEMENT OF ORAL ANTICOAGULATION

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Background: In the last decade treatment of patients under long-term oral anticoagulation (OAC) by selfmanagement has become an established principle in Europe and the US. We started an investigation to proof how regular INR is controlled by those who proceed selfmanagement for a longer time (up to >2 years), how many INRs are in the individual therapeutic range or a critical range (x<2,0 INR or x>4,0 INR). Further we looked for influences on quality of life (QOL).
Methods: 35 patients were investigated by questionaire about quality of life (QOL), INR-controll intervalls, management of complications or in case of critical INR. Additionally their original dosage-diaries were analysed. 26 patients could be examined. All pat. took part in a standardised training program according to the guidelines of ASA, Germany, and used prothrombinetime monitor COAGUCHEK S, Fa. Roche Diagnostics, Mannheim/Germany.
Results: Weeks of investigation: 1328 in total (25.5 PY), xmean=51,1week/Pat., range 4-109 weeks/Pat. Measurements: 1619 in total, xmean=62/Pat, range 4-195/Pat. INR in individual therapeutic range: 83,57%. INR out of individual therapeutic range but in general therapeutic range (2,0INR<X<4,0INR):16,42%. INR out of general therapeutic range(x<2,0INR or x>4,0INR): 2,9%. Interval of INR-measurement: 14,3% 2-3 times/week; 78.6% once/week; 7,1% every 1-2 weeks; in case of critical INRs in shorter intervals by every pat. Dosage: 1 pat. gets dosage of OAC by general practitioner, all others decide dosage by themselves but in case of critical INRs. Reported severe complications: one extended hematoma. QOL: higher QOL because pat. feel more safe, better informed, independent; everyone would start selfmanagement again.
Conclusions: In this long-term investigation we found that selfmangement of OAC therapy leads to a stable adjustment of INR-level which might help to reduce severe complications in OAC. Only about 3% of INRs were above 4.0 or below 2.0. The higher quality of life will also help the reach a better compliance and log-term outcome in pat. under long-term OAC.

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PITFALLS IN INR MEASUREMENT BY DIFFERENT PROTHROMBINETIME-MONITORS FOR CAPILLARY BLOOD

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Background: INR measurement for controll of oral anticoagulant (OAC) therapy can be done by capillary blood. Therefore different types of prothrombinetime (PT) monitors are available. Some systems are suitable for selfcontroll of INR by patients who had a special education in a training program for selfmanagement according to standards of ASA/Germany. In few special cases we found not plausible INR-results in comparison INR to a reference lab. So we looked for some special disturbance variables. Well known disturbance variables were excluded (hyperlipidemia, high hematocrit or polyglobulie, CSE-blocker).
Methods: PT monitors: COAGUCHEK S, Fa. Roche Diagnostics, Mannheim/Germany, Testprinciple: reflectance photometry; AvoSure, Fa. Menarini Diagnostics, Düsseldorf/Germany, Testprinciple: Fluorescens photometry. Reference method: mechanical systems to get rid off influences on photometry. Thomboplastine: Hepatoquick (Fa. Roche Diagnostics) Testsystems: KC10A coagulometer (Fa. Amelung) or Schnittger/Gross coagulometer. Controll of influences on extinction: free hemoglobin. Controll on influences of thromboplastine-kit: Phosphlipid-Antibodies.
Results: In two patients with increased free hemoglobin (2-3fold of normal range) INR differences between COAGUCHEK S and mechanical systems were up to INR (-1,4). In one case erythrocyte damage because of a prosthetic heart valve was found, the other had a severe infection. After normalizing of free hb a good correlation between Coaguchek S and reference method was found. In two patients with phopholipid-antibodies (GPL25-40) we found INR differences between COAGUCHEK S and AVOSURE compared to reference lab from –0,4 to –1,4INR.
Conclusion: Because our reference thromboplastine contains a high phospholipid surplus we suppose, that the investigated PT monitors COAGUCHEK S and AVOSURE get problems in case of phospholipid antibodies by less phospholipids in reagence in the ready-to-use teststripes.
Also disturbance variables on photometry like increased free hb seem to be difficult for this PT monitors.

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***TRAVELLER'S THROMBOSIS – LEGAL ASPECTS (IN GERMANY)**

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The legal aspects of traveller's thrombosis are still characterized by uncertainty. This depends on the fact, that clear legal statements demand exact medical facts, which in part are still missing. Furthermore, up to now there are no rulings of higher courts on this topic. There is only one decision by a regional court (LG Frankfurt/Main), which has not yet come into force, dismissing a traveller´s action.
The legal problems may occur in the following fields:
Liability of carriers (mainly airlines) in breach of their duty of care or information (1). Liability of physicians due to wrong information or malpractice (2).
1. The liability of airlines follows national law (Art. 24 Warsaw Convention), since thromboses are no accidents according to this treaty. In Germany, liability due to breach of duty of care has to be rejected, since to recent knowledge there is no evidence of a relevant increased risk of thrombosis compared to other means of transportation (bus, car) or correlated to the space between seats. Furthermore, the airlines offer the possibility to fly business or first class with more space and thereby offering better facilities for increased movement during the travel. Under German law there is also no duty to inform travellers that the risk of thrombosis might be lower in other booking classes. However, the airlines should inform the passengers about preventive behaviour during long-distance flights.
2. Physicians who give special medical advice for travelling, may face a danger of being made liable. In order to avoid medico-legal conflicts, they should be informed about travel-associated risks, outweigh the benefits and risks of any preventive measures and inform the patient accordingly.

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KININ RELEASE FROM HMW KININOGEN BY THE PLASMA HYALURONAN-BINDING SERINE PROTEASE

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Background: The hyaluronan-binding serine protease (PHBSP) was first observed as a "thrombin-like" activity in some PCCs and during purification of Vit K-dependent coagulation factors from plasma. This protease was identified as the active enzyme form of a plasma hyaluronan-binding protein. This protease was later described to activate FVII independent of tissue factor, to activate single chain plasminogen activators, and to inactivate FV and FVIII. In this study we tested the hypothesis that PHBSP has not only procoagulatory and profibrinolytic potencies but might also affect processes related to the contact phase system (kallikrein/kinin system).
Methods: Purified prekallikrein, FXI, FXII and HMW kininogen (HK), respectively, were incubated with purified PHBSP and the products were analysed by SDS-PAGE, Western blot and N-terminal sequencing. In a radioimmunoassay the kinetic of kinin release was investigated, MALDI-TOF-MS was applied to characterize the kinin generated by PHBSP. Plasma was treated with PHBSP to determine whether kininogen activation and kinin release is also found in plasma.
Results: No activation or cleavage of the proenzymes involved in the contact phase system was observed. HK, however, was in vitro cleaved by PHBSP in the absence of any charged surface, releasing the activated cofactor and the vasoactive nonapeptide bradykinin. The cleavage was comparable to that of plasma kallikrein, but clearly different to that of coagulation factors FXIa or FXIIa. The rate of kininogen cleavage at identical substrate to enzyme ratio was slower compared to plasma kallikrein. Upon extended incubation with PHBSP, partial removal of 7 kda from the N-terminus of the D5 domain was detected. These cleavage sites were distinct from plasma kallikrein or FXIa cleavage sites. We found that PHBSP was able to activate HK in plasma resulting in the release of bradykinin.
Conclusions: PHBSP represents a novel HMW kininogen-cleaving and bradykinin-releasing enzyme sharing significant catalytic similarities with plasma kallikrein. Since they are otherwise structurally unrelated, their similar activities could be directed to distinct sites. Since hyaluronic acid is a major component of the extracellular matrix, PHBSP function may be directed to this environment.

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REGULATION OF THE PLASMA HYALURONAN-BINDING SERINE PROTEASE

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Background: The plasma hyaluronan-binding serine protease (PHBSP) was first identified in some PCCs and was later shown to activate FVII, the plasminogen activator precursors, and HMW kininogen. The zymogen is the dominant form in plasma and undergoes in vitro rapid autocatalytic activation, a process enhanced in the presence of surfaces. There is only a limited knowledge about the regulation of PHBSP in vitro and in plasma. Therefore, we investigated the characteristics for the interaction of PHBSP with various plasma serpin- and Kunitz-type inhibitors, and addressed the question whether an inhibitor deficiency might affect the influence of PHBSP on hemostasis.
Methods: PHBSP was titrated with various inhibitors: the serpins alpha2-antiplasmin (a2AP), C1-inhibitor (C1-inh), alpha1-proteinase inhibitor (a1-Pi), AT III/heparin, and the Kunitz-type inhibitor inter-alpha-inhibitor (IaI). The rate of formation of stable enzyme/inhibitor complexes was measured kinetically and was visualized by gel retardation assays. The effects of PHBSP on coagulation of normal pool plasma and inhibitor-deficient plasma was studied by aPTT.
Results: From titration curves at variuos substrate concentrations was concluded that a2AP, C1-inh, ATIII/heparin and IaI are tight binding, competitive inhibitors, while a1-Pi was found to be a much weaker non-competitive inhibitor of PHBSP. By gel retardation assays complexes could be found with all inhibitors. The rate of complex formation was for all inhibitors within the seconds-range, except for a1-Pi that reacted much slower. The aPTT of normal pool plasma was not affected by pre-incubation with PHBSP for 1 min, but a2AP- or C1-inh-deficient plasmas showed a significant acceleration of coagulation. Longer incubation of plasma with PHBSP yielded the adverse effect.
Conclusion: PHBSP activity is strongly regulated by both, plasma inhibitors of the serpin- and the Kunitz-type. In vivo the function of PHBSP is most likely restricted to sites of reduced inhibitor activity, e.g. on protected cell surfaces or in the perivascular environment. C1-inh and a2AP apparently participate in the regulation of PHBSP-activity in plasma. Deficiency of these inhibitors clearly augmented PHBSP-induced affects on hemostasis. From the substrate specificity of PHBSP can be speculated if in inhibitor-deficiency disorders like hereditary angioedema or haemorrhagic disorders PHBSP could be involved.

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***DRUG COMBINATIONS IN THE MANAGEMENT OF THROMBOTIC DISORDERS. IMPACT ON FUTURE CLINICAL PRACTICE**

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Several factors have contributed the development of news anticoagulants and antithrombotic drugs in recent years. These include (in order of importance) the need to develop an alternate drug to substitute heparin in patients who are heparin compromised (HIT, ATIII deficient), drugs with improved pharmacologic profile , better alternate to current and oral anticoagulants and more cost effective drugs. Heparins, currently used oral anticoagulant drugs and aspirin are polypharmacologic agents with multiple sites of action at plasmatic and cellular sites, however, the development of newer drugs has primarily targeted specific sites and most of the newer antithrombotic/anticoagulant drugs are monotherapeutic. Such drugs as the antithrombin agents, anti-Xa drugs, receptor derived anticoagulants and specific enzyme inhibitors are mostly single targeted agents with a relatively narrow therapeutic spectrum in contrast to the conventional drugs. The therapeutic actions of heparins, warfarin and aspirin involve several sites and produce these effects in both the direct and indirect modulation of the hemostatic system. Regardless of the origin, thrombotic and occlusive disorders are polytherapeutic and involve the vasculature, blood cells and proteases with several amplification pathways. Therefore a single target drug may have only a limited effect on the overall pathogenesis of thrombotic syndrome. To produce comparable clinical response relatively stronger effects are needed with a monotherapeutic agent in contrast to the conventional polytherapeutic drugs. Experimental studies have shown that the ED_{50} of antithrombin drugs such as hirudin or synthetic agents such as argatroban can be markedly reduced with relatively weaker amounts of anti-Xa drugs. Similarily of the ED_{50} of anti-Xa drug such as the heparin pentasaccharide can be markedly reduced by the addition of antithrombin agents and activators of HC-II. These drug combinations may have some unexpected bleeding and vascular consequences which should be taken into account. From the pharmacokinetic standpoint the monotherapeutic agents such as the Argatroban or DX9065a (an anti-Xa inhibitor) demon-

strate predictable pharmacokinetic behavior even when combined, however their pharmacodynamic behavior is markedly different. A combined anti-Xa/anti-IIa approach is therefore much more effective and may be more optimal in the management of thrombotic syndromes. While the monotherapeutic approach may be useful in the management of venous thrombosis, in such indications as the acute coronary syndrome, thrombotic stroke and PAOD multiple drugs are combined with aspirin to achieve optimal outcome. However the pharmacodynamic consequences for the combination are not completely understood. Combined pharmacologic approaches will have a profound impact in antithrombotic and anticoagulant drug development in coming years. While these approaches may provide us with a stronger and broader therapeutic approaches, several developmental issues such as combined dosage optimization, pharmacologic antagonism and cumulative pharmacodynamic profile will require careful preclinical studies for the design of valid clinical trials in specific indications.

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NEWER INSIGHTS INTO THE MECHANISMS OF THE ANTITHROMBOTIC ACTIONS OF LMWH. STUDIES ON A LMWH, CLIVARINE, IN THE MANAGEMENT OF SURGICAL AND MEDICAL THROMBOTIC DISORDERS

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Despite the development of well-defined synthetic and biotechnology derived anti-Xa, anti-IIa and anti-tissue factor (TF) drugs, heparin (UH) and low molecular weight heparins (LMWHs) are still the anticoagulant/antithrombotic drugs of choice for the management of surgical and medical thrombotic disorders. Their reported adverse reactions such as heparin-induced thrombocytopenia have prompted the development of alternate anticoagulants such as hirudin, argatroban, hirulog and the pentasaccharide. However, none of these drugs have similar polypharmacologic actions as the heparins which target multiple sites at plasma and cellular levels. Current understanding of thrombogenesis has provided evidence that fibrinolytic deficit due to the upregulation of such mediators as PAI-1, TAFI and histadine rich glycoprotein (HRG) play an important role in the mediation of thrombogenesis. Equally well, the inflammatory process involving CRP, interleukins, CD-40 ligand and the modulation of their cellular receptors play a crucial role. Recently, the aberration of the TF pathway in terms of upregulation of TF and polymorphism in TFPI have been identified as key factors in thrombotic processes. Experimental approaches utilizing tissue culture isolated biochemical systems and experimental animal models do not truly provide a reliable database which can be correlated with human pathogenesis of thrombosis Thus, analysis of different thrombotic syndromes prior to, during and after treatment by using plasmatic and cellular based assays provides more direct evidence on the mechanisms involved in the mediation of thrombotic syndromes. Large-scale clinical trials have been conducted in specific indications where LMWHs have proven to be effective in the clinical management of such disorders as medical thrombosis, post-orthopedic surgical thrombosis and acute coronary syndrome. These trials provide a unique opportunity to not only study the pathogenesis of thrombosis but also its modulation by LMWH. To test the hypothesis that several mediators such as TF, TFPI, TAFI, thrombomodulin, PAI-1, ATIII, HCII, CRP and IL-6 may be altered during thrombogenesis, plasma from three well-defined large-scale clinical trials utilizing Clivarine for the prophylaxis and treatment of thrombosis were analyzed. The dosing of this LMWH ranged from 1750-4200 U. UH was used as a control in the treatment trials. Tests included the anti-Xa and global anticoagulant assays (Heptest, aPTT). In specific groups anti-phospholipid antibody titers along with anti-heparin/PF4 titers were measured. LMWH administration resulted in an increase in TFPI release and down regulation of functional TAFI. Similarly, CRP and soluble thrombomodulin were decreased during treatment. A relationship of these markers with the treatment outcome may reveal their role in the pathogenesis and potential sites of action of LMWH. Most notably, the relative anti-heparin/PF4 titers were much lower in all three trials in comparison to the reported values. The incidence of clinical thrombocytopenia was nearly absent in these groups. This presentation will provide an overview of the role of fibrinolytic deficit and the inflammatory process in the pathogenesis of thrombosis and its modulation with particular reference to the clinical results reported on the Plaster Cast, CORTES, and ECHOS trials. Experimental data on the action of Clivarine on the function of TAFI and other mediators will also be presented.

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INHIBITION OF HEPARINASE BY A SULFATED PENTOMANNAN (PI-88): POTENTIAL CLINICAL IMPLICATIONS

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PI-88 is a sulfated pentomannan with antiproliferative and antithrombotic actions mediated by interactions with growth factors and heparin cofactor II, the endogenous release of TFPI and inhibition of generated proteases. While PI-88 is resistant to the digestive effects of heparinase-I, PI-88 inhibits heparinase-I. Studies were designed to determine the effects of PI-88 in defined biochemical systems and in whole blood. Porcine mucosal heparin (PMH) solutions were supplemented with heparinase-I in the concentration range of 0.01-1.0 U/ml. PI-88 was supplemented to the buffered PMH solution (10 mg/ml) at a concentration of 250 mcg/ml prior to the supplementation of heparinase-I. After a 30 min incubation, the solution was heated to stop the depolymerization reaction. The anticoagulant activities of various inhibited mixtures were measured using the anti-Xa, anti-IIa, Heptest, activated partial thromboplastin time (aPTT), and activated clotting time (ACT). A concentration-dependent depolymerization of heparin was noted, rendering PMH non-anticoagulant at a heparinase concentration of 1.0 U/ml. Heparinase-I produced an almost complete neutralization of the anticoagulant effects of PMH as measured by the ACT, aPTT, and Heptest. Supplementation of PI-88 restored the anticoagulant effects of PMH. The effects of PI-88 on the anti-Xa actions of heparin were not as strong as with the other tests. The inhibitory actions of heparinase-I were dependent on the concentration of heparin. Low molecular weight heparins (LMWHs) were highly susceptible to the digestive actions of heparinase-I and PI-88 effectively inhibited actions of heparinase on these LMWHs. Heparin-derived oligosaccharides such as the chemically synthesized pentasaccharide and fragmented heparin oligosaccharides were less susceptible to the digestive actions of heparinase; however, heparinase-I was inhibited by PI-88 when these agents were used as substrates. GPC-HPLC analysis of heparinase-I digested oligosaccharides confirmed the digestive actions of heparinase and its inhibition by PI-88. Heparinase-I was also found to digest the anticoagulant effects of sulfated mucopolysaccharides, such as Ateroid, Orgaron and dermatan sulfate. This digestion was also inhibited by PI-88. This observation provides additional data on the potential therapeutic effects of PI-88 in proliferative and thrombotic actions.

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THROMBIN ACTIVATABLE FIBRINOLYTIC INHIBITOR IS DOWN-REGULATED BY DEFIBROTIDE IN PATIENTS AFTER HIP SURGERY: A POTENTIAL MECHANISM OF THE THERAPEUTIC EFFECT OF DEFIBROTIDE

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When activated, thrombin activatable fibrinolytic inhibitor (TAFI) modifies fibrin rendering it resistant to the lytic actions of plasmin. Thus, increased levels of TAFI produce a fibrinolytic deficit. TAFI levels are increased in patients with acute coronary syndrome, stroke, diabetic PAOD and hypertension. Defibrotide is a polydeoxyribonucleotide-derived antithrombotic/anti-ischemic agent, which has been shown to produce its therapeutic effect by endogenous activation of fibrinolysis. To test the hypothesis that defibrotide modulates TAFI levels, three groups of patients (n=10-20 each) treated with bridged defibrotide therapy (i.v. infusion followed by oral administration) were evaluated for TAFI, plasminogen activator-1 (PAI-1) and D-dimer. The treated groups were compared with a historic placebo group (n=17). Since hip surgery produces a strong activation of thrombogenesis, patients (n=13) who had undergone hip surgery were also studied. A marked elevation of TAFI (9.3± 2.1 mcg/ml) was observed in the patients in contrast to normal (5.1± 1.3 mcg/ml). A marked reduction in the functional TAFI level was observed during the post-surgical period in both treated groups ($p<0.032$). TAFI antigen levels were also lower in the treated groups. While a wide scatter was noted in the PAI-1 level, the treated groups showed a trend towards lower PAI-1 antigen levels. D-dimer levels were significantly higher in the untreated historic control ($p<0.043$). The antigen:functional TAFI ratio was much higher in the untreated group indicating a consumption of TAFI (1.3-1.8), whereas in the defibrotide treated group, it remained near 1.0. TAFI antigen levels were decreased after defibrotide treatment. These results support the hypothesis that one of the major mechanisms of defibrotide's action is through its modulation of the fibrinolytic process. These observations represent another potential mecha-

nism for the poly-therapeutic defibrotide in the control of thrombotic and ischemic processes in medical and surgical patients. Together with the release of tissue factor pathway inhibitor (TFPI), defibrotide is capable of producing its antithrombotic actions in orthopedic patients via a reduction in TAFI levels. Such therapeutic actions may also be observed in patients with other.

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HEPARIN CO-FACTOR II (HC-II) BINDING OLIGOSACCHARIDES AUGMENT THE ANTITHROMBOTIC EFFECTS OF ANTITHROMBIN BINDING HEPARIN DERIVED OLIGOSACCHARIDES

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Several heparin co-factor II (HC-II) activating oligosaccharides have been reported to interact with other oligosaccharides components of heparin. Hypersulfated heparin derived oligosaccharides and synthetic and biotechnology derived sulfated oligosaccharides such as the sulfated pentomannan produce strong activation of HC-II and mediate the antithrombin actions. On the other hand, antithrombin (AT) affinity heparin oligosaccharides produce solely the anti-Xa actions. High AT-III affinity oligosaccharides can be separated from low molecular weight heparins (LMWHs) and obtained by synthetic approaches. HC-II binding oligosaccharides can also be obtained by LMW dermatan sulfate preparations. A sulfated pentomann preparations (PI-88) is comprised of hypersulfated mannan derivatives (MW 2700) and is mainly composed of oligosaccharides (4-8 units). This agent mainly interacts with HC-II and does not exhibit any AT- binding. PI-88 also exhibits strong anticoagulant and antithrombotic effects (IV ED_{50} 1.25–2.50 mg/kg) in a rat model of jugular vein thrombosis. A comparable oligosaccharide mixture obtained from LMWHs by AT affinity chromotographic methods also exhibit similar antithrombotic actions (IV ED_{50} 0.25–0.70 mg/kg). In the same model AT-binding concensus oligosaccharide sequence demonstrated slightly stronger antithrombotic effects (IV ED_{50}, 0.1-0.5 mg/kg). The IV ED_{50} effects of High AT oligosaccahride and heparin pentasaccharide were markedly augmented by simultaneous administration of PI-88. Administration of 0.5 mg/kg of PI-88 shifted the ED50 of both the heparin oligosaccahrides (0.05–0.35 mg/kg) and heparin pentasaccharide ($\leq$0.25 mg/kg) suggesting a synergistic interaction. In vitro studies on thrombin and Xa generation assays confirmed these observations where PI-88 produced strong potentiation of the pentasaccharide. In whole blood studies the generation of F1.2 (Prothrombin fragment) was markedly suppressed by the combined agents. These studies clearly suggest that pharmacologic effects of AT binding oligosaccharides can be markedly augmented by simultaneous administration of AT binding oligosaccharides. The combined AXa/AIIa oligosaccharide components may therefore be better antithrombotic/anticoagulant drugs in contrast to the sole AT or AT III oligosacharides. Additional experimental evidence on the synergism between the anti-Xa and antithrombin oligosaccharides will be presented.

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NEW ASPECTS IN THE APPLICATION OF DDAVP AS A HEMOSTATIC DRUG

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The vasopressin analogon desmopressin (DDAVP) is known to act as a hemostatic drug in patients with certain coagulation disorders. Its mechanism is not yet completely understood. It probably releases coagulation factors from their endogenous deposits and therefore raises factor plasma-levels in treated patients for a short period of time.
In our trial we tested a total of 42 children with the diagnosis of von Willebrand disease Type I (vWD) and mild Hemophilia A for their response to DDAVP-application. The purpose of the test was to reveal whether these patients could undergo small surgery without substitution of factor-concentrates when treated with DDAVP shortly before the operation. Particularly patients with vWD Type I showed an excellent response to DDAVP application, which was documented in PTT (decrease to 78%), bleeding time (BT) (decrease to 75%), Factor VIII function (2.8fold increase), von Willebrand Factor Antigen (vWF-Ag) serum-level (2.75fold increase) and von Willebrand Factor Collagen-Binding-Activity (vWF-CBA) (4fold increase). Patients with mild Hemophilia A reacted in a lower extent in most of the documented parameters except bleeding time (PTT 86%, BT 72%, Factor VIII function 2.3fold, vWF-Ag 2.29fold, vWF-CBA 3.4fold increase). But they showed a longer half-life-time of Factor VIII function and vWF-Ag. The maximum of the DDAVP-effect in patients with mild hemophilia documented in PTT and bleeding time appeared 2 hours after DDAVP-application. Patients with vWD Type I showed their maximum effect 1 h after DDAVP-injection.
Interestingly in patients with vWD Type I as well as in patients with mild Hemophilia A the best response to DDAVP showed vWF-CBA. vWF-CBA increased to a higher level than vWF-Ag. It seems that DDAVP stimulated vWF-Ag shows a higher activity than the not-stimulated vWF-Ag.

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***INTEGRIN ACTIVATION IN TUMOR METASTASIS**

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Cancer cell adhesion, migration and invasion are critical during tumor metastasis. We found that human breast cancer cells express the adhesion receptor integrin $\alpha_v\beta_3$ in either an activated or a non-activated functional form. This was shown in *in vivo* and *in vitro* selected functional variants of the MDA-MB 435 breast cancer cell line. Importantly, activated - but not non-activated $\alpha_v\beta_3$ strongly promotes experimental metastasis of human breast cancer cells in immune deficient mice. Furthermore, the expression of activated integrin $\alpha_v\beta_3$ is associated with metastasis in clinical breast cancer, as shown in primary metastatic cells isolated from pleural effusions and peripheral blood samples of patients with stage IV breast cancer. Activated integrin $\alpha_v\beta_3$ endows tumor cells with functional qualities, that likely promote cancer cell escape from the primary tumor, dissemination in the vasculature, and invasion of target organs. In contrast to non-activated $\alpha_v\beta_3$, the activated receptor binds soluble ligands, supports tumor cell arrest during blood flow through tumor cell interaction with platelets, and permits or enhances tumor cell migration toward extracellular matrix proteins, that are constituents of primary tumor stroma, subendothelial matrix and basal lamina. Support of tumor cell-matrix interaction under dynamic flow conditions, as found in the vasculature, is a unique ability of integrin $\alpha_v\beta_3$ and requires receptor activation. This is shown in a melanoma cell model and buffer perfusion system. It is also true during blood flow, where the tumor cells do not arrest directly at a given matrix, but attach to activated platelets, which then mediate tumor cell arrest. The interaction requires binding of soluble plasma protein ligands as bridging molecules between tumor cells and platelets. The ability of metastasizing cancer cells to leave the primary tumor and colonize target tissue, after arresting in the vasculature during hematogenous dissemination, depends on directed invasive tumor cell migration. We found that activated integrin $\alpha_v\beta_3$ is required for breast cancer cell migration toward a fibrinogen/fibrin matrix, and enhances migration toward vitronectin and fibronectin. This process depends on a unique cooperation between activated tumor cell integrin $\alpha_v\beta_3$ and metalloproteinase MMP-9. We propose a model mechanism, in which activated $\alpha_v\beta_3$ binds the latent 92 kDa form of pro MMP-9 as a soluble ligand, and facilitates conversion of pro MMP-9 to active MMP-9 at the tumor cell surface. Active MMP-9 modifies a fibrinogen/fibrin matrix. Collectively this results in $\alpha_v\beta_3$ mediated migration of metastatic breast cancer cells toward this specific substrate. Together, the expression of integrin $\alpha_v\beta_3$ in an activated functional form provides a unique advantage for malignant tumor cells by promoting their metastatic phenotype and dissemination.

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IN VITRO EFFECTS OF DIRECT THROMBIN INHIBITOR ARGATROBAN ON THE PROTHROMBIN TIME AND INFLUENCE OF ORAL ANTICOAGULATION

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Therapy with oral anticoagulants (OA) is commonly monitored by the prothrombin time reagents (PT expressed as Quick or INR. Direct thrombin inhibitors (DTI) like argatroban also influence the PT during concomitant phases of therapy. Despite in vivo volunteer studies described in literature, it remains unclear, whether these interactions are of additive or hyperadditive manner. However, they make exact dose adjustment of OA impossible using PT reagents. It is only possible to make a statement, whether the anticoagulation under concomitant presence of both drugs is sufficient or not. Unlike heparins, there is no possibility to eliminate the additive effects of DTIs pharmacologically. Aim of the present study was to analyse dose-effect relationship of argatroban in vitro under the influence of oral anticoagulation and uninfluenced by OA. Blood samples of six healthy volunteers and ten patients on phenprocoumon were spiked with argatroban (500 to 3000 ng/ml) and prothrombin time was determined (Innovin, ISI: 1.09). Normal PT was 10.2±0.3 s (all data mean±SD). OA with phenprocoumon prolonged PT-values to 25.9±5.0 s. Argatroban had a concentration-dependent prolonging effect on the PT. A concentration of 2000 ng/ml increased the PT to 37.5±4.1 s and 140.6±50.0 s in plasma samples from healthy and from patients on OA therapy. Healthy plasmas exerted a sigmoidal dose-effect relationship

while plasma samples on OA showed an approximately linear one. Individual PT ratios could only reduce, but not cease these differences between healthy and OA-patients (mean ratios of 3.7 and 5.3 at 2000 ng/ml). This is also valid for the INR (3.6 vs. 12.9). This stands in contrast to ECT and PiCT. Not even normalising INR lead to a cessation of differences between both groups (3.2 and 5.2). This fact should be considered in clinical practice.

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ECARIN CLOTTING TIME UNDER INFLUENCE OF HIRUDIN, ARGATROBAN AND MELAGATRAN AND ORAL ANTICOAGULATION WITH PHENPROCOUMON

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Anticoagulant actions of direct thrombin inhibitors (DTI) are usually monitored by aPTT methods. The limitations of this method include a non-linear dose-effect relationship, a plateau effect, a great variability among different testing instruments or reagents and between different lots of the same reagent. The ecarin clotting time (ECT) has a linear dose response relationship for hirudins and is therefore more accurate for these drugs than the aPTT. The ECT is also prolonged to some extent during therapy with Vitamin K antagonists. Aim of the present study was to analyse in vitro concentration-effect relationships of three different DTIs during treatment with oral anticoagulants. Hirudin, argatroban and melagatran were added to plasma samples of healthy volunteers and to samples of patients orally anticoagulated with phenprocoumon to obtain information on additive effects. Plasma samples of 6 healthy donors and ten patients on phenprocoumon were spiked with different concentrations of the three DTIs and the ECT was tested in all samples. Normal test range was <55s. Hirudin showed linear concentration-response relationships with prolongation of ECT from 42±0.9 s (mean±SD) to 242±38.9 s for healthy plasma and from 52±3.6 s to 358±35.9 s for oral anticoagulation at 2000 ng/ml. In plasma samples of the healthy, argatroban exerted a concentration dependent non-linear dose-effect relationship with sigmoidal shape. A concentration of 2000 ng/ml prolongated the ECT to 345±17.2 s. In plasmas of patients on oral anticoagulation with phenprocoumon, the same concentration showed a linear prolongation to 433±44.9 s and the curve lost its sigmoidity. Melagatran (500 ng/ml) lead to an ECT of 411.0±20.9 and 489±28.9 s, respectively. Increasing doses of all three drugs tested lead to increasing differences between ECT values in healthy and orally anticoagulated plasma. Expression of results as individual ratio decreased differences for hirudin and abolished them for argatroban and melagatran between persons without and with oral anticoagulation. Although ECT is only slightly elevated during oral anticoagulation, the results presented here suggest that with increasing doses of a DTI, ECT results in seconds lose their monitoring value for dosage of DTIs (falsely high estimated doses). Therefore, introduction of individual ECT ratios seems convenient.

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INFLUENCE OF THROMBIN INHIBITORS HIRUDIN, ARGATROBAN, MELAGATRAN AND UNFRACTIONATED HEPARIN ON THE PICT ASSAY AND THE EFECT OF ORAL ANTICOAGULATION

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Immediate acting anticoagulants include heparins [UFH]), hirudins, argatroban and melagatran. They are commonly monitored by aPTT. Most inconvenient limitation of this method is a non-linear dose-effect relationship. As monitoring of direct thrombin inhibitors is far from being satisfactory, recently a new test method was developed: the Prothrombinase-induced Clotting Time (PiCT)with improved sensitivity against low molecular weight heparins . The PiCT coagulation values are prolonged in plasma samples from patients with factor I, II, V and X deficiencies. This study investigated dose dependent effects of UFH, hirudin, argatroban and melagatran on the PiCT during oral anticoagulation (OA). Plasma samples of 4 healthy persons and 6 patients on oral anticoagulation with phenprocoumon were spiked with 300 and 500 ng/ml of UFH, hirudin and argatroban. Melagatran was added in concentrations of 30 and 100 ng/ml. Linear concentration-effect relationships ocurred for UFH as well as all direct thrombin inhibitors. PiCT exerted highest sensitivity against melagatran, followed by argatroban, UFH and hirudin. Normal PiCT value was around 30 s in the healthy and 40 s under OA. OA had a strong additive effect on the PiCT in case of melagatran with increased variability, followed by argatroban and hirudin and no effect on dose-effect relationship of UFH (equimolar scale). Normalised presentation of the data ceased the differences. Equimolar scaling reveals that in healthy plasma UFH is the strongest drug, followed by melagatran, hirudin and argatroban. On OA, melagatran and UFH change places. According to these data, PiCT is a promising alternative to the aPTT not only for UHF and LMWH, but also for the direct thrombin inhibitors hirudin, argatroban and melagatran. Normalised ratio eliminates the effects of OA on PiCT. This would be a considerable advantage in monitoring concomitant therapy.

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DISTINGUISHING ACTIONS OF DIRECT THROMBIN INHIBITOR HIRUDIN AND ORAL ANTICOAGULANT PHENPROCOUMON DURING CONCOMITANT THERAPY WITH A MATHEMATICAL MODEL

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Oral anticoagulation is usually monitored by the prothrombin time test, commonly expressed as INR (international normalised ratio). Hirudin and phenprocoumon both influence the INR during concomitant therapy, which occurs during therapy switches. Therefore it is impossible to monitor the dosage of vitamin-K antagonists by prothrombin time reagents. It is only possible to state, whether the anticoagulation under concomitant therapy is sufficient or not. In contrast to heparins, there is no possibility to eliminate the additive effects of DTIs pharmacologically. Aim of the present study was to establish a mathematical model for the distinction of the effects of both drugs during concomitant therapy. Ex vivo samples of patients were spiked with hirudin (500 ng/ml) and individual dose-effect relationship graphs were plotted. Ex vivo ECT, hirudin ELISA and S-2238 chromogenic test, measured concentrations of hirudin. A mean value of the results of all three methods was calculated in each case. The plot line was shifted to the right by the measured individual hirudin-concentration. The interception on the y-axis was determined. This value was compared with the one measured before hirudin therapy. Empirical analyses lead to the conclusion that the shift had to be divided by 2.75 to obtain correct intersection values. Maximal prediction error between INR values corrected with this mathematical method and the ones on oral anticoagulation alone was 7.2%. Although larger number of cases will be required for the method's full validation, this could by a possible way to eliminate the effect of direct thrombin inhibitors during concomitant treatment with OA in order to dose correctly vitamin-K antagonists.

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ROLE OF COAGULATION FACTOR XII IN THROMBIN GENERATION INDUCED BY BLOOD CONTACT WITH MEDICAL GRADE POLYVINYLCHLORIDE

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Background and aims: The mechanisms leading to thrombin generation during blood contact with foreign surfaces, e.g. in extracorporeal circuits for hemodialysis or cardiopulmonary bypass, are not well understood. Especially, data concerning the interaction of the coagulation system with Polyvinylchloride (PVC), a polymer widely used for blood contacting medical devices, are rare and inconclusive. We therefore studied the role of factor XII in PVC-induced coagulation activation under flow conditions comparable with hemodialysis.
Methods: We used a modified Chandler model, medical grade PVC tubing loops (1/4″, volume 6.5 ml) filled with 6 ml heparinized (0.7 U/ml) human whole blood and rotated vertically at 37°C/20 rpm. After 30 min contact time, the blood was removed and mixed with 0.11 M citrate (1/9 v/v) or EDTA. Before and after blood-PVC contact, we determined platelet count, thrombin-antithrombin (TAT) complexes (ELISA) , factor XIIa (ELISA, Shield Diagnostics), FXII-C1 inhibitor and FXII-antithrombin complexes (house-made ELISAs, detection limits 0.5% of kaolin-activated plasma). For selective inhibition of the coagulation factors VII, X or XII, heparinized whole blood was mixed with antibody solutions containing one of the specific polyclonal antibodies or goat resp. sheep IgG or saline as controls. Factor activities were suppressed below 5% as shown by standard one-stage clotting assays with factor-deficient plasma.
Results: Blood contact with PVC tubing resulted in strong TAT complex formation (pre <1, after 30 min. 66±25 µg/l, n=4). The platelet count decreased moderately and reached 90±3% of the initial value. The FXIIa blood level did not increase after contact with PVC. Neither Factor XII-C1 inhibitor nor FXII-Antithrombin complexes were detectable. Blockade of factor VII did not affect TAT generation (68±23 µg/l), whereas inhibition of factor XII strongly suppressed TAT complex formation (8±5 µg/l, 10% of control). As expected, thrombin generation was almost completely inhibited by blockade of factor X (1,75±2 µg/l).
Conclusion: Our data strongly suggest that PVC triggers coagulation activation via factor XII, although we were not able to demonstrate increased factor XII activation products. Obviously, only minute amounts of activated FXII are necessary to start coagulation. The Factor VII/TF system does not seem to be involved.

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PLASMINOGEN ACTIVATOR INHIBITOR TYPE 1: DIFFERENTIAL INHIBITION OF GENE EXPRESSION BY STATINS

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Hintergrund: Erhöhte Plasmaspiegel des Plasminogenaktivator-Inhibitors Typ I (PAI-1) sind ein unabhängiger Risikofaktor für kardiovaskuläre Erkrankungen. Statine zeigen neben der Cholesterinreduktion pleiotrope Effekte. Nachfolgend werden Ergebnisse von Zellkulturexperimenten vorgestellt, die den Einfluß verschiedener Statine auf die PAI-1 Expression untersuchen sollten.
Methodik: Humane glatte Muskelzellen wurden mit unterschiedlichen Konzentrationen (0, 0.1, 1 und 10 uM) der verschiedenen Statine inkubiert.Die Versuche erfolgten jeweils in Gegenwart und Abwesenheit von 1 ng/ml TGF-ß, einem der stärksten Agonisten der PAI-1 Expression. Im Zellüberstand wurden PAI-1 und t-PA mittels ELISA sowie die Gesamtproteinmenge bestimmt. Die Quantifizierung der Expression des PAI-1 Gens erfolgte durch Northern Blotting.
Ergebnisse: Nach Inkubation mit den unterschiedlichen Statinen über 24 Stunden war eine Reduktion der PAI-1 Proteinkonzentration von 12 % bei Pravastatin (p=ns), 37 % bei Simvastatin (p=0,018), 39 % bei Lovastatin (p=0,003), 44 % bei Atorvastatin (p=0,003), 46 % bei Fluvastatin (p=0,014) und 50 % bei Cerivastatin (p=0,027) lediglich in Gegenwart von TGF-ß zu beobachten, einhergehend mit einer verminderten PAI-1 mRNA Expression. Die Gesamtproteinkonzentration wurde durch die Statine nicht beeinflußt.
Schlußfolgerung: Die klinisch eingesetzten Statine hemmen eine pathologisch erhöhte PAI-1 Expression in unterschiedlichem Ausmaß. Der Einsatz von spezifisch auf die PAI-1 Expression wirkenden HMG-CoA-Reduktasehemmer bei Patienten mit pathologisch erhöhter PAI-1 Expression könnte sich günstig auf die Entstehung und Progression kardiovaskulärer Erkrankungen auswirken. Die Entwicklung von Statinderivaten als PAI-1 Inhibitoren erscheint sinnvoll.

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HYPERCOAGULABILITY INDUCED BY A LONG HAUL FLIGHT-PROJECT: ECONOMY CLASS SYNDROME 2001

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Introduction: Long haul flights are suggested to be associated with an increased risk for deep vein thrombosis (DVT). Venous stasis through sitting in uncomfortable position, dry air with the risk of dehydration as well as the mild hypoxia on board might induce an overwhelming of the procoagulatory side of haemostasis. Aim of the study was to investigate the effect of a long haul flight on haemostasis.
Methods: 10 healthy volunteers with no risk and 10 subjects with a moderate risk for DVT were examined before, during a long haul flight from Vienna to Washington (9 h duration), during the return-flight, immediately after the arrival in Vienna, as well as 1 and 3 days after return. PT, aPTT, fibrinogen, platelet count, thrombin-antithrombin-complex (TAT), D-dimer, plasmin-alpha2-antiplasmin-complex (PAP), t-PA, PAI-1, protein C and S as well as factor VII and VIII were determined. Further we performed modified thrombelastography (ROTEG®).
Results: Compared to baseline all volunteers exhibited a significantly shortened aPTT, significantly increased factor VII and VIII activity, decreased t-PA and increased PAI-I serum levels, resulting in a significantly decreased t-PA/PAI-ratio. As measured by ROTEG® analysis maximum clot firmness and á-angle was significantly increased. We did not find significant changes in TAT, PAP, D-dimer, protein C and S. No differences between both groups were found.
Discussion: All participants presented an increase of the procoagulatory side of the haemostasis, indicated by increased factor VII and VIII concentrations, as well as a decreased fibrinolytic potential. Moreover, these changes were accompanied by increase in clot firmness as detected by ROTEG® measurements. In combination with other risk factors like dehydration, impaired venous blood flow, or even moderate trauma, there might be a increased risk for developing a DVT during long haul flights.

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HOMOZYGOSITY FOR THE FXIII 34LEU GENOTYPE IS A PROTECTIVE FACTOR FOR THE DEVELOPMENT OF ISCHEMIC STROKE BUT NOT FOR INTRACRANIAL HAEMORRHAGE

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Background: Coagulation factor XIII is a transglutaminase that cross-links fibrin monomers in the final stage of clot formation and is essential for clot stability and resistance to fibrinolysis. Recently, a frequent polymorphism in the FXIII A-subunit gene has been described leading to a Val to Leu amino acid exchange at position 34. This polymorphism is suggested to affect clot stability and has been associated with a decreased risk for venous thromboembolism and myocardial infarction. Its role in the development of stroke is still under investigation. To assess the importance of this polymorphism in stroke, we performed a case control study on 801 Austrian individuals.
Patients and Methods: 121 patients with arterial intracerebral haemorrhage (ICH) (mean age±standard deviation was 60±13.5 years; age range 29 to 94 years; 60 males, 61 females), 317 patients with ischemic stroke (61±14.7 years; range 22 to 95 years; 157 males, 160 females) and 363 healthy controls (58±14.3 years; range 23 to 86 years; 195 males, 168 females) were analysed for FXIII Val34Leu with a newly developed mutagenic separated PCR assay.
Results: Within the control group 191 individuals (52.6%) were FXIII 34Val/Val homozygous, 142 (39.1%) were heterozygous for FXIII 34Val/Leu and 30 (8.3%) were homozygous for FXIII 34Leu/Leu. The genotype distribution in the ICH group, in which 66 (54.6%) patients carried the FXIII 34Val/Val genotype, 47 (38.8%) were FXIII 34Val/Leu and 8 (6.6%) were FXIII 34Leu/Leu, was similar to the controls. Interestingly, in the ischemic stroke group 171 (53.9%) patients were FXIII 34Val/Val, 134 (42.3%) FXIII 34Val/Leu and only 12 (3.8%) were homozygous for FXIII 34Leu/Leu. Compared to the control group individuals carrying the FXIII 34Leu/Leu genotype had a significantly reduced risk to experience an ischemic stroke (OR=0.4; 95%CI: 0.2-0.9).The results were essentially the same in males and females. No difference was found between patients with ICH and controls.
Conclusions: Our data provide evidence that the FXIII 34Leu/Leu polymorphism may represent a protective factor for the development of ischemic stroke. However, it does not seem to be involved in the pathogenesis of ICH. Screening for this polymorphism could help to assess the individual risk of ischemic stroke.

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***DISSEMINATED INTRAVASCULAR MICROTHROMBOSIS AND COAGULATION ABNORMALITIES IN SEPSIS ASSOCIATED PURPURA FULMINANS**

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In contrast to meningococcal meningitis with a mortality of less than 1%, fulminant meningococcaemia with septic shock is associated with case fatality rate between 20–50%.
The extension of purpura fulminans in correlation to the body surface is associated with the mortality. Histological investigations of purpuric lesions showed widespread disseminated intravascular microthrombosis. Thrombotic occlusion of the microvasculature of extremities results frequently in autoamputation.
All patients with purpura fulminans have disseminated intravascular coagulation (DIC) and the extent of DIC correlates with the prognosis. Coagulopathy in meningococcal septicemia is characterized by raised prothrombin and partial thromboplastin times, increased levels of fibrin degradation products, reduced coagulation factors and thrombocytopenia. There appears to be an imbalance in the procoagulant and anticoagulant pathways. Levels of anticoagulant factors are reduced, including protein C, protein S, tissue factor pathway inhibitor, and antithrombin III. The procoagulant pathway is upregulated with expression of tissue factor and PAI-1.
Recent investigations of genetic polymorphisms in children with meningococcal disease showed that the 4G allele of a functional insertion/deletion-polymorphism in the promoterregion of the plasminogen activator inhibitor 1 (PAI-1) gene is significantly more frequent in deceased children. This 4G/4G polymorphism causes higher PAI-1 levels which could result in more effective inhibition of fibrinolysis.
These data suggest, that the imbalance in the coagulation system is causally related with mortality of meningococcaemia with septic shock.

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FIBRINOGEN KIEL III: A NOVEL GAMMA-CHAIN A108G SUBSTITUTION IN TWO UNRELATED PATIENTS WITH ACUTE ISCHAEMIC STROKE

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Background: Dysfibrinogenemia is a rare defect of haemostasis. It is mostly diagnosed by discrepant values for plasma fibrinogen concentration analyzed by functional and antigenic tests, and a prolonged thrombin time. Nevertheless, some alterations in the fibrinogen molecule detectable on the DNA level may escape diagnosis by routine coagulation assays. Clinical symptoms of dysfibrinogenemia vary from mild to moderate bleeding, recurrent abortions, venous and arterial thrombosis, and renal amyloidosis. About half of the patients show no clinical symptoms. In patients with ischaemic stroke we currently screen for mutations in the three genes coding for the fibrinogen molecule. Here we present our results for the mutation analysis of the fibrinogen gamma chain gene.
Patients: A total of 100 patients presenting with ischaemic stroke were included in this study.
Methods and Results: Genomic DNA was extracted from peripheral blood, and the coding region of the fibrinogen gamma chain including exon-intron boundaries was amplified by PCR. PCR products were subjected to single strand conformational polymorphism (SSCP) analysis by horizontal flat bed electrophoresis and subsequent silver staining. PCR products with an aberrant banding pattern were sequenced. In two of the 100 patients we detected a heterozygous mutation C->G in exon 4 resulting in an amino acid substitution A->G in codon 108. This mutation introduced a new recognition site for the restriction enzyme RSA I allowing the development of a new PCR-RFLP assay, which offers an appropriate procedure for rapid screening for this mutation.
Conclusions: In two unrelated patients with acute ischaemic stroke we found a novel fibrinogen gamma-chain A108G substitution. Studies in the two patients families as well as screening for this mutation in 100 healthy controls are under way. Results of these investigations may shed some light on the possible impact of this mutation in the genetic predisposition for thrombosis and/or ischaemic stroke.

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CELL SURFACE EXPRESSION OF CD63, CD62 AND FIBRINOGEN BINDING IN RESTING AND ACTIVATED PLATELETS ARE NO RISK FACTORS FOR SPONTANEOUS VENOUS THROMBOEMBOLISM

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Background: A constant in vitro hypersensivity of platelets in response to adenosine diphosphate (ADP) and epinephrine has been suggested as an inherited risk factor for arterial and even venous thrombosis. Additionally a protective effect towards VTE has been described for a gene polymorphism of the platelet thrombin receptor (PAR-1). Our aim was to determine phenotypic and functional alterations of platelet surface components as potential thrombotic risk factors in patients with a history of unexplained spontaneous venous thrombosis.
Methods and materials: 39 patients (f: 22, m: 17, mean age: 48,3 y) with a history of spontaneous venous thrombosis and no inherited or acquired thrombophilic risk factors were compared to a reference group of 29 healthy volunteers (f: 17, m: 12, mean age: 45,8 y). Flow cytometric technique was applied to analyse expression of CD62 (p-selectin), CD63 and fibrinogen binding to glycoprotein IIb/IIIa-receptor by two-colour staining with FITC- and PE-labeled antibodies in paraformaldehyde (1%) stabilised whole blood. Investigations were performed at least 6 weeks after the thrombotic event. In addition studies of platelet response to 5 mM ADP and 2 mM thrombin receptor activator peptide 6 (TRAP-6) were performed prior to FACS-analysis.
Results: CD62- and CD63 surface expression and fibrinogen binding to glycoprotein IIa/IIIb-receptor were higher in patients with a history of thrombosis but failed to reach statistical significance compared to the reference group (U-Man-Whitney-Test). In vitro stimulation with TRAP-6 and ADP induced no significant increase of CD62, CD63 and binding of fibrinogen in comparison to the control group.
Conclusion: Increased expression of CD62, CD63 and fibrinogen binding to resting and activated platelets does not seem to be a risk factor for spontaneous VTE.

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INTERMITTENT FACTOR V INHIBITOR AND LUPUS-ANTICOAGULANT IN A PATIENT WITH RECURRENT VENOUS THROMBOSIS

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Background: Acquired circulating anticoagulants are usually immunoglobulins directed to components of the coagulation system. They can be divided into non-specific or lupus like inhibitors, which react with certain types of antiphospholipids and antibodies to specific coagulation factors. The specifc inhibitors are frequently directed to factor VIII. Antibodies to factor V are rarely observed. While non-specific inhibitors are in common related to an increased risk of thrombosis and pregnancy loss, specific inhibitors can cause bleeding.
Patient: We report the case of a 61 year old man admitted with recurrent thrombosis of the left superficial and common femoral vein. Two months prior to admission prolongation of prothrombin time and partial thromboplastin time had been observed for the first time. Further coagulation investigations confirmed reduced factor V activity (11%) caused by a factor V inhibitor. Additional laboratory investigations of thrombophilia revealed a lupus anticoagulant and a positive anticardiolipin IgG type antibody raising additional suspicion of an antiphospholipid-syndrome. Despite decreased factor V-activity body-weight adapted anticoagulation was started (Fraxiparin®, Nadroparin, 2× 0,8 ml s.c.). Within the following two months factor V reduction resolved spontaneously and lupus anticoagulant and anticardiolipin antibodies disappeared. Therefore anticoagulant therapy was successfully changed to phenprocoumon without bleeding complications.
Conclusion: To our knowledge this is the first reported case of the combined appearance of a specific and a non-specific inhibitor in a patient with an antiphospholipid syndrome and venous thrombosis. Despite presence of reduced factor V activity therapeutic anticoagulation did not cause any bleeding complications. Careful integration of both clinical features und laboratory findings in the evaluation of such a patient is necessary.

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HOMOZYGOUS AND COMBINED HETEROZYGOUS FACTOR V G1691A AND PROTHROMBIN G20210A MUTATIONS AS PREDICTORS OF VENOUS THROMBOSIS DURING PREGNANCY

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We have previously demonstrated that heterozygous carriers of factor V G1691A (factor V Leiden (FVL)) or prothrombin G20210A mutation have a low risk for pregnancy-associated thrombosis. The objective of this study was (1) to evaluate the relative risk for venous thromboembolism (VTE) associated with homozygous or combined defects of FVL or prothrombin during pregnancy and the puerperium and (2) to determine the positive predictive value of these hereditary risk factors. We performed a genetic analysis of factor V G1691A and prothrombin G20210A in 192 women with VTE during pregnancy and the puerperium and in 313 normal women. An incidence of one thromboembolic event in 1500 pregnancies was assumed. In the control group, there were no homozygous individuals and no carriers of a combined FVL and prothrombin mutation. The prevalence of heterozygous carriers of FVL in healthy controls was 8%, that of prothrombin G20210A 2.1%. The number of homozygous individuals among controls was calculated using Hardy-Weinberg equilibrium. The number of combined defects was estimated on the basis of the prevalence of combined defects. Among patients with VTE during pregnancy and the puerperium, the prevalence of homozygous FVL was 2.6% (n=3/115, other defects excluded), as compared with 0.17% calculated for normal women (OR 15.3, 95% CI 8.8-41), that of combined heterozygous FVL and heterozygous prothrombin mutation 9% (n=11/123, other defects excluded) as compared with 0.15% calculated for normal women (OR 65, 95% CI 34-121), and that of combined homozygous FVL and heterozygous prothrombin mutation 1.8% (n=2/114, other defects excluded) as compared with 0.0033% calculated for normal women (OR 537, 95% CI 78-999). In univariate analysis, the probability of pregnancy-associated thrombosis in carriers of homozygous FVL was 1.0% (95% CI 0.32-1.3), in carriers of combined heterozygous FVL and heterozygous prothrombin mutation 3.8% (95% CI 2.1-6.7), and in carriers of combined homozygous FVL and heterozygous prothrombin mutation 25% (95% CI 6.2-61.7). In conclusion, women with combined defects have a very high risk for pregnancy-associated thrombosis. This subgroup of individuals should be considered for thromboprophylactic therapy. Since in caucasians the estimated prevalence of combined factor V G1691A and prothrombin G20210A mutation is approximately 1 in 1000 women, a genetic screening for risk markers of VTE among pregnant women should be considered.

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INCREASED RISK FOR POSTOPERATIVE HEMORRHAGE AFTER INTRACRANIAL SURGERY IN PATIENTS WITH DECREASED FACTOR XIII – IMPLICATIONS OF A PROSPECTIVE STUDY

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Introduction: Coagulation Factor XIII (F XIII) encymatically cross-links fibrin monomers and enhances clot stability through incorporation of alpha-2-antiplasmin and fibronectin. Postoperative intracranial hematoma due to hemostatic abnormalities and hyperfibrinolysis remain severe complications after intracranial surgery. The clinical relevance of decreased F XIII after neurosurgical procedures with respect to hematoma formation is unclear. Therefore the aim of the present study was to prospectively investigate the incidence of postoperative decreased F XIII and to evaluate the association with significant postoperative hematoma.
Methods: 910 neurosurgical procedures were performed in 876 patients. Prothrombin time (PT), partial thromboplastin time, (PTT), platelets (PLTS), fibrinogen (F) and Factor XIII (F XIII) were monitored pre- and postoperatively and analysed to elucidate causes of major postoperative hematoma (defined requiring surgical evacuation).
Results:. 39 (4.3%) of the 910 prospectively included procedures were complicated by the development of a postoperative hematoma. Of these 39 patients 13 (33.3%) had a postoperative F XIII less than 60% compared to 61 (7%) out of 867 patients without hematoma ($p<0.001$ Fisher's exact test). F XIII was significantly lower in the hematoma group (97.89±24.98% preoperatively and 71.45±20.57% postoperatively) compared to the non hematoma group (113.66±27.31% and 93.46 ±24.37%, respectively, $p<0.001$ t-test). Therefore the relative risk to develop a postoperative hematoma is 6.6-fold increased for patients with postoperative F XIII less than 60% and 3.8-fold for patients with F XIII less than 80% preoperatively, respectively.
Conclusions: This is the first prospective study investigating the association of F XIII with postoperative hematoma formation after intracranial surgery. The results indicate that the role of postoperative decreased F XIII is currently underestimated. F XIII testing and specific replacement may reduce the risk of postoperative hematoma.

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DIFFERENT COAGULATION PROFILES IN MALIGNANT AND BENIGN BRAIN TUMOR PATIENTS

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Objective: Hemostatic abnormalities are common in patients with brain tumors which can pose intraoperative and postoperative management difficulties. The aim of this prospective study was to further evaluate the coagulation profile of patients with brain tumours undergoing surgery.
Method: From may 2000 to september 2001 132 patients with primary brain tumors and patients with cerebral metastasis admitted to our department and planned for surgery were included in this prospective study. Preoperative plasma level of prothrombin time (PT), partial thromboplastin time (PTT), antithrombin activity (AT), fibrinogen (Fib), factor XIII activity (FXIII), total tissue factor pathway inhibitor antigen (TFPI), von Willebrand factor antigen (vWFAg), C1-esterase inhibitor activity (C1-Inhibitor), Ristocetin cofactor activity (vW:RCo), D-Dimer (DD), tissue plasminogen activator antigen (tPA) and plasminogen activator inhibitor antigen (PAI) were tested in preoperative blood samples of all patients. Data were compared to the histological diagnosis which was classified as benign (tumors WHO grade I and II) and malignant (tumors WHO grade III and IV) for primary brain tumors. Cerebral metastasis were classified as malignant brain neoplasms.
Results: In patients with primary malignant brain tumors (n=34) and metastatic brain lesions (n=21) TFPI, AT, vWFAg, vW:RCo, C1-Inhibitor and tPA were significantly ($p<0.05$) elevated compared to patients with benign tumors (n=77), whereas PTT was significantly lower in patients with malignant tumors, respectively. PT, Fib, FXIII, DD and PAI did not show significant differences between both groups.
Conclusions: This is the first study describing the correlation of all these parameters with respect to malignancy in brain tumor patients. Further studies are needed to clarify the pathogenic mechanism and the clinical relevance of these data.

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IN NEONATES HIGHER DOSES ORGARAN MAY BE NEEDED TO ACHIEVE EFFECTIVE DOSES

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Background: Heparin induced thrombocytopenia (HIT) type II is a serious complication of heparin therapy, which may cause life-threatening thromboembolism. The platelet counts often fall below 100000/µl after onset of heparinization and will return to normal values after cessation of heparin.
Patient: A male neonate (3,5 kg) was admitted to our hospital because of a hypoplastic left heart syndrome. On his 16th day of life the Norwood I procedure was performed. Unfractionated heparin u was given since his birth for fluid therapy. Nine Days after surgery the thrombocytes dropped to 48,000/µl and HIT II was diagnosed and prophylactic treatment with Orgaran was started. Initially Orgaran was given as infusion and later additional by single doses twice a day. The anti-Xa-levels were determined twice a day and Danaparoid was used for the standard curve. We must increas the Orgaran doses step by step from 11 IE/kg/d to 71 IE/kg/die. Despite high doses of Orgaran the anti-Xa-activity was only two times in the desired level (0.4-0.7). No bleeding and thromboembolic complications occurred and the patient could be discharge with an anticoagulant therapy with aspirin and persantin.
Discussion: HIT is a rare but important differential diagnosis of thrombocytopenia even by children. We observed that our neonate required much higher doses of Orgaran to reach an adequate anti- Xa-level as was recommended.

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***REDUCTION OF BLOOD LOSS AND BLOOD TRANSFUSIONS AFTER CARDIAC SURGERY BY APPLICATION OF PLASMA COAGULATION FACTOR XIII**

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Objective: In a pilot study we could demonstrate that application of coagulation factor XIII reduces postoperative blood loss and need for transfusions after cardiac surgical operations. As the pilot study did only cover 22 patients, the aim of the present study was to reproduce these results in a larger patient population and to investigate if there is a dose dependant effect of the factor XIII medication.
Methods: 75 patients after coronary artery bypass grafting were included in a prospective, randomized, double blinded, placebo controlled study. Immediately after antagonization of heparin (needed for extracorporeal circulation) by protamine sulfate 25 patients received 2500 IE of factor XIII, 25 patients received 1250 IE factor XIII and 25 patients received placebo. Standard postoperative regimen concerning coagulation and volume replacement remained unchanged in either group. Groups were also not different concerning age, height, weight, extracorporeal circulation time, and routine laboratory parameters.
Results:

placebo group	1250 IE group	2500 IE group
Factor XIII before ECC (%)	107±26 108±21	113±19
Factor XIII after ECC (%)	65±17 63±14	63±11
Factor XIII after application. (%)	71±23 85±13	103±25
Drain volume 6h post-op. (ml)	271±133 261±111	227±114
Drain volume 12h post-op. (ml)	445±191 413±164	383±163
Drain volume 24h post-op. (ml)	738±257 692±244	655±224
Drain volume 36h post-op. (ml)	970±319 878±296	802±261
Drain volume 48h post-op. (ml)	1224±241 1002±416	970±350
Red cell packs (#)	3.4±2.4 2.6±2.3	2.6±1.9
Fresh Frozen Plasma (#)	0.4±1.0 0.6±1.6	0.1±0.6
Platelet transfusion (#)	0.1±0.5 0	0

There were no early bypass occlusions found in the 2500 IE group but 1 in the 1250 IE group and 2 in the placebo group.
Conclusions: We could reproduce and thus confirm our pilot study's results that plasma level of factor XIII are below normal values after extracorporeal circulation and that application of factor XIII reduces postoperative bleeding and need for blood transfusions. The effects are dependent on the plasma level of factor XIII, which itself is dependent on the amount administered directly after the operation. As a conclusion, the application of factor XIII should be in mind when treating postoperative bleeding after cardiac surgery.

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TEMPORARY INCREASE IN THE RISK OF RECURRENCE DURING PREGNANCY IN WOMEN WITH A HISTORY OF VENOUS THROMBOEMBOLISM

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There are two controversial recommendations to manage pregnant women with previous venous thromboembolism (VTE) (1) administration of prophylactic heparin or (2) clinical surveillance. In the general population pregnancy increases the risk for VTE about 5fold. To investigate, whether pregnancy temporarily increases the risk of recurrent thrombosis, we evaluated the recurrence rate during pregnancy in women with a history of VTE and compared it to the non-pregnant period. 109 women had at least one pregnancy (in total 180 pregnancies) without prophylaxis after a single VTE. To compare the risk of recurrent VTE during to the risk outside pregnancy we evaluated the time periods during pregnancy and when patients were not pregnant. Cox regression was used to estimate the relative risk of VTE due to pregnancy. The median age of our study population was 38 years (range 22–76 years) at inclusion and 24 years (range 14–42 years) at first VTE. In 39 women (40%) no thrombosis risk factor was identified. A natural inhibitor deficiency was present in 11%, factor V:R506Q in 38%, prothrombin gene G20210A in 8%, hyperhomocysteinemia (HHC) in 12%, elevated factor VIII in 12% and lupus anticoagulant in 3%, combinations were present in 24%.
43/109 women had a first recurrent event during a total observation period of 1014 years, 73 years with and 941 outside pregnancy. 8 events occurred during pregnancy, 5 during the first, 2 during the second and one during the third trimester. 4 women had a detectable abnormality (4 with heterozygous factor V:R506Q, two of these with additional HHC. In all women with recurrence during pregnancy a temporary risk factor had been present at first event. 35 women had a recurrent event without being pregnant. 25 recurrent events occurred without a temporary risk factor, 2, while on OC, posttraumatic or during immobilisation, respectively, and 4 after surgery. Recurrence rate per patient-year was 10.9% during pregnancy and 3.7% in the non-pregnant period. Using Cox regression analysis the estimated relative risk during pregnancy was 3.5 (95% CI 1.6–7.8, p=0.002).
Our data suggest that pregnancy leads to a temporary and more than three-fold increase in the risk of recurrent thrombosis. Temporary prophylactic administration of LMWH during pregnancy might reduce the risk. It remains to be established in well designed trials whether prophylactic anticoagulation is able to decrease the rate for pregnancy associated recurrent VTE.

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LOKAL ACTIVITY OF UROKINASE, IMMUNOHISTOCHEMICAL EXPRESSION OF UROKINASE AND UROKINASE-RECEPTOR IN AN ANIMAL MODEL OF ATHEROSCLEROSIS

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It has been shown that urokinase (UPA) and urokinase-receptor (UPA-R) play a critical role in cell proliferation and migration, e.g. tumor growth and invasion. We studied the course of time of immunohistochemical expression of UPA and UPA-R and the UPA-activity in an animal model of traumatic atherosclerosis. In 15 rabbits actin positive neointimal plaques were induced by balloon-denudation of the illiac artery. 5 healthy animals served as controls (c). 2, 4 or 8 days after the trauma the arteries were harvested, immunohistochemical staining for UPA and UPA-R (APAAP-technique) and visual scoring of the relative staining intensity in the media of the vessel were performed and the activity of UPA in homogenised arteries was measured using a chromogenic assay. Two days after trauma the immunohistochemical UPA- and UPA-R-expression in the media of the artery and the UPA-activity increased, reaching statistical significance at day 4 after trauma (mean±std in the relative staining intensity for UPA in traumatised rabbits 1,6±0,65 vs. 0,5±0,68 in controls, for UPA-R 0,75±0,25 vs 0±0 in controls and UPA-activity 952±189 vs 517±81 in controls). Eight days after trauma there was no more significant difference in immunohistochmical UPA- and UPA-R-expression and UPA-activity between traumatised animals and controls. Immunohistochemical UPA-and UPA-R expression and local UPA-activity show a typical time-pattern after vascular trauma underlining its importance for the proliferation and migration of vascular smooth muscle cells.

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RETROSPECTIVE INVESTIGATIONS IN NEUROSURGICAL PATIENTS SUFFERING FROM INTRACEREBRAL OR INTRASPINAL HEMORRHAGE OCCURING DURING THERAPY WITH PHENPROCOUMON

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Phenprocoumon is one of the most common oral anticoagulants for long-term treatment of thromboembolic events. Nevertheless, serious complications such as life threatening bleeding events may occur. The present study includes 46 patients (23 male and 23 female; mean age 64,9 years with a range from 33 to 84); all of them were treated with phenprocoumon and were admitted to the hospital because of acute cerebral or intraspinal hemorrhage. The indication for treatment with phenprocoumon was examined in a retrospective study. In 42 of our 46 patients (91%) treatment with phenprocoumon was obviously justified according to current guidelines; indication in these patients included atrial fibrillation, replacement of aortic or mitral valve, congestive cardiomyopathy, pulmonary embolism (PE) or deep vein thrombosis (DVT)within the past 6 months, mitral valve stenosis, peripheral and cerebrovascular occlusive disease, atrial septal defect and atrial thrombus. In 4 of our 46 patients (9%) there was obviously no indication for treatment with phenprocoumon. The first patient (59 years old) suffered from subarachnoid hemorrhage while taking phenprocoumon over 30 years after the first DVT. The second patient (47 years old) presented with hemorrhage of the basal ganglia and was treated with phenprocoumon over 18 months after the first DVT combined with PE.The third patient (72 years old) was admitted to the hospital because of multiple intracerebral hemorrhage; he also was treated with phenprocoumon after his first DVT 18 months ago and died 3 days after admission to the hospital. The fourth patient (49 years old) suffered from intracerebral hemorrhage during therapy with phenprocoumon over 36 months after the first DVT; further laboratory investigation in this patient revealed heterozygous factor V Leiden mutation. In none of these 4 patients hypertensive crisis or vascular malformation as possible aggravation factors of the bleeding event could be confirmed. Our data show that the indication for therapy with phenprocoumon should be critically reexamined from time to time to avoid potentially life threatening bleeding events, especially in patients after DVT. This may be even the more important in elderly patients with additional risk factors such as hypertension or diabetes.

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ALPROSTADIL (PROSTAGLANDIN E_1) IN THE THERAPY OF PATIENTS WITH RETINAL VEIN OCCLUSIONS

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Thrombotic retinal vein occlusion (RVO) are the second - after diabetic retinopathy - of the most frequent causes of vascular retinal disorders. Thrombotic central retinal vein occlusions happen more often in the elderly and rather in man.. The therapy of retinal vein occlusions is based on anti-inflammatory , fibrinolytic, and antithrombotic drugs as well as drugs which inhibit aggregation of platelets. In previous trials prostacyclin (PGI_2) was proved effective in the therapy of RVO. The aim of our present study was to assess the effectiveness of stable analogue alprostadil – prostaglandin E_1 in the RVO patients.
Prostaglandin E_1 was administrated to 15 patients (including 9 females and 6 males) with RVO for 2 weeks in the 3 hours lasting venous infusion at a dose of 40 µg of alprostadil. Mean follow-up was 15 months (within the range of 11-37 months). Ophthalmological examination including fluorescein angiography, physical examination and basic laboratory test were performed before and after the therapy. In six patients the influence of the therapy with alprostadil on deformability of red blood cells was studied using a shear stress Rheodyn diffractrometer. Blood for all tests was taken before the beginning of the therapy, after a week and after a fortnight.

The results were evaluated on the basis of ophtalmoscopic and angiographic examination of retina and visual acuity (distant and close). Reduction of oedema , resorption of effusions and normalizing of blood flow, were considered as improvement. Alprostadil therapy revealed improvement in 11 patients (73,4%), no improvement in 2 patients (13,3%), and worsening in 2 cases (13,3%).Two weeks of the alprostadil therapy resulted in an increase of red blood cell deformability.
It seems that alprostadil acts via prostacyclin receptor and shows many favourable prostacyclin like effects.
Our results show that the treatment with alprostadil leads to a significant clinical improvement in patients suffering from RVO.

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A DIAGNOSTIC TOOL FOR DETECTION FOR NON-MUSCLE MYOSIN HEAVY CHAIN (NMMHC) OF GIANT PLATELET DISORDER PATIENTS

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Purpose: Hereditary giant platelet disorders (i. e. May-Hegglin Anomaly (MHA), Sebastian Platelet syndrome (SPS), Fechtner syndrome (FS)) are a heterogeneous group of dominant inherited thrombocytopenias. They are characterized by a triad of hematological manifestations: granulocyte inclusion bodies, thrombocytopenia and large platelets. Additionally, FS is associated with nephritis, cataract, neurosensorial deafness. Often those syndromes are misdiagnosed as chronic autoimmune thrombocytopenia. Recently, we identified mutations of the MYH9 gene encoding the 224 kDa heavy chain of the non-muscle myosin (NMMHC-A) in eight patients (four different families). In the present study we assessed the distribution of NMMHC-A protein in granulocytes in these patients by immunocytochemistry.
Methods: Immunocytochemical detection of the NMMHC was performed using air-dried peripheral blood smears and anti-NMMHC-A polyclonal antibodies (Biomedical Technologies, Stoughton MA). Blood smears fixed and permeabilized in –20°C cold acetone for 5 min, air dried over night or stored at room temperature for up to 4 weeks were incubated with anti-NMMHC-A polyclonal antibodies diluted 1:5000 for 60 min in a moist chamber. Antibody binding was detected by an alkaline phosphatase method (streptavidin-biotin complex LSAB2 kit, DAKO Corporation, Carpinteria, CA). Levamisole was added to the chromogen for alkaline phosphatase to inhibit endogenous enzymatic activity. Slides were counterstained with Mayer`s Hematoxylin and mounted with the aqueous–based Faramount Medium (DAKO).
Results and Conclusion: In normal controls NMMHC-A protein is dispersed in granulocytes and platelets. In hereditary giant platelet disorders the protein is clustered in granulocytes resembling the inclusion bodies typically seen in May-Grünwald Giemsa staining. No obvious differences in NMMHC-A protein distribution in platelets was seen. The immunocytochemical method is a sensitive assay for detection of granulocyte inclusion bodies associated with MYH-9 disorders.

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PREDICTIVE VALUE OF PARTIAL THROMBOPLASTINE TIME (PIT) AS A PREOPERATIVE SCREENING TEST FOR BLEEDING COMPLICATIONS IN CHILDREN

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Background: Determination of PTT is frequently performed as a preoperative routine diagnostic test in order to identify patients at elevated bleeding risk during or after surgery.
Aim: This study was designed to assess the predictive capacity of preoperative PTT-determination for bleeding complications of surgery.
Methods: Records of 100 children scheduled for ENT surgery were retrospectively analyzed with regard to indications of bleeding disorder by family history, patient history and physical examination. Patients with indications of bleeding disorder by history or physical examination, liver disease, or anticoagulant therapy were excluded.A PTT of 28 to 40 seconds was considered as a normal test result.
Results: 14 patients had a prolonged PTT (maximum, 75 seconds). Four patients experienced unexpected bleeding during or after surgery; one patient with minimal prolonged PTT (40.2s), three patients with regular PTT. Based on these results, the sensitivity of PTT (test positives among bleeding patients) was 25%, the specificity (test negatives among healthy) was 75%. The positive predictive value (bleeding cases among patients with prolonged PTT) was 7%. The negative predictive value (healthy among patients with normal PTT) was 93%. The efficiency, as calculated correct results among all test results, was 71%.
Conclusion: The low predictive capacity of PTT as a routine screening test for surgical bleeding complications in children of our study confirms results of earlier studies performed among adults. ENT surgery aimed at reducing the probability of respiratory tract infections which can cause prolongation of PTT should not be delayed because of mildly elevated test results.

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REDUCED THROMBIN FORMATION CAPACITY IN SEVERE HAEMOPHILIA IS ASSOCIATED WITH HYPOSTIMULATION OF PRO- AND ANTICOAGULANT PATHWAYS

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Haemophiliacs of identical severity frequently suffer from variable haemorrhagic phenotypes. To investigate the hypothesis, that individual differences in the ability to effectuate compensatory coagualtion activation account for differing bleeding phenotypes of severe haemophiliacs, we performed extensive coagulation analyses. Twenty-one males with severe haemophilia (13 A, 8 B) were investigated and the results were compared to those of healthy controls.
Procoagulant factor activity analysis yielded comparable results for factor I, V, VII, X and XI in both groups. Activities of factor II, VIII (haemophilia B only), IX (haemophilia A only) and XII and of HMW kininogen and of prekallikrein were significantly lower in haemophiliacs. Von Willebrand factor was significantly higher in the haemophilia group. Anticoagulant factor analysis showed similar antithrombin activities in both groups, protein C and S activities and antigens were significantly lower in haemophiliacs. The endogenous thrombin potential (ETP) is a global function test of the plasmatic coagulation system. Median ETP in haemophiliacs was 157 AU (141-169) and significantly lower ($p<0.0001$) compared to the ETP of 217 AU (203-258) in controls. Correlation analysis revealed a significant positive correlation of factor II ($r=0.65$; $p=0.002$) and IX ($r=0.64$; $p=0.019$) activities with ETP values. Insignificant positive associations were observed for HMW kininogen and prekallikrein activities. No clear associations were found for the other procoagulant factor activities. In addition, significant positive correlations were observed between protein C and S activities or antigens and ETP values ($r=0.45$; $p=0.04$ for protein C activity; $r=0.46$; $p=0.04$ for protein S activity).
From the close correlation with lowered ETP values we conclude, that the reduced activity of 'unaffected' procoagulant factors in severe haemophilia is caused by a permanent hypostimulation of feedback activation mechanisms, primarily of the intrinsic cascade. The decreased activities of the natural inhibitors protein C and S are most probably due to a permanent hypostimulation of the thrombin-thrombomodulin pathway, caused by the strongly reduced thrombin formation capacity in severe haemophilia. As no correlation of any test result with clinical findings could be demonstrated, our findings do not offer an explanation for different haemorrhagic phenotypes in severe haemophilia.

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DYSFUNCTIONAL COAGULATION REGULATION IN SEVERE HAEMOPHILIA IS ASSOCIATED WITH COMPENSATED HYPERFIBRINOLYSIS AND ABSENCE OF PLATELET ACTIVATION

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Haemophiliacs of identical severity frequently suffer from variable haemorrhagic phenotypes. To investigate the hypothesis, that individual differences in the ability to effectuate compensatory activation account for the differing bleeding phenotypes of severe haemophiliacs, we performed extensive coagulation analyses. Twenty-one males with severe haemophilia (13 A, 8 B) were investigated and the results were compared to healthy controls.
TPA, PAI1 and TPA-PAI1 complex concentrations were elevated significantly in haemophiliacs, to exactly double the concentrations of respective test results in controls. Plasminogen and plasmin inhibitor activities were within the normal range in haemophiliacs, albeit significantly lower compared to controls. Plasmin-plasmin inhibitor complex concentrations were similar in both groups. Platelet function was assessed by thrombelastography, PFA 100 test, adhesion, aggregation and flow cytometric analysis of native and stimulated platelets. Clot formation capacity, assessed by thrombelastography and PFA 100 test, was reduced significantly in haemophiliacs. All other platelet function tests yielded similar results in both groups. No elevated baseline or post-stimulation platelet reactivity was observed in haemophiliacs.

We hypothesize, that ineffective haemostasis in haemophiliacs evokes a protracted stimulation of the coagulation system. From raised TPA concentrations we infer, that a simultaneous activation of the fibrinolytic systems occurs. As the regulation of the fibrinolytic system is unimpaired, this inadvertent activation is counterbalanced by raised PAI1 activity. The resultant stimulus on fibrinolytic activity is minimal, indicated by only slightly decreased plasminogen and plasmin inhibitor activities and normal plasmin-plasmin inhibitor complex concentrations. Although tissue-factor based coagulation initiation is thought to be normal in haemophilia, coagulation propagation is severly disturbed. The process of coagulation propagation is indispensable for the generation of thrombin in 'haemostatic' concentrations. As these haemostatic amounts of thrombin cannot be generated in haemophilia and as thrombi is a major stimulant of platelet reactivity, no 'compensatory' platelet activation can be achieved. This explains, why platelet reactivity remains neutral, in spite of a severe haemorrhagic condition.

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PHARMACODYNAMICS AND PHARMACOKINETICS OF THE ORAL DIRECT THROMBIN INHIBITOR *XIMELAGATRAN

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Thrombin is the central molecule in coagulation and is therefore a key target in the prevention of thromboembolic disorders. The current therapeutics for anticoagulation are heparins, which are indirect thrombin inhibitors, and coumarins which modulate the synthesis of vitamin K-dependent proteins. As the only oral anticoagulant in clinical use, coumarins fulfil a very important clinical need for patients requiring long-term anticoagulation. However, coumarins have both pharmacodynamic and pharmacokinetic drawbacks, such as narrow therapeutic index, slow on- and offset of action and a large inter- and intra-individual variability. Ximelagatran is the first new oral anticoagulant in advanced clinical investigations since more than half a century. As compared to coumarins Ximelagatran has an advantages pharmacodynamic and pharmacokinetic profile: a more shallow does-response curve with a consequently wider therapeutic index between effect and bleeding, rapid on-/offset of action, no food-drug or drug-drug interaction, and a low variability observed in the anticoagulant response.
Based on these properties a large clinical programme in various indications has been set up and have led to the design of studies evaluating the effects of Ximelagatran given in fixed doses without routine anticoagulation monitoring. Thus, oral direct thrombin inhibitors, such as Ximelagatran, are set to provide a further breakthrough in the prophylaxis and treatement of thrombosis.

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***THE PRODRUG PRINCIPLE USED IN ORAL ANTICOAGULANTS AS EXAMPLIFIED BY THE ORAL, DIRECT THROMBIN INHIBITOR XIMELAGATRAN, AND IT'S ACTIVE FORM MELAGATRAN**

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Introduction**:** The lack of good and reproducible oral absorption is a problem for the development of many thrombin and factor Xa inhibitors. This is often due to the basic functionality selected to fit the arginine side-pocket of thrombin or factor Xa. Melagatran is a direct thrombin inhibitor, highly charged at gastro-intestinal pH, with insufficient oral bioavailability in man. To improve the gastro-intestinal absorption of new inhibitors focus has been on reducing the equilibrium dissociation constant (pKa) of the basic functionality. We found that, by using the prodrug principle, i.e. addition of protecting residues, we were able to build on melagatran's good pharmacodynamic properties and suitable pharmacokinetic properties after parenteral administration.
Results**:** The presented studies describe the new oral anticoagulant ximelagatran, a prodrug with two protecting residues added to melagatran. Absorption properties *in vitro*: Ximelagatran is uncharged at intestinal pH while melagatran is charged. Ximelagatran is 170 times more lipophilic (octanol water partition coefficient) than melagatran. As a result, the *in vitro* intestinal permeability coefficient across Caco-2 cells is 80 times higher for ximelagatran than for melagatran.
Pharmacokinetic studies in healthy volunteers: Ximelagatran is rapidly metabolised to melagatran in man. The oral bioavailability of ximelagatran, measured as the AUC of melagatran in plasma, is 20 % compared to 3-7 % with melagatran. The variability in AUC is much lower with ximelagatran (coefficient of variation, CV 20%) than with melagatran (CV 38%). When ximelagatran is administered together with food the AUC for melagatran is not clinically significantly changed, while administration of melagatran with food decreased the AUC with about 80%, representing a bioavailability of ≈1%.
Pharmacodynamic properties: Ximelagatran is inactive towards human α-thrombin compared to melagatran [inhibition constant (*Ki*) ratio, 185 times], a potential advantage for patients with silent gastro-intestinal bleeding. Ximelagatran has been shown to be an effective and safe anticoagulant for the prevention of venous thromboembolism in patient following orthopaedic surgery, for the initial treatment of patients with deep venous thrombosis and for stroke prevention in patients with atrial fibrillation.
Conclusion: By the use of the prodrug principle ximelagatran endows the direct thrombin inhibitor melagatran with pharmacokinetic properties required for oral administration without compromising its promising pharmacodynamic properties.

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UPA-SILICA-PARTICLE SYSTEM (SP-UPA) TO INVESTIGATE UPA-UPAR INTERACTION AND TO TEST SYNTHETIC PEPTIDE ANTAGONISTS

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The serine protease uPA (urokinase-type plasminogen activator) and its cell surface receptor (uPAR, CD87) are important key molecules for tumor cell invasion and metastasis. Binding of uPA to uPAR on tumor cells exerts various cell responses such as migration, adhesion, proliferation, and differentiation. Hence, the uPA/uPAR system is a potential target for tumor therapy. Here, we present a new technology involving micro silica particles coated with uPA (SP-uPA) and reacting with recombinant soluble uPAR (suPAR), to rapidly assess the antagonistic potential of uPA-peptides by flow cytofluorometry (FACS). For this, we used silica beads of 10 um in diameter, the size of a tumor cell. HMW-uPA was covalently coupled to the bead surface using the EDC/NHS method. Recombinant, soluble uPA receptor (suPAR) was added, and the binding to uPA identified by addition of monoclonal antibody HD 13.1 recognizing uPAR and cyan dye (cy5) labeled antibody to mouse IgG as the secondary antibody. Thereby, it was possible to test naturally occurring ligands (HMW-uPA, ATF) as well as various synthetic uPA-derived peptides. The results obtained with the noncellular SP-uPA/uPAR system are highly comparable to those obtained with a cellular system involving FITC-uPA and the promyeloid cell line U937 as the source of uPAR.

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***TRAVELLERS' THROMBOSIS 2001 – SUMMARY OF AN EXPERT MEETING IN VIENNA**

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On June 9, 2001, an expert meeting was held in Vienna in order to define the term "travellers' thrombosis", to define patient populations at various levels of risk, and to give guidance for prevention of travellers' thrombosis.
Travellers' thrombosis has been defined as any venous thromboembolic event timely related to a journey lasting several hours in a sitting position in individuals who did not have signs of acute venous thromboembolism before travelling.
Individuals travelling several hours in a sitting position seem to be at low risk if they do not have additional risk factors.
A moderate risk has been defined for these travelling individuals and in case of pregnancy or post-partum or in case of accumulation of two of the following predisposing risk factors: Age over 60, clinically relevant cardiac disease, documented thrombophilia/family history of venous thromboembolism, large varicose veins or chronic venous insufficiency, oral contraceptives or hormone replacement therapy, obesity (BMI>30), dehydration.
A high risk has been defined for travellers with one of the following risk factors: History of previous venous thromboembolism, manifest malignant disease or other severe illness, plaster cast immobilisation of the lower extremity, recent surgery with high risk for thrombosis.
The recommended modalities for prevention are tailored to the individual risk constellation. Despite the lack of data from randomised trials, the use of an anticoagulant drug such as low molecular weight heparin may be recommendable for high-risk patients. This recommendation, however, is only based on extrapolation from results of clinical trials in different patient populations where these substances have been studied.

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***ORAL DIRECT THROMBIN INHIBITION – POTENTIAL FUTURE INDICATIONS**

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Thrombin is well known as the pivotal enzyme in the formation of fibrin clots and platelet activation and it has also been recognized for its central role as a growth stimulator and initiator of non-anticoagulant cellular effects. Thus, it may be interesting to speculate on potential future indications for anti-thrombin therapy.
With the emergence of orally administered direct thrombin inhibitors, such as Ximelagatran, that are efficacious, well tolerated, and do not require monitoring, a breakthrough in the prophylaxis in major orthopedic surgery is imminent. In the meantime, prolonged prophylaxis beyond discharge has become standard of care in several countries and therefore, it is an urgent clinical need to assess the efficacy and safety of Ximelagatran in this indication. Long-term prophylaxis also seems to be beneficial in cancer patients with and without surgery. In cancer patients, the risk for thrombosis is significantly increased by tumor procoagulants and the release of tissue factor, accompanied by comorbid predisposing factors such as prolonged bed rest, age and concomitant therapy such chemotherapy administered via indwelling central venous catheters. In these patients, the antithrombotic activity as well as potential anti-cancer effects of an oral thrombin inhibitor deserve further investigation.
Large clinical development programs are under way in treatment of venous thromboembolism, and in patients with atrial fibrillation or acute coronary syndromes. If it can be demonstrated, that the efficacy and safety of Ximelagatran will be at least equal to that of the conventional anticoagulants in these indications, it would significantly facilitate the management of thrombotic disorders as well as, possibly, lead to better results. Thus, Ximelagatran may one day replace heparins and vitamin K antagonists in prevention and treatment of venous and arterial thromboembolism.
Furthermore, Ximelagatran could contribute to improvements of antithrombotic therapy in indications such as vascular bypass surgery, peripheral artery disease, myocardial infarction, ischemic stroke, various types of thrombophilia, stent implantation, and treatment of patients with implantation of prosthetic heart valves. Some of these indications are presently studied with conventional anticoagulants. Thus, it may be premature to speculate whether an oral thrombin inhibitor like Ximelagatran could replace other anticoagulations in these clinical settings.

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***PREVENTION OF SEVERE VENOUS THROMBOEMBOLISM IN PATIENTS UNDERGOING TOTAL HIP OR KNEE REPLACEMENT. A RANDOMISED COMPARISON OF LOW MOLECULAR WEIGHT HEPARIN (REVIPARIN) WITH UNFRACTIONATED HEPARIN. THE ECHOS TRIAL**

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Peri- and postoperative prevention of venous thromboembolism (VTE) in patients undergoing major orthopedic surgery has become standard of care and low molecular weight heparin (LMWH) has been used most frequently in this indication. However, no prospective data are available comparing preoperative onset of LMWH-prophylaxis to 7500 IU unfractionated heparin (UFH) bid and using severe venous thromboembolism (proximal deep vein thrombosis, pulmonary embolism and/or death) as primary endpoint. Furthermore, in previous trials the efficacy and safety of LMWH has been studied in either total hip replacement (THR) or total knee replacement (TKR), but not in a mixed patient population.
We performed a prospective, randomised, double-blind trial with a parallel group comparison of a low molecular weight heparin, reviparin sodium, and standard unfractionated heparin in patients undergoing elective THR or TKR. Both patient populations were randomised in two strata. Joint replacement was performed with cemented or non-cemented prostheses. 2018 patients were randomly allocated to receive once daily subcutaneous injections of reviparin sodium, 4200 anti-Xa I.U. per day plus matching placebo (n=1014) or twice daily 7500 IU unfractionated heparin sodium (n=1007). Patients were eligible for the trial if they were scheduled for elective total hip or total knee replacement. All patients underwent systematic bilateral phlebography on day 14. Phlebograms from 1628 patients were blindly adjudicated by a central adjudication committee.
Results: The primary composite endpoint (incidence of venographically diagnosed proximal DVT, pulmonary embolism, and/or death) was significantly lower in the reviparin group compared with UFH and a significant reduction in VTE was maintained in the follow-up period up to 6-8 weeks. The secondary endpoints was the total incidence of DVT (i.e. distal + proximal). Primary safety endpoint was the incidence of major bleeding events; no difference was seen between the two treatment groups.
Conclusions: Prophylactic treatment with reviparin significantly reduces the incidence of severe VTE compared with UFH in patients undergoing elective hip or knee replacement without increasing the bleeding risk.

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***ADVANCES IN STROKE THERAPY AND PREVENTION**

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Stroke is the second leading cause of death worlwide (WHO 1999). Despite being a disease of the elderly, only half of strokes occur at ages over 75 years. Recurrence rates of stroke vary between 5%/year (microvascular strokes) and 15%/year (atherothrombotic stroke in patients at multiple vascular risks).
Systemical thrombolysis with rt-PA and multimodal management on a stroke unit are the hallmarks of acute stroke therapy. Thrombolysis within three hours of stroke onset reduces the risk of death or permanent disability by 10% as compared to non-treatment, taking into account a risk of intracerebral hemorrhage of about 8%. Thrombolysis is underused because of the bleeding hazard, delayed onset-to-needle times and the reluctance to treat too severe and too mild stroke syndromes. Neuroprotectives did not reduce the neurological deficits by themselves, neither did they work as an adjunct to thrombolysis. Within hours of stroke onset, the maintenance of high mean blood pressure, normal blood glucose levels, low-to-normal body temperature, swallowing assistance and physical therapy aid to prevent stroke deterioration. This is what stroke units may achieve.
The only evidence-based reduction of acute stroke recurrence within the first weeks after stroke is provided by aspirin 100-300 mg. Although incompletely studied, heparin and heparinoids at all dosages and time intervals after acute stroke had no net benefit. Minor risk reductions in stroke recurrence or stroke progression were offset by an increased cerebral bleeding risk. Clopidogrel (in atherothrombotic high-risk patients) and coumadins (after cardioembolic stroke) have clearcut longterm benefits over aspirin, but have not been studied within weeks after stroke. There is no clinical experience with GPIIb/IIIa-antagonists or the combination of different platelet inhibitors (clopidogrel +aspirin, dipyridamol + aspirin) in acute stroke. Ongoing studies are adressing the role of NO-stimulating/ cholesterol reducing drugs (statins), heparin in stroke subtypes, and antiinflammatory medication.

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***COUMARIN-INDUCED SKIN NECROSIS**

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Skin necrosis is a rare but serious complication of oral anticoagulant treatment. It occurs almost exclusively in patients with venous thrombosis between the 3rd and 10th day after starting anticoagulation. Possible causal relations as well as treatment modalities are discussed in this review.

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ANALYSIS OF PLATELET HEMOSTATIC CAPACITY IN PATIENTS WITH CEREBRAL ISCHEMIA BEFORE AND UNDER ASS-MEDICATION USING THE PFA-100

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Introduction: Evidence from recent studies shows that the antiplatelet effect of ASA may be highly variable. Therefore it appears necessary to measure the prevalence of ASA-Non-Responders in patients with cerebral ischemia (TIA, PRIND, Stroke).
Methods: We studied groups with a first ischemic event (n=57), patients affected by recurrent stroke whilst during medication of 100mg ASA (n=50), patients in clinically stable status for at least 6 months after a previous cerebral ischemic event (n=37), and a healthy control group (n=39). The patients of the both groups with acute cerebral ischemic events were examined directly by admission. Platelet Hemostatic Capacity was determined by measuring closure time (ct) using PFA-100 collagen/epinephrine and collagen/ADP cartridges. ASA-Non-Responder was defined as having a normal ct in PFA/Epi (85-165 sec).

Results:

Patients with first events:

	admission	after 5 d	after 10 d	after 30 d	after 90 d
∅CT PFA/Epi	130 sec	205 sec	206 sec	256 sec	221 sec
CT <85	1	0	0	0	0
CT 85–165	54	19	12	2	6
CT>165	2	31	24	25	18

Patients with recurrence:

∅CT PFA/Epi	230 sec
CT≤165	14 (28%)
CR>165	36

Patients with stable clinic:

	first analysis	after 30 d	after 90 d
∅CT PFA/Epi	254 sec	251 sec	254 sec
CT≤165	6 (16%)	5 (13%)	4 (12%)
CR>165	31	32	29

Healthy volunteers:

∅CT PFA/Epi	132 sec
CT<85	0
CT 85–165	38
CT>165	1

Conclusions: There is a normal platelet function in patients with first cerebral ischemic events, apart from sporadic exceptions. In approximately 30% of the patients medication with 100 mg ASA per day did not lead to a measurable prolongation of the closure time. Accordingly 28% of patients with recurrence of an cerebral ischemic event during medication of 100 mg ASA likewise have a normal closure time. The relevance of these data is demonstrated by the fact that clinically stable patients in only 15% do not respond to ASA. The data indicate that 10-15% of cerebral ischemic events may be related to an insufficient effectiveness of the ASA prophylaxis and therefore monitoring of the effectiveness of ASA therapy seems appropriate.

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***PLATELET TRANSFUSION IN CARDIOVASCULAR SURGERY**

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Numeric and qualitative platelet abnormalities are very common during cardiovascular surgery and are estimated to determine the majority of non-surgical transfusion requirements. Depending on diagnostic criteria, 4%-32% of patients undergoing cardiopulmonary bypass (CB) suffer from excessive bleeding with a 3%-7% incidence of mediastinal reexploration. Thrombocytopenia is mainly caused by blood loss, haemodilution and increased turnover of activated platelets during CB. Platelet dysfunctions may originate from extracorporal circulation, hypothermia or drugs. Their features comprise loss or changes of platelet glycoproteins, disturbed signal transduction, changes in platelet adhesion molecules and prolonged bleeding time.
Indications for platelet transfusion can be prophylactic or therapeutic. Platelet concentrates should be given in patients with bleeding complications at a platelet count of <50 G/l or in individual cases depending on the severity of bleeding at >50G/l (1). Patients undergoing emergency surgery treated with platelet inhibiting agents may be candidates for prophylactic platelet transfusion and/or additional platelet function stabilising therapy (i.e. desmopressin, aprotinin, tranexamic acid). Cardiovascular surgery in patients with inherited platelet disorders is very rare but a clear indication for preoperative and prolonged postoperative platelet transfusion therapy. When platelets are given for treatment of bleeding the usual initial dose is one therapeutic unit (2-4 x 10^{11} platelets). Subsequent transfusions may be required to stop bleeding by maintaining platelet counts above 50 G/l (2). In prophylactic settings the dose depends on the severity of platelet dysfunction. There is a debate whether preoperative testing of platelet function should be included into routine screening programs. At present there is no convincing evidence that these tests are superior to other coagulation profile tests in predicting excessive blood loss.
M. Contreras: Final statement from the consensus conference on platelet transfusion. Transfusion 1998, 38: 796-7
Leitlinien zur Therapie mit Blutkomponeneten und Plasmaderivaten. Herausgegeben vom Vorstand und Wissenschafltichen Beirat der Bundesärztekammer 2001

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***THRIVE – THE NEW REGIMEN OF XIMELAGATRAN FOR INITIAL AND LONG-TERM TREATMENT OF VENOUS THROMBOEMBOLISM**

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The treatment of choice in patients with venous thromboembolism (VTE) is the initial administration of subcutaneous low-molecular-weight heparin (LMWH) followed by oral anticoagulation. APTT-adjusted intravenous heparin is less effective and safe as compared to LMWH. The limitations of therapy are laboratory controls, recurrent thromboembolism, and bleeding complications. In addition, after termination of oral anticoagulation, VTE may re-occur in up to 25% of patients within 2 years. Oral thrombin inhibitors with good bioavailability may offer an alternative due to a lacking need to control anticoagulant effect as well as an improved efficacy and safety profile.
Anticoagulation with the oral direct thrombin inhibitor Ximelagatran is currently investigated in the THRIVE (Thrombin Inhibition in Venous Thromboembolism) program including treatment of acute VTE and prophylaxis of recurrent events of VTE in multinational, prospective, and double-blind clinical studies.
The initial treatment of VTE and the prophylaxis of recurrent events are investigated in a prospective, randomised, double-blind, double-dummy, controlled study comparing twice-daily oral Ximelagatran with subcutaneous Enoxaparin plus oral Warfarin over 6 months. The available overall results will be presented.
After termination of oral anticoagulation for prophylaxis of recurrent events new VTE are observed in up to 25% of patients within 24 months. To investigate the reduction in the recurrent VTE after cessation of oral anticoagulation, twice daily oral Ximelagatran is compared to placebo in a prospective, randomised, double-blind clinical trial over 18 months. Based on clinical symptoms, a 6% re-occurrence of VTE is estimated during placebo and a 2% recurrence during treatment with Ximelagatran. The inclusion of patients into the study is terminated. Available interim results will be reported.

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2-YEARS FOLLOW UP OF PATIENTS WITH ACUTE DEEP VEIN THROMBOSIS ON THE INCIDENCE OF RECURRENT THROMBOEMBOLISM, MORTALITY AND BLEEDING COMPLICATIONS

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Patients with venous thromboembolism (VTE) are currently followed up for 3 to 6 months for the re-occurrence of VTE, mortality or major bleeding events. Longer follow up periods is available from cohort studies aiming to define the development of postphlebitic syndrome, the clinical significance being debated. We have followed up for 2 years patients with VTE treated initially with body weight independent fixed dose of subcutaneous low-molecular-weight heparin (LMWH) or aPTT-adjusted intravenous unfractionated heparin (UFH).
Data were available from 203 of 265 and 220 of 273 patients initially randomised to LMWH Certoparin and UFH, respectively (Thromb Haemost 2000; 83:652-6). VTE re-occurred in 9.4% and 12.3% of the two treatment regimens being on or off vitamin-K antagonists. 11.8% and 14.1% of patients died within the 2-years observation period independently from the presence of cancer. Bleeding episodes occurred in 10.3% and 11.8% of patients initially randomised to LMWH and UFH, respectively. The data demonstrate that the initial effects of reduced incidences of recurrent VTE events, mortality and bleeding episodes persist for at least 2 years in patients treated initially for acute VTE with LMWH compared to UFH.

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***CLINICAL RELEVANCE OF COAGULATION PARAMETERS DURING TREATMENT WITH NEW ANTITHROMBOTICS**

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Specific factor Xa and thrombin inhibitors as well as activated protein C are currently developed for clinical use or have recently been proved. The main importance for development of new antithrombotic drugs is due to the requirement for laboratory monitoring. Laboratory methods for measuring conventional anticoagulants are very difficult to standardize or are poorly standardised. Therefore, one of the aims of new antithrombotic drugs, i.e. of factor Xa and thrombin inhibitors, is to avoid laboratory monitoring routinely. The clinical efficacy of activated protein C should be monitored by specific coagulation parameters, such as D-dimer. Pentasaccharide, the newly developed specific factor Xa inhibitor can be monitored by measuring the inhibition of factor Xa by chromogenic or coagulation test systems, which determine specifically the inhibition of this serine protease. APTT and activated clotting time are not influenced by pentasaccharide in therapeutic dosages. Standardisation of methods using the pentasaccharide has still to be performed.
Direct thrombin inhibitors, of which melagatran are currently in phase III clinical trials, can be monitored using ecarin clotting time and APTT. They're some minor differences between melagatran, hirudin, and argatroban on these coagulation parameters. Hirudin is currently evaluated in an inter-laboratory control study, which identifies the methods for quantification of the concentration and the biological effects of thrombin inhibitors. It is likely, that ecarin-clotting time is the method, which determines all thrombin inhibitors by a linear prolongation of the coagulation times with increasing dosages. That is in contrast to APTT and ECT, which determine the inhibitors only in low concentrations, whereas higher concentrations do not prolong anymore these coagulation parameters. Currently, clinical trials are performed without monitoring. However, over- or underweighted patients, children, impairment of liver and renal function and extracorporeal circulation may necessitate measurement of the new antithrombotic drugs.

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REDUCTION OF RECURRENCE OF VENOUS THROMBOEMBOLISM OVER 6 MONTHS IN PATIENTS TREATED INITIALLY WITH SUBCUTANEOUS BODY-WEIGHT INDEPENDENT LOW-MOLECULAR-WEIGHT HEPARIN CERTOPARIN

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Body weight adjusted subcutaneous as well as body weight independent low-molecular-weight heparin (LMWH) has been proven to be at least as effective and safe as aPTT-adjusted intravenous unfractionated heparin (UFH) for the initial treatment of patients with acute venous thromboembolism. However, there is no evidence that the initial benefit may last for a 6-month follow-up during oral anticoagulation. Two randomised prospective clinical trials with almost identical designs were pooled including patients with proven proximal deep-vein thrombosis (DVT) receiving fixed-dose, body-weight independent subcutaneous LMWH Certoparin (8,000 anti-factor Xa U b.i.d) for 10 to 14 days or aPTT-adjusted intravenous UFH for 5 to 12 days. Oral anticoagulation was initiated between days 2 and 7 and continued for 6 months. Primary endpoint was the recurrence of clinical VTE during 6 months. Secondary endpoints were major bleeding and mortality. 1758 patients randomly received either UFH (n=865) or LMWH (n=893) after objective confirmation of the diagnosis. They aged 61.5±14.6 (UFH) and 60.7±14.4 (LMWH) years and weighted 80.6±15.1 (UFH) and 81.3±15.7 kg (LMWH), respectively. VTE re-occurred in 5.1% and 3.1% of patients during 6 months (risk reduction 0.62, confidence interval 0.39-0.98, p=0.041, Fishers exact test). Major bleeding complications and death occurred more frequently in patients randomised to UFH compared to LMWH: 1.7% versus 0.8% and 3.6% versus 2.1% (not significant). The initial treatment of acute DVT in adults with fixed dose, body weight-independent subcutaneous LMWH Certoparin is more effective than UFH to reduce the recurrent events of venous thromboembolism.

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***LMWH CERTOPARIN REDUCES SIGNIFICANTLY THE COMBINED OUTCOME OF RECURRENCE VENOUS THROMBOEMBOLIC EVENT, MORTALITY AND MAJOR HEAMORRHAGE IN PATIENTS WITH ACUTE DVT**

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Both, body-weight independent (Thromb Haemost 2000, 85:652-656) as well as body weight adjusted subcutaneous low-molecular-weight heparin (LMWH) has been proven to be at least as effective and safe as aPTT-adjusted intravenous unfractionated heparin (UFH) for the initial treatment of patients with acute venous thromboembolism. However, there is no evidence that the initial benefit may last for a 6-month follow-up during oral anticoagulation. Two randomised prospective clinical trials with almost identical designs were pooled including patients with proven proximal deep-vein thrombosis (DVT) receiving fixed-dose, body-weight independent subcutaneous LMWH Certoparin (8,000 anti-factor Xa U b.i.d) for 10 to 14 days or aPTT-adjusted intravenous UFH for 5 to 12 days. Oral anticoagulation was initiated between days 2 and 7 and continued for 6 months. Primary endpoint was the recurrence of clinical VTE during 6 months. Secondary endpoints were major bleeding and mortality. 1758 patients randomly received either UFH (n=865) or LMWH (n=893) after objective confirmation of the diagnosis. They aged 61.5±14.6 (UFH) and 60.7±14.4 (LMWH) years and weighted 80.6±15.1 (UFH) and 81.3+15.7 kg (LMWH), respectively. VTE re-occurred in 5.1% and 3.1% of patients during 6 months (risk reduction 0.62, confidence interval 0.39-0.98, 2p=0.041, Fishers exact test). Major bleeding complications and death occurred more frequently in patients randomised to UFH compared to LMWH: 1.7% versus 0.8% and 3.6% versus 2.1% (not significant). The initial treatment of acute DVT in adults with fixed dose, body weight-independent subcutaneous LMWH Certoparin is more effective than UFH in reducing the re-occurrence of VTE.

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OXIDIZED PHOSPHOLIPIDS AS THE REGULATORS OF PLATELET RECEPTOR ACTIVITY

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Introduction: Activated platelets promote thrombus formation, and release vasoactive mediators which induce vasoconstriction and remodeling of the vessel wall. The relation between platelets and lipids responsible for the biological activity of modified lipoproteins are still under investigation. The aim of the study: The influence of native and ex vivo oxidized lipoproteins enriched with oxidized 1-palmitoyl-2-arachidonoyl-snglycero-3-phosphorylcholine (ox-PAPC) on platelet membrane receptor expression and aggregation was studied. Methods: Influence of native and oxidized lipoproteins (5-100 mg protein/ml); oxPAPC (0.5-50 mg/ml); ADP (1-10 mM) as well as the specific phosphatase 1 and 2A inhibitor okadaic acid (3-10 mM) on washed platelet receptors activity and aggregation was measured. Platelet receptors CD62p, CD41, CD36 and CD42b were examined by flow cytometry.
Results: Flow cytometry revealed that lipoproteins increased CD41 expression. Preincubation of platelets with oxPAPC alone, significantly upregulated CD62p and CD41 receptors (higher dose) but potently inhibited anti-CD36 MoAb binding. Lipoprotein/ oxPAPC mixture increased expression of CD42b and down-regulated CD36 receptor. Okadaic acid increased anti-CD41 and decreased anti-CD36 and anti-CD42b MoAbs binding. Neither OxPAPC nor okadaic acid induced platelet aggregation.
Conclusion: The platelet CD36 is the main receptor responsible for binding of oxidized lipoproteins, particularly its oxPAPC epitope. The effect of the threonine/serine phosphatase inhibitor okadaic acid on CD36 and CD41 argue for the participation of phosphorylation-dependent reorganization of cellular trafficking and microtubule organization by oxPAPC.

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ANTI-FACTOR VIII (FVIII) ANTIBODY SECRETING PLASMA CELLS PERSIST IN SPLEEN AND BONE MARROW OF HEMOPHILIC MICE AFTER TERMINATION OF TREATMENT WITH HUMAN FVIII

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The induction of neutralizing anti-FVIII antibodies in patients with hemophilia A is a major complication of replacement therapy with FVIII but the mechanisms by which the anti-FVIII immune response is regulated are not fully understood. We asked whether anti-FVIII antibody-secreting cells (ASC) could play a part in the long-term persistence of this response. To answer this question, we used hemophilic E-17 mice, which we and others have recently shown develop an antibody response to human FVIII similar to that of patients. We analysed the characteristics and the anatomic distribution of ASC during and after treatment with FVIII. Hemophilic mice were treated with four intravenous doses of FVIII. Anti-FVIII antibodies in blood plasma as well as anti-FVIII ASC in spleen, lymph nodes and bone marrow were analysed after each dose of FVIII and up to 22 weeks after termination of FVIII treatment. ASC first appeared in the spleen where they were detectable after two doses of FVIII. They increased in frequency up to the last dose and decreased after termination of FVIII treatment. ASC in bone marrow were detectable after three doses, increased in frequency up to the last dose and stayed constant for at least 22 weeks after termination of treatment. The IgG-subclass distribution of ASC was similar in spleen and bone marrow and matched the subclasses of anti-FVIII antibodies in blood plasma. The long-term persistence of ASC correlated with the long-term presence of anti-FVIII antibodies in the absence of any further FVIII-antigen challenge. Therefore, persistent anti-FVIII ASC might be responsible for maintaining anti-FVIII antibody titers over a prolonged period even in the absence of an exogenous FVIII-antigen challenge. These ASC could be either long-living ASC as described by Slifka et al. (Immunity 1998; 8:363-72) and Manz et al. (Nature 1997; 388:133-4) or cells continuously formed by antigen-driven differentiation of memory B cells as described by Ochsenbein et al. (PNAS 2000; 97:13263-8).

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A ROLE FOR THE PLATELET P2X1 RECEPTOR ION CHANNEL IN COLLAGEN INDUCED PLATELET ACTIVATION STUDIES IN P2X1 KNOCK-OUT MICE

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The functional role of the platelet P2X1 receptor, an ATP-gated cation channel, in hemostasis and thrombosis has been difficult to assess due to the lack of potent and selective antagonists and also due to rapid desensitization of this receptor during preparation of platelets for in vitro studies. Therefore we resorted to P2X1 knockout (KO) mice in order to investigate the role of this receptor in hemostasis and thrombosis. In order to prevent desensitization of the P2X1 receptor during preparation of washed platelets, high concentration of apyrase (ATP-diphosphohydrolase) (0.9 U/ml) was added in course of the washing process as well as in the final resuspending buffer. Under these conditions, the stable and selective P2X1 receptor agonist alpha, beta-MeATP induced a transient calcium influx and a very slight and transient aggregation in wild type (WT) mouse platelets, whereas no calcium influx and aggregation could be detected in P2X1 KO mice. Platelet aggregation induced by ADP (0.1 mM), thrombin (0.1 U/ml) or the TXA2 analogue U46619 (2×10^{-3} mM) was similar between WT and P2X1 KO mice, indicating that the P2X1 receptor does not have a significant role in platelet aggregation induced by these agonists. In contrast, P2X1 KO mouse platelets displayed a decreased response to collagen, reflected by an increased lag phase, an increased time for shape change and a reduced amplitude of aggregation. This difference was more pronounced in response to low collagen concentration (1.25 µg/ml). Similarly, selective desensitization of the P2X1 receptor with alpha, beta-MeATP resulted in strong inhibition of human platelet aggregation induced by low concentration of collagen. The bleeding time, which reflects in vivo primary hemostasis, was significantly although mildly prolonged in P2X1 KO mice (349±44 s, n=20) as compared to the WT mice (259±45 s, n=18) (p=0.1625). No difference in sensitivity to thromboembolism induced by intravenous infusion of a mixture of collagen and adrenaline was observed between P2X1 KO and WT mice, as reflected by similar platelet consumption. Overall these results indicate a role for the P2X1 receptor in the early phase of collagen-induced platelet activation. Whether the P2X1 receptor plays a role in vivo in platelet interaction with subendothelial collagen of damaged vessels remains to be assessed.

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AUTOLOGOUS PLATELET PREPARATION FOR TOPICAL USE IN MAXILLOFACIAL SURGERY

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Purpose: Alveolar bone regeneration is frequently necessary prior for the placement of implants in maxillofacial surgery. Recent reports indicate that high levels of peptide growth factors presence in platelet rich plasma (PRP), may enhance wound healing and the formation of new bone when used in combination with bone grafts. To assess the effect of topical autologous platelet concentrate on the bone regeneration, patients undergoing maxillofacial procedures were studied.
Methods: Autologous platelet concentrate was obtained from PRP derived from 450 ml of CPD anticoagulated blood. After storage for 24 hours, PRP was concentrated by differential centrifugation and adjusted to 10^{10} platelets per ml. Platelet gel was generated intraoperatively from 3 ml platelet suspension by the use of 0,5 ml 10% calcium gluconat and 0,5 ml autologous native blood (thrombin). This mixture was added to approximatly 3 cm^2 autologous bone graft. The platelet gel and bone preparation was performed by using autologous materials alone.
In this study, thirteen consecutively treated patients were followed clinically and radiographically in a peroid of 10 months. Core biopsies of grafted areas were obtained to determine the osteoid and bone formation.
Results: In twelve patients areas healed without complications and clinical signs of sinusitis. One patient showed localized resorption of the bone graft. The final bone graft controlled by computerized tomography showed remarkably dense bone in sufficient quantity. Histological evaluation of biopsy specimens revealed numerous areas of osteoid and bone formation without evidence of inflammatory cell infiltrate.
Conclusions: These findings suggest that topical use of autologous concentrated platelets may improve the process of bone regeneration.

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THE EFFECT OF ALPHA-TOCOPHEROL AND ITS WATER-SOLUBLE ANALOG TROLOX ON NITRIC OXIDE FORMATION IN ENDOTHELIAL CELLS

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Background: Reduced generation of endothelium-derived nitric oxide (NO), leads to vasomotor dysfunction and disordered thromboregulation and has been implicated in a number of vascular diseases. Tocopherol seems to be involved in the regulation of vascular homeostasis but the underlying mechanisms are poorly characterized. The present study investigates whether alpha-tocopherol or its short-chain, water-soluble analog trolox affect calcium-dependent NO synthesis in endothelial cells.
Methods: NO generation after ionomycin stimulation (2 µM, 15 min) of human umbilical vein endothelial cells was detected as formation of its co-product citrulline and as intracellular accumulation of its effector molecule cGMP. The expression of endothelial NO synthase (eNOS) in cell lysates or subfractions was analyzed by Western blotting. The intracellular concentration of the eNOS cofactor tetrahydrobiopterin (BH4) was calculated from biopterin levels measured with HPLC after oxidation of dihydrobiopterin and BH4 in cellular extracts under acidic and basic conditions. GTP cyclohydrolase I (GTP-CH), the key enzyme of BH4 synthesis, was analyzed by real-time RT-PCR.
Results: Preincubation of cells with alpha-tocopherol or trolox (10-200 µM, 24 h) potentiated ionomycin-stimulated citrulline- and cGMP-formation up to 1.5-fold or 2.2-fold, respectively, without changing the expression or the subcellular distribution of eNOS. The effect of alpha-tocopherol was maintained when intracellular BH4 levels were increased by ascorbic acid (100 µM, 24 h) whereas the effect of trolox on NO formation was abolished by coincubation with ascorbate. Accordingly, trolox but not alpha-tocopherol led to a 2-fold increase of endothelial BH4 concentration. This was not due to an enhanced expression of GTP-CH. However, the increase of BH4 levels by trolox was associated with a decrease of more oxidized biopterin derivatives suggesting that the compound led to a chemical stabilization of BH4.
Conclusion: The stabilizing effect of trolox on BH4 may be related to its distribution in the cytosolic compartment. Tocopherol which is localized in cellular membranes may affect eNOS by a different mechanism, possibly via its ability to inhibit the activity of the protein kinase C and thus to modulate the phosphorylation state of the enzyme. The potentiation of NO synthesis represents an additional molecular mechanism of protective effects of alpha-tocopherol in the vascular system.

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PREVALENCE OF MOLECULAR RISK FACTORS FOR VENOUS AND ARTERIAL THROMBOSIS IN VARIOUS INDIAN TRIBES OF COSTA RICA

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Cardiovascular diseases and venous thrombosis are multifactorial diseases. Risk factors result from genetics, environment and behavior. There are known molecular risk factors (gene mutations/polymorphisms) which increase the relative risk for the disease and others, who have protective effects. In order to estimate the role of these factors in a certain population, it is necessary to know their prevalence. We determined the prevalence of of the markers Factor V Leiden (FVL), FV HR2 (His1299Arg), FV-IVS16, FII 20210G>A, methylenetetrahydrofolate reductase (MTHFR) 677C>T and the insertion/deletion (I/D) polymorphism in the angiotensin-I converting enzyme (ACE) gene in a sample of 732 Amerindians from six different tribes: Chorotega, Guaymi, Cabecar, Bribri, Huetar and Guatuso .For comparison we analyzed 170 Caucasians from northeast Germany.
The prevalence of established risk factors is lower in Amerindians of Costa Rica than in Caucasians (D-allele of ACE) or even absent (FVL, FII) and others (MTHFR, FV-HR2 allele) have a extremely high prevalence. For many markers (HR2, MTHFR, ACE) intertribal heterogeneity is obvious reflecting the evolutionary history of these tribal groups and a different degree of admixture with other populations.

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***GREIFSWALD REGISTRIES OF FACTOR IX (HAEMOPHILIA B)-, FVII- AND FX-DEFICIENCIES**

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Clotting factors IX, VII and X are plasma glycoproteins which play an important role in the coagulation cascade. Bleeding disorders of variable severity result from abnormalities in the expression or gene mutations in coding regions, which lead to loss of the function of the proteins.
In the multicentric study “Greifswald registry haemophilia B” (started in 1986) the mutation from more than 220 haemophilia B patients were analysed and registered.122 different causativ FIX lesions in different parts of the FIX gene were detected. This mutation analysis is the basis for the genetic counselling in Haemophilia B families.
The “Greifswald study FVII deficiency” was started in 1995.The aims of this study are to characterize the molecular defects of the FVII gene in patients with FVII deficiency, to find genotype-phenotype correlation and provide an genetic counselling service on this basis. 189 unrelated patients/families with FVII deficiency were analysed, 62 different FVII gene lesions were detected.
In 1998 the “Greifswald registry of FX congenital deficiency” was started. More than 20 patients/families were analysed, 17 different mutations were detected, 11 of them were novel mutations. The registry gives the unique possibilty to characterize the causative mutations as basis for genetical counselling and to study genotype-phenotype correlation by genomic diagnosis and clinical evaluation.
The results of the registries will be shortly reported and the clinical relevance will be discussed.

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MOLECULAR ANALYSIS OF FACTOR VII GENE IN FACTOR VII DEFICIENCY PATIENTS OF VENEZUELA

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Factor VII (FVII) deficiency is a rare autosomal recessive disorder with a high variability of both clinical expression and mutation spectrum. The FVII gene spans 13kb and is located on chromosome 13. We characterized the molecular defects in unrelated patients with FVII deficiency from Venezuela. Nine patients with reduced or low FVII activities (and their families) are under investigation. All coding regions, the promoter and exon/intron boundaries of the FVII genes were analysed by sequencing the patients DNA. Here we report the results of the molecular analysis of 15 unrelated patients.
Ten different mutations were detected :-60T>C in the promotor,deletion Phe24, Pro134 Leu*, IVS6+1G>T and IVS7+1G>A* in the donor splice sites, Gly283Ser*, Pro303His, Arg304Gln, Phe328Ser. Three of them (*) are novel, previously unreported mutation (according to data base FVII mutation 2001). The novel mutation Gly283Ser was found in four unrelated patients. This mutation was the most frequent one in the studied group. Haplotyping using five different FVII polymorphisms showed indentical haplotypes, indicating the same origin (founder effect). The phenotype of the two homozygous patients and three compound heterozygous patients are discussed.

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PREVALENCE OF FVLEIDEN AND FVHR2 POLYMORPHISM IN YUPKA AND WAYUU AMERINDIANS FROM VENEZUELA

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FVLeiden is well known as risk factor for venous thrombosis in Caucasiens. Recently a specific factor V gene haplotype (HR2) was defined by five restriction polymorphisms in exon 13 and a sequence variation of exon 16. The exon 13 markers included the Rsa I polymorphic site, the rare allele of which (R2) has been found to be associated with partial FV deficiency. The HR2 haplotype causes a mild APC resistance phenotype and interacts with FV Leiden (FVL) to produce a severe APC resistance phenotype (Bernardi et al.1997). In this study we determined the prevalence of FVL and FVHR2 in two tribes of Indians from Venezuela: Yupka Indians from the Perija region (n: 189) and Wayuu Amerindians (n: 98) from the Venezuelan Goajira. In comparison we studied Caucasiens from North Eastern Germany (n:170). For the DNA analysis blood samples soaked onto filter paper cards (Herrmann et al.1997) were used for PCR.
Only one of 286 Indians studied from Venezuela carried FVL. However the prevalence of HR2 haplotype was significantly higher in both tribes of Venezuelan Indians (allele frequency 0.291 in Yupka Indians and 0.253 in Wayuu Indians) compared to Germans (0.079). Heterozygous carriers for the R2 allele are frequent in both Indian tribes (39,2% and 32,3%, resp.) compared to German blood donors (15,9%). 22 homozygotes (R2R2, 7.7%) in Indians were detected.
The geographic distribution of FVL and of FVHR2 supports the hypothesis, that the HR2 haplotype is older than FVL and represents a very ancient set of mutations, dating back to a time antecedent the migration of man out of Africa. The younger FVL mutation has the origin in the European Caucasian population and is virtually absent in Indians. The one FVL carrier among 286 Indians is probably caused by admixing with caucasiens. Whether the observed homozygosity of the R2 allele in Indians is associated with a higher risk for venous thrombosis has to be estimated in further clinical studies.

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PHARMACOKINETICS OF PROTEIN C CONCENTRATE CEPROTIN™ IN TREATMENT OF A CHILD WITH SEVERE HOMOZYGOUS PROTEIN C DEFICIENCY

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Background: Severe homozygous protein C (PC) deficiency may be associated with early onset of thrombotic and thrombohemorrhagic complications and DIC in newborns. Symptomatic individuals require lifelong thromboprophylaxis usually with coumarin-type oral anticoagulants. For surgery coumarin prophylaxis is removed to achieve normal coagulation properties. To avoid recurrence of thrombotic complications PC has to be substituted. Therefore, recently a highly purified PC concentrate has been available for clinical trial being soon introduced in the market.
Patient and Methods: A 10 month old boy from Saudi Arabia with connatal skin necroses, intracranial and intraocular thrombohemorrhages due to severe homozygous PC deficiency was prepared for neurosurgery to drain a hydrocephalus. Anticoagulation was switched from warfarin to low molecular weight heparin enoxaparin 10-12.5 mg b.i.d. subcutaneously. PC was applied by i.v. infusion of PC concentrate Ceprotin™. During the first application of 50 I.E. per kg b.w. Ceprotin the pharmacokinetics of PC were examined by PC activity (PCa) and antigen (PC ag) determinations from samples obtained before and 1, 4, 8, 12, 24, 48 and 72 h after infusion. Efficacy of LMWH anticoagulation was monitored by anti Xa, clotting and fibrinolytic activity by D-Dimer fibrin split products.
Results: Under warfarin/INR 2.4 were PCa 1% and PC ag 2% . D-Dimer was 0.4 µg/l.
Peak PC levels 1 h after application were: PCa 69%, PC ag 85%. After 24 h decreases of PCa 11%, PC ag 12%, after 48 h PCa 5%, PC ag 4%, and after 72 h: PCa 2%, PC ag 2% were observed indicting estimated PCa and PC ag elimination halflifes of 7 to 10 h. INR within 48 h was

found to be normal (=1.0). D-Dimer levels during the first 48 h were also low (0.2-0.8 µg/ml) but with decreases of PC and INR concomitantly increased to 5.9-11.2 µg/ml during the next 30 h. Anti Xa levels increased from 0.19 to 1.06 anti-Xa-I.U./ml.
Interpretation: Ceprotin effectively replaces warfarin anticoagulation in severe homozygous PC deficiency by inhibiting coagulation. Due to the short halflife of PC clotting and secondary fibrinolysis turnover increase despite therapeutic anti-Xa levels when PC is low.

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PLATELET ACTIVITY, REACTIVITY AND PLATELET-LEUKOCYTE CONJUGATES FORMATION AFTER SHORT TERM EXERCISE
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Background: Maximal exercise influences the platelet function, but no data are available about platelet activity and reactivity and the formation of platelet-leukocyte conjugates after short term exercise. The aim of the study was to investigate changes after maximal exercise with a duration up to 90s.
Methods: In random order, 15 healthy male subjects (age: 24±2 years, rel. VO2 peak: 56.0±8.2 ml/min/kg) underwent 3 maximal isokinetic cycle exercises, with a duration of 15, 45, and 90 s on a SRM ergometry system and a control-day. Blood samples were repeatedly taken after a 30 min rest [A], directly before [B], directly [C], 15 min [D] and 1h [E] after the exercise for measuring the expression of CD62P, with or without TRAP-6 stimulation, and the formation of platelet conjugates with white blood cells (platelet-granulocyte [Pla-Gra], platelet-lymphocyte [Pla-Lym], platelet-monocyte [Pla-Mon]).
Results: In comparison to the pre-value taken before exercise, no significant change of CD62P unstimulated was investigated, while after TRAP-6 stimulation the percentage of CD62P positive cells in [D] were higher after 15 s (p=0.041) and 90 s (p=0.004). The percentage of platelet-conjugates were increased, too in [D], in Pla-Gra after 45 s (p=0.02), 90 s (p=0.014), Pla-Lym after 45 s (p=0.03), 90 s (p=0.001), Pla-Mon after 45 s (p=0.02), 90 s (p=0.046) and additionally in [C] in Pla-Lym after 90 s (p=0.026).
Conclusions: Maximal short term exercise increases platelet reactivity (15, 90 s) and the formation of platelet conjugates with white blood cells (45, 90 s). This can be shown particularly 15 minutes after finishing the exercise and is regressed 1 hour after exercise. It seems that the investigation of CD62P without stimulation alone is unable to describe the in vivo activation of platelets completely.

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BLOOD COAGULATION AND FIBRINOLYSIS AFTER SHORT-TERM EXERCISE
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Background: Exercise alters blood coagulation and fibrinolysis but until now, no data are available about the influence of extremely short-term exercise on blood coagulation and fibrinolysis. The aim of the study was to investigate changes after maximal exercise with a duration up to 90 s.
Methods: In random order, 15 healthy male subjects (age: 24±2 years, rel. VO2 peak: 56.0±8.2 ml/min/kg) underwent 3 maximal isokinetic cycle exercises, with a duration of 15, 45, and 90 s on a SRM ergometry system and a control-day. Blood samples were repeatedly taken after a 30min rest, directly before, directly, 15 min and 1 h after the exercise for measuring intrinsic or extrinsic total thrombin potential (TTP-in; TTP-ex), intrinsic or extrinsic endogenous thrombin potential (ETP-in; ETP-ex), aPTT, PT, F1+2, TAT, PAP, tPA antigen, PAI-1 antigen and D-dimer.
Results: In comparison to the pre-value taken before exercise, a significant increase of TTP-in, (15 s, 8%; 45 s, 11%; 90 s, 15%), and F1+2 (15 s, 10%; 90 s, 11%) and a shortening of the aPTT (15 s, 10%; 45 s, 17%; 90 s, 20%) directly after exercise were measured. In addition, markers of fibrinolytic activation were distinctively increased in relation to exercise duration (PAP: 15 s, 75%; 45 s, 172%; 90 s, 261%), (tPA antigen: 15 s, 83%; 45 s, 149%; 90 s, 293%), while other parameters e.g. PAI-1 antigen und D-dimer remained unchanged.
Conclusions: Maximal short-term exercise only leads to a small increase of thrombin formation (F1+2) and TTP-in but not to a change of ETP-in. In contrast, these exercises up to 90 s lead to an intensive activation of fibrinolysis which is directly dependent on exercise duration. It is to point out that there already exists a clear activation of fibrinolysis after 15 s of exercise duration.

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***THROMBOSIS PROPHYLAXIS IN CANCER PATIENTS. WHEN? HOW? HOW LONG?**
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Cancer patients are highly susceptible to thromboembolic complications, which significantly affect the morbidity and also the mortality of the disease. During the course of their illness some 15% of tumor patients will experience a thromboembolic event. In lung cancer a percentage of 29% has been reported. Because of the frequency of cancer in our population and the duration of the illness a general recommendation for thromboprophylaxis in cancer with heparin or oral anticoagulants can not be given. Anticoagulant prophylaxis should be individualized with respect to the risk situation of the cancer patient. In surgery every patient has to be considered to be a high risk patient and should be treated prophylactically with low molecular weight heparin (LMWH) in a dosage for high risk situations. Thrombosis prophylaxis is also indicated for immobilized, bedridden patients for the duration of their immobilization, for patients with a high tumor load with induction chemotherapy or radiotherapy, and patients with a large tumor of the pelvis. If there is a known thrombophilia or the history of a previous thromboembolic event a prolonged thromboprophylactic strategy during active illness can be adviced. Less clear are the data in support of routine use of anticoagulants in cancer patients with indwelling central venous catheters, during chemotherapy and radiation therapy. There is controversy whether the terminal cancer patient should receive prophylaxis or not. This decision can be only made in the individual case. Prevention or treatment of a thromboembolic event can be viewed as palliative if symptoms of acute thrombosis can be relieved. The thromboprophylactic treatment of choice in this decade is the application of LMWH which must be injected subcutaneously only once daily and can be easily given also over prolonged periods in the hospital and at home. No laboratory monitoring as for the prothrombin time is necessary.

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INFORMED CONSENT ABOUT HEPARIN-INDUCED THROMBOCYTOPENIA: PILOT-STUDY OF WRITEN INFORMATION IN 460 TRAUMA PATIENTS
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Background: The need to obtain the patients' informed consent for diagnostic and therapeutic invasive procedures and for some forms of drug treatment such as blood products or chemotherapy is widely accepted. Informed consent on rare adverse effects of heparin prophylaxis might cause anxiety about thrombosis prophylaxis.
Methods: 460 consecutive trauma patients needing thrombosis prophylaxis were given a one page information sheet explaining the main benefits (prevention of thrombosis) and risks (haemorrhage and thromboembolic complications due to heparin-induced thrombocytopenia (HIT)) of heparin treatment. Afterwards patients were interviewed by a standardised questionnaire about their previous knowledge of complications, whether they thought it right to be informed about them, whether they were understandable, whether it made them feel more comfortable with the prospect of heparin treatment and whether it improved their perception of the treating doctors.
Results: 99.1% of patients found the written information understandable for a medical layperson, and all of them thought it right to be informed, with 17.4% judging their previous knowledge about complications associated with heparin treatment as poor. Their feeling about the imminent heparin therapy were "very good" in 33.5%, "good" in 56.5%, satisfactory in 8.7% and "adequate" in 0.4% and "bad" in 0.0%. Over 97% judged similar information to be useful for all specialities.
Discussion: Informed consent has important legal implications. Severity and frequency of possible adverse effects in relation to the expected benefit usually determine the need for explaining the risk/benefit-ratio to the patient. The severe adverse effect of heparin treatment, HIT with thrombosis, is usually not included in informed consent. We found that a short written information about HIT is well accepted by patients, without causing anxiety or confusing them, and improves their perception of the treatment. Notably, not a single patient refused thrombosis prophylaxis with heparin as a result of the information about HIT.

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***A CELL BASED MODEL OF HEMOSTASIS**

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Based on our work and that of many other workers, we have developed a model of coagulation in vivo. Many workers have demonstrated mechanisms by which cells can influence the coagulation process. Nonetheless, the prevailing view of hemostasis remains that the protein coagulation factors direct and control the process with cells serving primarily to provide a phosphatidylserine containing surface on which the procoagulant complexes are assembled. By contrast, we propose a model in which coagulation is regulated by properties of cell surfaces. This model emphasizes the importance of specific cellular receptors for the coagulation proteins. Thus, cells with similar phosphatidylserine content can play very different roles in hemostasis depending on their complement of surface receptors. We propose that coagulation occurs not as a "cascade", but in three overlapping stages: 1) initiation, which occurs on a tissue factor bearing cell; 2) amplification, in which platelets and cofactors are activated to set the stage for large scale thrombin generation; and 3) propagation, in which large amounts of thrombin are generated on the platelet surface. This cell based model explains aspects of hemostasis that a protein-centered model does not, including why factor VIII or IX deficiency results in such a severe bleeding tendency and how therapy with high-dose factor VIIa overcomes the hemostatic defect in hemophilia.

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HEPARIN ANTAGONIZES ANTITHROMBIN-MEDIATED INHIBITORY ACTION ON ENDOTOXIC MICROCIRCULATORY DYSFUNCTION IN VIVO

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A recent clinical sepsis trial (Kybersept trial) reported a significant reduction in 90-day mortality by antithrombin (AT) exclusively in the subgroup of patients without simultaneous low-dose heparin prophylaxis. Patients additionally receiving heparin did not benefit from AT treatment. Herein, we studied whether this clinically observed heparin-antithrombin antagonism may be caused by a heparin-mediated inhibition of AT action on endotoxin-induced microcirculatory dysfunction. In Syrian hamsters normotensive endotoxemia was induced by 2mg/kg endotoxin (LPS, E. coli) i.v.. In a first group of animals, AT (AT, 250 IU/kg i.v., n=6) was given 5min before LPS administration. A second group of animals (Heparin+AT, n=5) received AT (250 IU/kg i.v.) combined with unfractionated heparin (sodium heparin, 100 IU/kg/24h, i.v.). Additional animals (LMWH+AT, n=5) received AT (250 IU/kg i.v.) combined with low molecular weight heparin (nadroparin 5ml/kg, s.c., 2h before LPS). LPS-treated animals, which received only saline, served as con-trols (Control, n=6). Using dorsal skinfold preparations, endotoxin-induced microvascular leukocyte-endothelial cell interaction (LE) and alteration of functional capillary density (FCD) were studied by intravital video fluorescence microscopy. In controls, LPS induced a massive increase in LE with a maximum at 8 h and an impressive decrease in FCD over a 24-h period. Both LPS effects were effectively prevented by AT treatment ($p<0.01$), whereas Heparin+AT and LMWH+AT animals showed microcircu-latory alterations comparable to that in controls. Thus, our study indicates a relevant in vivo antagonism of the beneficial action of AT on endotoxin-induced microcirculatory dysfunction by heparin. This may explain the clinical finding that the reduction of sepsis-induced mortality by AT is reversed by concomitant heparin treatment.

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***CLINICAL MEANING OF LABORATORY TEST TO MANAGE ANTITHROMBOTIC REGIMEN**

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Antithrombotic therapeutic management is hampered both by bleeding complications and by recurrent or persistent thrombotic events. Failure of thrombolytic regimen or anticoagulant therapies may result from underdosing in a certain number of patients, on the other hand standard doses may vice versa result in overdosing in other patients. Especially if drugs with uncertain predictability of the individual response are used the control by laboratory tests seems to be mandatory.
However, in many clinical situations only bed-side or point-of-care tests will be useful. One reasons is the limited time for therapeutical consequences resulting from the test data (e.g. during catheter interventions). Furthermore, regimen like single-shot thrombolysis do not allow does-adjustments after injection. A further major clinical limitation is the lack of scientific data on prospective patient management according to test results.
A ideal test should be

- rapidly available
- easy to perform
- fast and reproducible
- clinically evaluated with respect of the examined regimen and hard endpoints
- not expensive.

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***ENOXAPARIN IN COMBINATION WITH A FIBRIN-SPECIFIC THROMBOLYTIC DRUG FOR ACUTE MYOCARDIAL INFARCTION – RESULTS OF THE ASSENT-3 TRIAL**

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Current thrombolytic regimen are limited by a lack of optimum reperfusion in a certain number of patients with acute myocardial infarction. The new single-bolus thrombolytic drug tenecteplase was investigated in the "Assessment of the Safety and Efficacy of a New Thrombolytic Regimen (ASSENT-3)" trial in combination either with the low-molecular-weight heparin enoxaparin or with abciximab.
6095 patients with acute myocardial infarction were randomly assigned to either full-dose tenecteplase combined with enoxaparin (for 7d), half-dose tenecteplase combined with abciximab and low-dose unfractionated heparin or full-dose tenecteplase and unfractionated heparin (for 2d). There were fewer efficacy endpoints for the combination strategies (11.4% vs 15.4% for enoxaparin vs. unfractionated heparin, highly significant). The same observation resulted for the combined efficacy and safety endpoints (13.8%/14.2%/17.0% for enoxaparin plus tenecteplase / abciximab plus tenecteplase/unfractionated heparin plus tenecteplase). The combination of tenecteplase combined with enoxaparin seems to be a safe regimen, which additionally reduces ischemic complications in acute myocardial infarction. Furthermore its ease of administration favours very early treatment in emergency units or in the prehospital setting.

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***PHARMACOLOGICAL DIFFERENCES OF GPIIB/IIIA ANTAGONISTS**

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The heterogeneous chemical nature of the GPIIb/IIIa ($alpha_{IIb}$ $beta_3$) antgonists, which are in clinical use, determines several differences of their pharmacological characteristics. As a humanized F(ab) fragment of IgG, abciximab has a high molecular weight and primarily distributes within the intravascular space. About half of the administered amount of drug binds to platelet $alpha_{IIb}$ $beta_3$. High affinity and a slow rate of dissociation are responsible for a long lasting inhibition of platelet aggregation by abciximab. Eptifibatide, a cyclic heptapeptide, and the fibanes, which are nonpeptide compounds, share a lower molecular weight. In contrast to abciximab, inhibition of platelet aggregation by these drugs is shorter and correlates with plama concentration. While the potency of all inhibitors to prevent platelet aggregation is increased by lowering extracellular Ca^{2+}, this is most evident for eptifibatide. Abciximab also binds with high and eptifibatide with lower affinity to vascular $alpha_v$ $beta_3$ integrins. Experimental evidence suggests that this decreases vascular neointima formation, but clinical support is missing. Abciximab, but not eptifibatide and the fibanes, also interacts with leukocyte $alpha_M$ $beta_2$ integrins, possibly producing antiinflammatory effects. However, abciximab´s affinity to $alpha_M$ $beta_2$ integrins appears to be lower than to $alpha_{IIB}$ $beta_3$ and $alpha_v$ $beta_3$. Some investigators noticed that GPIIb/IIIa inhibitors, besides inhibition of aggregation, may stimulate to a limited extent platelet functions via integrin outside-in signaling, potentially translating into a limited prothrombotic effect. Comparative experimental studies indicate that there may be differences among the three classes of compounds.

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***THE ROLE OF HEMOSTASIS IN CARDIAC TRANSPLANT ARTERIOSCLEROSIS**

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The major long-term problem following cardiac transplantation is the development of cardiac transplant arteriosclerois in the transplanted heart. Its prevalence increases to approximately 80 percent at 5 years after transplantation. Transplant arteriosclerosis is associated with smooth muscle cell proliferation, affecting large epicardial coronary vessels and penetrating smaller intramyocardial branches.
Transplant arteriosclerosis is believed to be the result of immunological and non-immunological mechanisms, whereby an initial coronary endothelial injury leads to a deleterious disturbance of the local hemostatic vessel wall equilibrium. One change in endothelial cells that occurs apparently independently of immunological dammage is predisposing the artery to fibrin deposits, which are not seen in stable grafts but are present in patients who subsequently develop a poor clinical outcome. Histological examinations characterizing intraluminal thrombus and fibrin formation along the graft vessel wall intima suggest that the intravascular activation of the coagulation system might be closely involved in lesion formation. Thereby, the increased thrombogenicity within the micro- and macrovasculature of transplanted hearts is related on the one hand to an aberrant expression of tissue factor within the coronary intima and on the other hand to the loss of anticoagulant and fibrinolytic pathways. These changes involve arteries, veins, and capillaries, and thus indicate a panvascular process within the allografts. The hypothesis that endothelial tissue factor expression causes a shift in the prothrombotic endothelial phenotype accociated with atherogenic mechanisms is supported by the observation that agents that suppress intraluminal tissue factor expression prove an effective therapy to retard or prevent transplant arteriosclerosis.
The data just summarized suggest that transplant arteriosclerosis represents an extreme case of an - at least in part - hemostatically driven arterial hyperplasia. Analysis of usual atherosclerotic lesions also shows evidence of chronic or acute coagulation activation. Therefore, by studying the extreme cases such as transplant arteriosclerosis, one probably can gain insight into the pathophysiological mechanisms that contribute to the multifactorial form of ususal atherosclerosis.

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ANTITHROMBIN REDUCES EXPERIMENTAL CARDIAC TRANSPLANT VASCULOPATHY

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Background: Intravascular clotting activation has been implicated in the pathogenesis of cardiac transplant vasculopathy (TVP). We recently identified in rat heart transplants the expression of tissue factor (TF) within the coronary intima which was associated with neointimal thickening. In this study we evaluated the effect of antithrombin (AT) on the development of TVP in Lewis to Fisher rat cardiac allografts.
Methods: Transplant recipients were randomized to a control group (n=6) and a AT-treated group (n=6). AT was administered at a dose of 500 IU/kg i.v. every second day from day -1 to +20 post transplantation. The degree of rejection, transplant vasculopathy, and transplant function were evaluated 120 days after transplantation.
Results: AT significantly attenuated the development of TVP in the graft microcoronary bed, but was less effective in large coronary arteries. In small-sized coronary arteries (<2 smooth muscle cell layers), both the severity and frequency of TVP were significantly reduced with AT when compared to controls ($p<0.05$). AT treatment of transplanted animals was safe as it did not lead to increased perioperative bleeding. Transplant rejection was not affected by AT.
Conclusions: AT decreased neointimal hyperplasia in this rat cardiac transplant model. The anti-proliferative benefit may either rely on a direct effect of AT on thrombin, reversing the intravascular hypercoagulable state, or on recently described anti-inflammatory properties of AT.

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THROMBIN GENERATION IN HEPARIN INDUCED THROMBOCYTOPENIC PATIENTS AND HITTS. MODULATION BY THROMBIN INHIBITORS

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Heparin induced thrombocytopenic syndromes are associated with hemostatic activation including the upregulation of coagulation processes, fibrinolytic deficit and endothelial dysfunction. Platelet microparticles along with leukocytes also contribute to the overall thrombotic process. In order to characterize the activation of coagulation, markers of thrombin generation were measured in plasma samples from 136 clinically diagnosed and apparently untreated patients. These patients were subsequently managed by pharmacologic and plasmapheresis approaches. The control group consisted of normal healthy males and females (n=130, age span 26-46 years). Tissue factor antigen, prothrombin fragment F 1.2, thrombin-antithrombin complex, fibrinopeptide A and D-dimer levels were measured using sandwich based ELISA techniques from commercial vendors. Functional AT III levels were measured using a chromogenic substrate method. Although a wide scatter in the levels in HIT patients was apparent, the cumulative data analysis revealed a marked increase in almost all of the markers in comparison to the control group. Functional AT III levels decreased 30% in the HIT group. The cumulative data is given below.

Marker	Control (n=136)	HIT (n=130)	% Change
Tissue Factor (pg/mL)	61±29	170±60	178 (+)
Prothrombin F1.2 (nM)	1.8±0.6	3.1±1.6	72 (+)
Thrombin-antithrombin complex (ng/mL)	2.6±1.7	5.6±3.2	115 (+)
Fibrinopeptide A (ng/mL)	2.0±1.2	7.9±3.7	295 (+)
D-dimer (mcg/mL)	0.48±0.31	1.7±0.27	254 (+)
Functional ATIII (% NHP)	89±19	63±24	30 (–)

In a substudy, it was shown that fibrinopeptide A and TAT levels in HIT patients decreased after treatment with argatroban or hirudin. Further analysis showed that thrombin generation was inhibited to a argatroban greater degree with in comparison to hirudin when both drugs were used at their recommended dosages. These results clearly indicate that HIT/HITTS syndrome results in the generation of thrombin as evidenced by the increase in plasma levels of various molecular markers. Furthermore, thrombin inhibitors with broad spectrum anti-protease activities such as argatroban produce a stronger inhibition of thrombin generation. Additional investigations are warranted to demonstrate the therapeutic efficacy of thrombin inhibitors in HIT syndrome. Measurement of these markers may be of crucial value in the prognosis of HIT.

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BETA-2-GLYCOPROTEIN I – ANTIBODIES IN PATIENTS WITH VENOUS THROMBOEMBOLISM WITHOUT A LUPUS ANTICOAGULANT

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Antibodies against beta-2-glycoprotein I (beta2GPI) are frequently found in patients (pts) with antiphopholipid syndrome. Their role in the pathogenesis of development of thrombosis in pts without antiphospholipid syndrome has not clearly been established. We investigated 698 pts (437 women, 261 men) with a history of venous thromboembolism (VTE). The mean age at first VTE was 42+16 years (range 2–88 years), 8 pts had VTE before the age of 15 years. Pts with lupus anticoagulants (n=17) were excluded from further evaluation. 51 pts had antithrombin-, protein C- or protein S-deficiency, 23 had homozygous, 189 heterozygous FV:R506Q and 52 heterozygous G20210A prothrombin mutation. 161/681 (24%) pts had a history of recurrent VTE. 113 healthy individuals served as a control group. All pts were screened with a qualitative enzyme linked immunoassay (ELISA) for the detection of IgG-, IgM- or IgA-beta2GPI antibodies (QUANTA LiteTM beta2GPI-Screen, Inova Diagnostics Inc., San Diego, USA). For quantitative determination of anti-beta2GPI-IgG, -IgM and -IgA a cut off level of 0.22 optical density (OD) of the anti-beta2GPI-Screen assay was defined. This cut off level comprised each individual of the control group with a level of IgG-, IgM- or IgA-beta2GPI antibodies higher than the 95th percentile. In 19/681

(2.8%) pts with a history of VTE the anti-beta2GPI-screen value was above the 95th percentile of the control group (>0.48 OD). Anti-beta2GPI-IgG, anti-beta2GPI-IgM or anti-beta2GPI-IgA were above the 95th percentile of the control group in 2.1%, 4.6% and 3.8%, respectively. Thus, the prevalence of elevated levels of beta2GPI antibodies was not higher in pts than in controls. There were no statistically significant difference with regard to age at first VTE in pts with and without elevated anti-beta2GPI antibodies. In pts with a history of recurrent VTE the anti-beta2GPI-screen level was not statistically significantly higher than in pts without recurrence. Pts with pulmonary embolism had significantly higher anti-beta2GPI screen and IgG levels ($p<0.5$) than those without, however there was a big overlap. We conclude from the present data that the determination of antibodies to beta2GPI using the upper mentioned assays does not add further clinically relevant information on the risk profile of pts with a history of VTE without the lupus anticoagulant. Whether beta2GPI can provide additional information in certain subgroups of pts remains to be established.

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***FIRST RESULTS OF THE BERLIN THROMBOPHILIA STUDY**

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Factor V Leiden (FVL) and Factor II (FII) G20210A represent common risk factors for thromboembolic (TE) events. While the heterozygous genotypes are associated with the occurrence of TE-events, additional risk factors can be identified in most children. The homozygous genotype for FVL may result in unheralded, catastrophic TE-events even in small children without known additional risk factors. There is a lack of prospective data, how common TE-events are in such children. We have therefore initiated a prospective cohort study for the homozygous and double heterozygous genotypes for FVL and FII 20210. The probands and the heterozygous controls are identified by neonatal screening that involves >98% of the neonates born in Berlin. After informed consent has been obtained the probands are enrolled into the study. From January 1999 to September 2001 we have screened a total of 85304 samples by multiplex PCR analysis of DNA contained in filter cards. We have identified 5338 heterozygotes for FVL (6.3%) and 1132 for FII G20210A (1.3%). This confirms previously published data about the gene frequency of FVL, although FII G20210A appears relatively uncommon in this population. We detected 116 homozygotes for FVL, 19 for FII G20210A, and 94 double heterozygotes. Thus, we detected a significant higher number of homozygotes and double heterozygotes than expected when calculated on the basis of the respective gene frequencies.
72 FV Leiden homozygotes, 8 FII 20210 homozygotes, and 46 double heterozygotes were enrolled into the study and matched to 125 and 49 heterozygous controls, respectively. Definite or probable TE events were noted in 3/72 probands with homozygous Factor V Leiden (anterior cerebric artery, renal vein, adrenal vein) and in 1/46 probands with the double heterozygous genotype (renal vein), but in none of the controls. There are no differences in mental or motor development between the probands and the heterozygous controls.
In conclusion, during its first 33 months this prospective neonatal cohort study shows that TE-events in infancy are significantly associated with the homozygous and double heterozygous risk genotypes. In contrast, this study indicates that there is no major prenatal loss of fetal carriers of these genotypes and may even confer a prenatal advantage in fetal carriers. Putative subclinical TE-events do not impair motor or mental development during the first 2 years of life.

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STAPHYLOKINASE-DIPETALIN-FUSION PROTEINS WITH FIBRINOLYTIC AND ANTICOAGULANT PROPERTIES

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Besides interventional cardiology, progress in the therapy of thromboembolic diseases can be attributed to drug intervention in blood coagulation and fibrinolysis. Fusion proteins were constructed consisting of His-tagged staphylokinase (H6-Sak) which stimulates fibrinolysis via activation of plasminogen, and of the two-domain Kazal-type thrombin inhibitor dipetalin (Dip-I+II), derived from the assassin bug Dipetalogaster maximus. A functional and structural characterization of fusion proteins with retained bifunctionality is presented. The functional tests comprise global clotting (aPTT, TT, PT) and plasminogen activation (chromogenic substrate and fibrin plate assays) as well as thrombin-induced platelet aggregation and vascular relaxation. NMR spectroscopy was used to determine the 3D protein structures of the domains. Sak in fusion with Dip possesses full plasminogen activating potential. Furthermore, the presence of Sak does not interfere with dipetalin's thrombin inhibitory potency. To effectively inhibit thrombin-induced blood coagulation and cellular effects, the linked dipetalin domain-I and -II are required. The anticoagulant effects of H6-Sak-Dip-I+II and Dip-I+II are in the nanomolar range comparable to those observed for hirudin. This is supported by Ki-values of 0.91 and 0.78 pmol/l for H6-Sak-Dip-I+II and Dip-I+II, respectively. The thrombin-induced aggregation of human blood platelets as well as thrombin-induced vascular relaxation can be blocked by H6-Sak-Dip-I+II and Dip-I+II in the nanomolar range. [1H-15N]-HSQC spectra of the fusion proteins in comparison to the spectra of Sak and Dip-I show that the modules in the fusion proteins exhibit their native folds. Three areas of Sak identified to be important for biological function are not afflicted by pronounced chemical shift changes in the NMR spectrum of the fusion protein. In conclusion, the novel fusion protein H6-Sak-Dip-I+II, which possesses both fibrinolytic activity and anticoagulant potency without interference in the action of either of the fusion partners, might be of benefit for therapeutical use.

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DOSAGE ADJUSTMENT OF ARGATROBAN JUSTIFIED IN COMBINATION WITH GP IIb/IIIa INHIBITORS: LABORATORY AND CLINICAL IMPLICATIONS

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Argatroban a direct thrombin inhibitor is now approved by the USFDA for prophylaxis of thromboembolism and for percutaneous coronary interventions (PCI) in patients with Heparin-Induced Thrombocytopenia (HIT) and Thrombosis Syndrome (HITTS). To determine the potential interactions of antithrombin drugs with glycoprotein IIb/IIIa inhibitors by celite Activated Clotting Time (ACT), argatroban alone was profiled in the concentration range of 0-10 mcg/ml, and then in combination with a fixed final concentration of 5 mcg/ml of tirofiban in normal healthy volunteers (n=6). Serial blood samples were also collected from patients with HIT undergoing PCI while receiving argatroban and tirofiban. The celite ACT values with argatroban alone at concentrations of 0, 2.5, 5, and 10 mcg/ml were 118.3±8.08, 260.3±35.9, 340.6±37.6 and 420.0±44.1 seconds, respentively. When a fixed concentration of tirofiban was added, the clotting times increased to 115.3±9.5, 272.0±24.6, 400.3±55.2 and 518.3±63.6 seconds, respectively. This synergistic interaction was confirmed clinically when argatroban was administered to HIT patients undergoing PCI at a dose of 300 mcg/kg IV bolus followed by 10 mcg/kg/min infusion plus tirofiban 10 mcg/kg IV bolus+0.015 mcg/kg/min infusion. The Hemochron ACT values at baseline, 5-10 min post argatroban, 30 min post argatroban and 10 min post-tirofiban were 131.0±31.1, 270.5±13.4, 261.0±12.7 and 345.0±40.3 seconds, respectively. The corresponding values using HemoTec ACT were 123.0±14.8, 367.0±32.5, 324±7.07 and 411.0±10.9 seconds, respectively. The corresponding values obtained by using Ecarin Clotting Time (ECT) cards (Pharmanetics, Raleigh, NC.) in one of the patients were 77.0, >700.0, 383.6 and >700.0 seconds, respectively. This data clearly indicates that there is a synergistic effect of tirofiban on the anticoagulant effect of argatroban. The specific elevation of the ECT is highly suggestive of the release of protein/site bound argatroban, warranting studies to investigate the competitive binding of these two drugs when given simultaneously. The increased anticoagulant effect of argatroban under the antiplatelet umbrella may be beneficial PTCA, atherectomy and rotablation procedures. However, in other situations the dosage of argatroban may be optimized or adjusted for the level of anticoagulation desired.

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SUCCESSFUL USE OF ECARIN CLOTTING TIME TEST IN DOSAGE OPTIMIZATION OF REFLUDAN IN PATIENTS WITH HEPARIN-INDUCED THROMBOCYTOPENIA UNDERGOING OFF-PUMP CORONARY ARTERY REVASCULARIZATION

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Refludan (lepirudin-rDNA for injection) is the first direct antithrombin drug approved by the USFDA for anticoagulation in patients with Heparin-Induced Thrombocytopenia (HIT). We have used a modified Ecarin Clotting Time (ECT) performed on a fibrometer and on a kinetic centrifugal analyzer (ACL300 plus) on whole blood and citrated plasma for the monitoring of refludan for the therapeutic anticoagulation in four patients with HIT undergoing off-pump coronary artery revascularization. Activated Clotting Time measurements were made on native whole blood. Two patients with normal renal function were administered refludan at 0.4 mg/

kg IV bolus followed by an infusion of 0.15 mg/kg/hr, one patient on hemodialysis received 0.2 mg/kg IV bolus followed by an infusion of 0.02 mg/kg/hr, while undergoing off-pump coronary artery revascularization. The fourth patient with normal renal function who underwent a new FDA approved procedure using the Anuerex or Guidant endovascular abdominal aortic aneurysm device to repair aneurysm, received refludan at a dose of 0.4 mg/kg IV bolus followed by 0.15 mg/kg/hr infusion. Blood samples were drawn at baseline, 5 min post bolus of refludan and every 15 minutes during the procedure. ACT was performed immediately on the citrated whole blood samples. The ACT values ranged between 300-400 seconds following the bolus of refludan and were maintained above 250 seconds for adequate anticoagulation. The ECT was ideally maintained above 400 seconds during the surgical procedure. Additional boli of refludan were given as when necessary in order to maintain the ECT >400 seconds. The calculated circulating concentrations of refludan based on the liquid ECT and APTT were 3.51±1.35 and 2.02± 1.19µg/ml respectively. This study indicates that liquid ECT assays provide a reliable means of determining the circulating levels of refludan. Based on this study it is projected that off-pump surgical procedures can be performed at circulating levels of refludan in the range of 3-5 µg/ml. Furthermore, this study shows that ECT can be effectively used to monitor and optimize the dosage of refludan in patients with HIT undergoing off-pump coronary artery revascularization and abdominal aortic aneurysm repair.

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MOLECULAR GENETIC ANALYSIS OF THE PATIENTS WITH SEVERE DEFICIENCY OF COAGULATION FACTOR XIII USING DHPLC

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Introduction: Mutations of the Factor XIII (FXIII) A-subunit have been found in FXIII deficiency, a rare autosomal recessive disorder characterized by a tendency for spontaneous bleeding and impaired wound healing. FXIII is a plasma transglutaminase consisting of two catalytic A-subunits and two carrier protein B-subunits. It catalyzes the formation of covalent bounds between fibrin monomers, thus stabilizing the fibrin clot and increasing its resistence to fibrinolysis. FXIII A-subunit gene is located on chromosome 6p24-25 and contains 15 exons while B-subunit gene is situated on chromosome 1q31-32 and consists from 12 exons.
Patients, materials and methods: Four patients suffering from severe FXIII deficiency were subjected to molecular genetic analysis. All exons including the promotor region were amplified. Heteroduplexes of patient and wild type DNA were formed and loaded on the WAVE DNA Fragment Analysis System (Transgenomics, San Jose, USA). The melting temperature of specific DNA sequence was choosen as recommended by Stanford Genome Technology Center (http://insertion. stanford.edu/dhplc.html). Exons with abnormal patterns in the dHPLC analysis were sequenced using an ABI sequencer system.
Results and discussion: Our technique allowed to identify 3 out of 4 homozygous mutations causing FXIII deficiency. All three mutations have been detected within the A-subunit gene revealing one small insertion in exon 9 (Cins at codon 400), one small deletion (Cdel at codon 248) in exon 6 and one splice site mutation (IVS5-1 G>A). In one case the mutation remained unknown. This patient was homozygous for a polymorphism (SNP) in exon 5 (Tyr204Phe). No homozygous persons for this SNP have been found so far in normal population. However, considering the only slight reduction of FXIII activity in heterozygous carriers of the Tyr204Phe polymorphism it is unlikely that this SNP is causative for the severe FXIII deficiency. The small insertion and the splice site mutation have been described by Vreken et al (1995) and Aslam et al (1998). The small deletion (Cdel at codon 248) is reported for the first time. This deletion results in a frameshift and premature stop signal at codon 266. From these findings we conclude that dHPLC technology is a highly sensitive method well suited to the molecular analysis of FXIII deficiency.

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THE HUMAN PLATELET ANTIGEN-1 POLYMORPHISM DOES NOT INFLUENCE THE ANTIPLATELET EFFECTS OF GLYCOPROTEIN IIb/IIIa INHIBITORS

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The HPA-1 polymorphism may have an impact on administering glycoprotein (GP) IIb/IIIa inhibitors to patients with acute coronary syndromes. We have, therefore, studied the hypothesis that the HPA-1 polymorphism may influence the effectiveness of GPIIb/IIIa inhibitors. Platelets were isolated from citrated blood and ADP (30 µM)-induced fibrinogen binding was measured by flow cytometry. Abciximab (0.03-3 µg/ml), tirofiban (0.3-30 nM), or eptifibatide (0.01-1 µg/ml) were incubated for 15 min with the samples prior to stimulation with ADP. IC_{50} values for the inhibition of fibrinogen binding were determined from each experiment. All subjects were genotyped by GALIOS and automated fluorescence correlation spectroscopy. Although a marked variability in the inhibitory effects of all three GPIIb/IIIa inhibitors was confirmed, there were no significant differences between the genotypes with respect to the inhibition of fibrinogen binding. Thus, the HPA-1 polymorphism does not explain the interindividual variability in the platelet inhibitory effects of the three GPIIb/IIIa inhibitors approved for clinical use.

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MEASUREMENT OF LIGAND-INDUCED BINDING SITE EXPRESSION IN THE PRESENCE OF GP IIb/IIIa INHIBITORS

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Upon binding to GPIIb/IIIa, fibrinogen induces conformational changes on this receptor leading to the generation of ligand induced binding site (LIBS) epitopes. It has been postulated that generation of LIBS epitopes may functionally compromise the long-term use of GP IIb/IIIa inhibitors. LIBS-1 and LIBS-6 expression was measured by flow cytometry following in vitro supplementation of tirofiban, eptifibatide, YM 337 or abciximab to platelet rich plasma (PRP) and ex vivo in PRP collected from patients undergoing percutaneous coronary intervention (PCI; n=10/group) at baseline and 2 hours post-administration of tirofiban, eptifibatide and abciximab. In the in vitro studies, 1 mcg/mL eptifibatide caused a significant increase in LIBS-1 expression (median fluorescence (MFI): 43.8±5.9 vs. 24.2±4.0 for saline; p=0.016). A small increase in LIBS-1 expression was observed in the presence of 1 mcg/mL tirofiban (MFI: 26.8±4.4; N.S.). At a concentration of 5 mcg/mL, neither abciximab nor YM-337 caused a significant change in LIBS-1 expression compared to saline (MFI: abciximab=24.5±3.7, YM 337=24.8±5.4). LIBS-6 expression induced by in vitro supplementation of eptifibatide, tirofiban, YM 337 and abciximab was not observed. An increase in platelet activation measured as P-selectin (CD62) expression was not observed following incubation of platelets with eptifibatide, tirofiban or abciximab (MFI: 16.3±1.5, 15.4±2.2, 16.3±1.9 vs. 14.9±1.9 for saline). It was observed that eptifibatide therapy was associated with a statistically significant increase in LIBS-1 expression compared to baseline (MFI: 43.1±18.1 vs. 22.8±6.7, p=0.027). This expression was comparable to that observed following in vitro supplementation of 1 mcg/mL eptifibatide.LIBS-1 expression was not induced during infusion of tirofiban compared to the patients own baseline (MFI: 34.4±5.3 vs. 33.3±7.4). LIBS-1 expression was lower following treatment with abciximab (MFI: 19.7±5.0 vs. 24.5±5.2; N.S.). LIBS-6 expression was not altered in patients treated with eptifibatide (MFI: 13.3±2.2 vs. 16.5±2.6), tirofiban (MFI: 26.2±5.8 vs. 23.3±5.9) or abciximab (MFI: 19.1±4.6 vs. 20.5±6.5. Although expression of LIBS-1 is different for the various GPIIb/IIIa antagonists tested, the clinical implications of these.

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COMPARATIVE PHARMACOKINETICS AND PHARMACODYNAMICS OF TINZAPARIN AND HEPARIN AT A FIXED DOSE (75 U/KG) IN PRIMATES

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The pharmacokinetics (PK) of low molecular weight heparins (LMWHs) is conventionally reported in terms of their anti-Xa effects. Using this approach, the PK parameters show marginal differences among different batches of the same product. Methods such as the global anticoagulant assays (aPTT, thrombin time (TT), Heptest), amidolytic assays (anti-IIa) and the endogenous release of TFPI, reveal assay-dependent differences in the pharmacodynamics of LMWHs and unfractionated heparin (UFH). In this study, the pharmacodynamics of Tinzaparin (T) were compared to that of unfractionated heparin following 75 U/kg intravenous (IV) and subcutaneous (SC) dosages. Drugs were administered to groups of primates (n=4-8) and blood samples were collected over a period of 24 hours. TFPI levels were measured by ELISA. The responses in all assays were calculated in terms of area under the curve (AUC). Plasma drug levels were extrapolated from calibration curves prepared by supplementing T and UFH to baseline plasma. The results are presented in the following table.
All data is represented as AUC (U*min/mL) except for TFPI which is reported as ng*min/mL. Bioavailability (BA) was calculated as AUCSC/AUCIV. As evident from these data, the PD effects of T and UFH were

assay-dependent. The IV studies showed T to be comparable to UFH as measured by aPTT and anti-IIa activties. While T exhibited a 1.4-fold higher effect in the Heptest assay, the AUC determined using anti-Xa data was nearly equivalent to that observed with UFH. In the SC studies, T exhibited comparable effects in the aPTT and anti-IIa assays. However, it showed much higher activities in the anti-Xa and Heptest assays. TFPI levels increased ~5-fold following IV and ~1.5-fold following SC administration of T. The cumulative BA index of T was much higher for T (0.57) compared to UFH (0.34). The data show that the PK and PD effects measured using different assays widely differ. For a proper PD analysis, multiple assays should be considered as both UFH and LMWHs are polycomponent in nature. Additional factors, such as enhancement of profibrinolytic effects and the release of endogenous mediators, may also contribute to the polypharmacologic actions of these drugs.

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MONOCYTE-PLATELET ASSOCIATE FORMATION IN PATIENTS WITH STENOSED CAROTID ARTERIES

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Background: Platelet activation is involved in the pathogenesis of cerebrovascular disease. Haemostasis and inflammation are highly interwoven processes. In the past we found that the majority of circulating platelets and leukocytes from patients with acute ischemic stroke exhibit signs of enhanced activation. We therefore studied 62 patients with high grade internal carotid artery stenosis (>50% reduction in diameter). Methods: Colour-coded Duplex-ultrasonography of carotid plaques was used to distinguish between plaques with a bright echo, known to consist predominantly of calcification and echolucent plaques. The latter are associated with an increased risk of future stroke in symptomatic individuals. Flowcytometry was used to analyse the activation status of leukocytes and the rate of associates between platelets and leukocytes. Age-matched healthy blood donors served as controls.
Results: Patients' monocytes expressed ex vivo about 2 times more thrombospondin-1 (TSP-1) than controls ($p<0.001$) and Mac-1-integrin (CD11b) expression on patients' monocytes was significantly increased ($p<0.01$). More than 50% of all patients' monocytes had platelets bound to their surface in comparison to about 15% in controls. Leukocyte activation status was compared between patients with echorich and echolucent plaques. While for most measured parameters reflecting the grade of leukocyte activation patients with echorich plaques did not differ significantly from controls, the monocytes of patients with echolucent plaques were highly activated. TSP-1 binding to monocytes from patients with echolucent plaques was significantly higher ($p<0.001$) than in controls and to patients from patients with echorich plaques ($p<0.01$). CD11b expression on monocytes from patients with echolucent plaques was significantly increased on monocytes ($p<0.005$) in comparison to controls. In the blood of patients with echolucent plaques significantly higher members of associates between monocytes and platelets were found in comparison to the blood from patients with echorich plaques ($p<0.05$) and to controls ($p<0.001$). Discussion: Activation of monocytes and monocyte-platelet associate formation might be not just indicators of processes that causes stroke, but rather involved in the processes triggering of accelerating cerebral ischemia. Follow up of the patients will prove whether high number of platelet-monocyte associates, or high-grade of monocyte activation, will predict future strokes.

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***PROBLEMS IN THE DEVELOPMENT OF FACTOR Xa AND THROMBIN INHIBITORS**

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Based on their pivotal role in the blood coagulation cascade, in the last years the serine proteases thrombin and factor Xa (FXa) came into the focus of interest for the development of new anticoagulant/antithrombotic drugs. The direct inhibition of these key enzymes may offer new ways to affect the blood clotting process as well as other actions of coagulation enzymes which may be important for the pathogenesis of various cardiovascular disorders. It has been shown that both FXa and thrombin inhibitors exert strong anticoagulant actions in vitro and in vivo, are antithrombotically effective in various experimental thrombosis models and also inhibit the proliferation of vascular smooth muscle cells in cell culture systems as well as in in vivo-models. However, despite of the effectiveness of FXa and thrombin inhibitors, there are still numerous unresolved problems and open questions for these new classes of drugs. Pharmacokinetic characteristics such as oral bioavailability, biological half-live, metabolic transformations or excretory routes are important factors for the clinical use of the inhibitors. Interactions with other drugs or endogenous factors as well as additional mechanisms of action have to be taken into consideration. Furthermore, the efficacy/safety profile and the possible occurrence of undesired side effects such as bleeding complications has to be evaluated. Other important points are the monitoring of the therapeutic effect and the neutralisation of the drugs in case of overdose or side effects. Thus, further experimental and clinical studies are required to demonstrate the inhibitory profile of FXa and thrombin inhibitors, their effectiveness and especially their superiority over other commonly used drug regimens for cardiovascular indications.

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BLOCKADE OF GPIIb/IIIa RECEPTORS BY XV 454 PREVENTS ADP-INDUCED PLATELET AGGREGATION IN RATS IN VITRO AND IN VIVO BUT DOES NOT EFFECTIVELY INHIBIT VASCULAR SMOOTH MUSCLE CELL PROLIFERATION IN THE RAT CAROTID ARTERY

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Aim of the study: Using an experimental model of vascular smooth muscle cell (VSMC) proliferation in the carotid artery of rats it was investigated whether a GPIIb/IIIa receptor antagonist, the compound XV 454, may have an antiproliferative potency. Methods: The inhibition of ADP-induced platelet aggregation by XV 454 in vitro was investigated in rat plasma which was substituted with increasing concentrations of the compound. In rats XV 454 was administered as i.v. bolus injection and at definite times ADP-induced platelet aggregation was measured in ex vivo samples. VSMC proliferation was induced in the rat carotid artery by damage of the vessel wall with atraumatic external vessel clamps and evaluated 5 and 14 days, resp., later by measuring either the [3H]thymidine incorporation/µg protein or the number of 5-Bromo-2′-deoxyuridine (BrdU)-positive proliferating cells and the total cell number stained by hematoxylin (HE). Results: The studies showed that XV 454 inhibited the ADP (20 µM)-induced platelet aggregation in vitro at concentrations between 25 and 800 ng/ml (=maximum inhibition) and in vivo at doses of 1, 2.5 and 5 mg/kg i.v. At the highest dose of 5 mg/kg i.v. a maximum platelet aggregation inhibition was measured 5 and 15 min p.i. Then the effect declined over time and was not longer measurable at 60 min p.i. The enhanced thymidine incorporation/µg protein measured in damaged carotid arteries of saline-treated rats was slightly reduced in animals treated with XV 454 at a dose of 2.5 mg/kg i.v. which was the medium effective dose for the inhibition of platelet aggregation in vivo. The significant increase in the number of proliferating (BrdU labelled) and total (HE stained) cells as well as in the proliferation index (PI=number of proliferating cells to total cell number in per cent) seen in damaged vessels of control animals both 5 and 14 days after induction of VSMC proliferation was not significantly reduced by i.v. bolus injection of 2.5 mg/kg XV 454. Conclusion: XV 454 is an effective platelet function inhibitor in rat plasma as shown by the inhibition of ADP-induced platelet aggregation in vitro and in vivo. At the dose and route administered the GPIIb/IIIa receptor antagonist does not clearly inhibit the proliferation of smooth muscle cells in the carotid artery of rats.

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ANTICOAGULANT, ANTITHROMBOTIC AND HAEMORRHAGIC EFFECTS OF TRIABIN, A HIGHLY POTENT EXOSITE INHIBITOR OF THROMBIN

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Aim of the study: Characterisation of recombinant triabin, an anion-binding exosite thrombin inhibitor originally isolated from the saliva of the blood-sucking bug Triatoma pallidipennis.
Methods: 1. Anticoagulant actions were studied in vitro in human, rabbit and rat plasma and in vivo after i.v. bolus injection into rabbits by measuring clotting times in global clotting assays (TT, APTT, PT, Heptest). 2. Effect on platelet functions was investigated in vitro and ex vivo by measuring agonist-induced platelet aggregation. 3. Effect on thrombin generation was evaluated by measuring plasma concentrations of the prothrombin fragment F1.2 in blood from healthy volunteers incubated with triabin. 4. Antithrombotic actions were studied in the Wessler model of jugular vein thrombosis in rats. 5. Haemorrhagic effects were investigated by measuring the bleeding time (BT) after both transection and standardised incision of the rat tail.

Results: 1. Triabin at concentrations from 0.25 to 5 µmol/l prolonged clotting times in vitro in both human, rabbit and rat plasma in a concentration-dependent manner. TT and Heptest were the most sensitive assays in which a very steep course of the concentration-response curves was observed. APTT and PT were less sensitive but showed more pronounced species differences. The i.v. injection of triabin at a dose of 2.5 mg/kg caused a strong prolongation of TT whereas PT and APTT were less influenced. 2. In rabbits both in vitro and in ex vivo plasma samples triabin inhibited the thrombin-induced platelet aggregation but did not influence ADP-, collagen- and PAF-mediated platelet reactions. 3. In human whole blood triabin at 20, 40 and 80 µg/ml inhibited the generation of thrombin in a concentration-dependent manner and, thus, also prolonged the time until spontaneous clotting of blood occurred. 4. The inhibitor at doses between 0.25 and 2.5 mg/kg i.v. reduced or prevented the formation of thrombi in the jugular vein of rats in a dose-dependent manner. 5. After transection of the rat tail triabin given i.v. at doses of 0.5 mg/kg and higher prolonged BT to more than 30 min. After standardised incision triabin at 2.5 mg/kg prolonged BT to 6.9 min (controls: 2.6 min) and at 5 mg/kg to more than 30 min in 3 of 5 rats. Conclusion: Triabin is a highly potent thrombin inhibitor with a novel specific mechanism of action that exerts strong species-dependent anticoagulant and antithrombotic effects.

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***FRONTLINE AND FAMOUS: UNDERSTANDING THROMBOSIS AND CANCER**

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Thrombosis appears to be a common complication in cancer patients, yet little is known about current perceptions with regard to thrombosis amongst clinicians who treat cancer nor about current patterns of practice. The FRONTLINE survey has been undertaken to provide the first comprehensive global assessment on current views in the field. One of the most interesting observations from clinical trials comparing intravenous unfractionated heparin with subcutaneous Low Molecular Weight heparin in the initial treatment of deep vein thrombosis has been the consistent advantage in terms of reduced mortality 3 months after therapy in cancer patients who received low molecular weight heparin (LMWH).
FAMOUS (Fragmin Advanced Malignancy Outcome Study) is the first prospective placebo controlled double blind study to evaluate the potential for Low Molecular Weight Heparin (Fragmin 5,000 units once daily) to prolong survival in patients with advanced solid tumour malignancy without underlying thrombosis.

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***MANAGEMENT OF VENOUS THROMBOEMBOLISM IN CANCER PATIENTS: THE CORTES SUBGROUP ANALYSIS**

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Deep vein thrombosis is a common problem in cancer patients and its treatment remains complicated by higher rates of recurrent two dosing regimes of the Low Molecular Weight Heparin Reviparin administered subcutaneously with intravenous unfractionated heparin in 1137 patients with acute Deep Vein Thrombosis. Patients underwent venography at time of presentations and three weeks after commencement of therapy. Analysis of patients with known cancer presenting with thrombosis demonstrates that the phlebographic response to therapy (defined as 30% of greater improvement in the Marder score between the two venograms) was seen in 50% of non-cancer but only 39% of cancer patients (p=0.026). Interestingly, phlebographic response was seen in only 21% of cancer patients who received unfractionated heparin but in 2% of those who received twice daily Reviparin and 50% of those who received the once daily regimen. Recurrent venous thromboembolism was also more common in cancer patients 10.2% vs 3% in non-cancer patients (p=0.001). These findings were consistent with a demonstration of a greater derangement of laboratory parameters of haemostasis – higher levels of Prothrombin Fragments 1+2, D-Dimer, factor XIIa and TFPI in cancer patients. This subgroup analysis indicates that thrombosis in the cancer patient is a treatment challenge where LMWH may offer considerable benefits.

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RHOGTPASE-DEPENDENT PLATELET-NEUTROPHIL INTERACTION AFFECTED BY HMG-COA REDUCTASE INHIBITION WITH ALTERED ADENOSINE NUCLEOTIDE RELEASE AND FUNCTION

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Platelet activation and aggregation is considered a crucial step in the initiation and aggravation of arterial thrombosis. Adenosinediphosphate (ADP) from activated platelets is recognized as major factor in thrombus formation and is a potent stimulator of oxygen free radical release from neutrophils. The aim of the present investigation was to determine in vitro the direct effects of HMG-CoA reductase inhibitors on adenosinetriphosphate (ATP) and ADP secretion by platelets and its impact on subsequent oxidative burst activity in neutrophils. Human neutrophils and platelets were isolated from peripheral blood of healthy donors. Levels of platelet-derived ATP and ADP were measured by high performance liquid chromatography, oxygen free radical release and chemotaxis of neutrophils were measured fluorometrically and Rho-GTPases were studied by Western blot analysis. Thrombin-activated platelets primed neutrophils for enhanced oxygen free radical release upon triggering with formyl-Met-Leu-Phe, which was reduced by cerivastatin and simvastatin treatment of platelets. The two statins decreased the amount of adenosine-derivate generation in these cells, and Rho-GTPases that are required for the signaling of thrombin and ADP in platelets and neutrophils were decreased in the cytoplasma when cells are co-incubated with the statins. Data demonstrate that by inhibiting Rho-GTPase activity simvastatin and cerivastatin inhibit platelet ADP and ATP release and the consecutive augmentation of neutrophil oxygen free radical release. Thus, statins affect platelet-neutrophil interactions by altering Rho-GTPase-dependent adenosine nucleotide function.

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REVERSAL OF THROMBIN-INDUCED DEACTIVATION OF CD39/ATPDASE IN ENDOTHELIAL CELLS BY HMG-COA REDUCTASE INHIBITION

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There is increasing evidence for functional crosstalk between inflammatory and hemostatic pathways. Adenosine triphosphate (ATP) and adenosine diphosphate (ADP), known to activate platelet, leukocyte and endothelial cell functions, are hydrolysed by endothelial CD39/ATPDase which provides an endogenous anti-thrombotic and anti-inflammatory mechanism. Since CD39/ATPDase is down-regulated in endothelial cells by inflammatory mediators and this may be affected by HMG-CoA reductase inhibitors, we examined the role of cerivastatin and simvastatin in regulating endothelial CD39/ATPDase expression, metabolism of ATP and ADP, and function of regulated adenine nucleotides and adenosine in platelets. Thrombin-stimulated endothelial cells in vitro were treated with the statins and hydrolysis of exogeneous ADP and ATP was assessed by measuring adenine nucleotide metabolites in HPLC and malachite green assays. Platelet aggregation studies were performed using endothelial cell supernatants as triggers. CD39/ATPDase surface expression by endothelial cells was determined immunologically by FACS and thrombin-induced dissociation of Rho-GTPases was assessed by Western blotting. Treatment by simvastatin or cerivastatin restored impaired metabolism of exogenous ATP and ADP in thrombin-activated endothelial cells to adenosine or inorganic phosphor and pyrophosphate by preventing thrombin-induced down regulation of CD39/ATPDase. Dissociated Rho-GTPases that are normally increased by thrombin in endothelial cells were decreased when cells were concomitantly treated with the statins. In platelet aggregation studies, ATP and ADP supernatants of thrombin-activated endothelial cells were less stimulatory in the presence of statins than in their absence. Data show that statins preserve CD39/ATPDase activity in thrombin-treated endothelial cells involving alteration by statins of Rho-GTPase function. Preservation of ATP and ADP metabolism to AMP and adenosine may directly contribute to the observed anti-thrombotic and anti-inflammatory actions of statins.

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SYNDECAN-4 ON HUMAN PERIPHERAL BLOOD LYMPHOCYTES AND MONOCYTES MEDIATES EFFECTS OF ANTITHROMBIN ON CHEMOTAXIS

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Antithrombin inhibits chemokine-induced migration of neutrophils by activating heparan sulfate proteoglycan-dependent signaling. Whether antithrombin affects migration of other types of leukocytes is unknown. We investigated the effects of antithrombin on spontaneous and chemokine-triggered migration of lymphocytes and monocytes from human peripheral blood in modified Boyden chamber micropore filter assays. Lymphocyte and monocyte populations from human peripheral blood were purified using magnetic antibody cell sorting. Signaling mechanisms in antithrombin-dependent migration were studied by Western blot analyses of protein kinases and Rho activation, or were tested functionally by using signaling enzyme blockers. Expression of heparan sulfate proteoglycan core protein was studied by RT-PCR and flow cytometry. As antithrombins, the concentrate Kybernin®P from human plasma and a monoclonal antibody-purified preparation therefrom were used. Pretreatment of lymphocytes and monocytes with antithrombin inhibited chemotaxis toward optimal concentrations of interleukin-8 or Rantes at concentrations of antithrombin as low as 10 nU/ml; in the absence of the chemokines, direct exposure of cells to gradients of antithrombin stimulated migration. Effects of antithrombin were abolished by pretreating cells with heparinase-1, chondroitinase, sodium chlorate and anti-syndecan-4 antibodies. Expression of syndecan-4 mRNA and protein in monocytes and lymphocytes was demonstrated in RT-PCR and anti-syndecan-4 immunoreactivity assay, respectively. In the presence of pentasaccharide, antithrombin lost its activity on the cells. Antithrombin induced chondroitinase- and heparinase I-dependent phophorylation of protein kinase C-alpha and dissociation of Rho-GTPase. Data indicate that antithrombin directly inhibits chemokine-stimulated migration of monocytes and lymphocytes via effects of its heparin-binding site on cell surface syndecan-4 by activation of protein kinase C and Rho signaling.

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FACTOR VII ACTIVATING PROTEASE (FSAP) REGULATES THE PROLIFERATION OF HUMAN VACULAR SMOOTH MUSCLE CELLS

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The factor VII activating protease (FSAP) is a recently described serine-protease, which can activate single chain plasminogen activators and factor VII independently of tissue factor. FSAP is present in human plasma as a single-chain zymogen, and under neutral conditions in a purified system autoactivation leads to the active two-chain form. Since the protease presents a strong affinity for glycosaminoglycans including heparin, this study was conducted to characterize the vascular cell activities of FSAP. Binding experiments indicated a specific interaction of FSAP with human vascular smooth muscle cells (HVSMC) with maximal binding around the physiological plasma concentration of FSAP (12 µg/ml). Active FSAP, but not the inactive zymogen, inhibited platelet derived growth factor (PDGF-BB)-mediated HVSMC proliferation, as deduced from DNA synthesis assay and cell counting. Fetal calf serum-mediated HVSMC proliferation was not affected by FSAP. The inhibitory activity of FSAP could be neutralized by a functional blocking anti-FSAP monoclonal antibody, aprotinin or by physiological concentrations of zinc ions, indicating that the active enzyme is required for the inhibitory effect. HVSMC-bound FSAP did not inhibit proliferation by degrading PDGF-BB, but by some as yet undefined mechanism. In contrast to long-term effects of cell proliferation, the short term PDGF-BB-induced mitogen activated protein-kinase phophorylation in HVSMC was not interfered by FSAP. These results unravel a new function of FSAP as inhibitor of PDGF-BB-dependent activities that require further characterization with respect to the unidentified substrate for FSAP possibly involved in mitogenic activities of these cells.

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C-TERMINAL THROMBOSPONDIN-1 (TSP-1) PEPTIDE INDUCES MONOCYTE PROCOAGULANT ACTIVITY

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Background: Haemostasis and inflammatory processes are linked by a crosstalk between platelets and leukocytes. The glycoprotein thrombospondin-1, the most abundant alpha-granule protein secreted from activated platelets, is thought to be important for integrin-mediated cell activation through binding of its C-terminal cell-binding sequence RFYVVMWK to its receptor on platelets and many other cells.
Methods: The C-teminal TSP-1 peptide RFYVVMWK was synthesized and used to study the effect on human monocytes, isolated from fresh buffy coats by elutriation. Flow cytometrical methods were used to analyse activation dependent expression of CD11b, MRP8/14 and tissue factor (TF) on isolated monocytes and the binding of FITC-labelled thrombospondin-1, FITC-labelled fibrinogen and FITC-labelled coagulation factor VIIa to monocytes. Monocyte derived microparticles were identified by their size and positive reactivity for an anti CD14 antibody.
Results: The TSP-1 peptide activated the monocytes; it induced in a dose dependent manner the expression of the Mac-1-integrin (CD11b), a protein necessary for monocytes to exit the vasculature and for migration into infected/damaged tissue. Moreover it induced the binding of thrombospondin-1 protein itself and of fibrinogen. These proteins are able to mediate associate formation between activated monocytes and quiescent platelets inducing activation of platelets. RFYVVMWK-treated monocytes expressed MRP8/14 on the cell surface, a protein known as a marker for early inflammation. Moreover RFYVVMWK induced monocyte microparticle formation. TSP-1 peptide activated monocytes and monocyte derived microparticles expressed high concentrations of tissue factor (TF) on their surface and bound coagulation factor VIIa in a TSP-1 peptide dependent manner.
Conclusions: TSP-1 may play a pivotal role as a linker between haemostasis and inflammation. The C-terminal of TSP-1 induced a fulminant activation of monocytes, making them proinflammatory and procoagulant. Abnormal TF expression induced by TSP-1 might have broad-ranging importance in a variety of human diseases including disseminated intravascular coagulation in patients with sepsis, and arterial thrombosis in patients with coronary heart disease.

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***MODIFICATION OF THE MANUFACTURING PROCESS OF REFACTO®: THE 3rd. GENERATION ALBUMIN-FREE PRODUCT**

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ReFacto® (BDDrFVIII), the first albumin-free formulated rFVIII was approved in 1999 in the EU and in 2000 in the US. It is produced by a genetically engineered Chinese hamster ovary (CHO) cell line. The CHO cell line secretes BDDrFVIII into a defined cell culture medium to which human serum albumin (HSA) is added. The medium contains no other proteins derived from animal sources. The rigorous five-step column chromatography purification process which includes a monoclonal antibody purification step results in a product with high specific activity. In addition a solvent-detergent step has been introduced to further improve viral safety. ReFacto® is formulated without HSA.
An improved manufacturing process has been developed which eliminates any human- or animal-derived raw materials. The master cell bank has been changed in order to remove fetal bovine serum and human serum albumin, but uses the same cell line as the current process. The cell culture media no longer contains human serum albumin, and requires only one protein (recombinant insulin derived from *E. coli*). The monoclonal antibody used for the affinity chromatography step in the purification of ReFacto® has been replaced by a chemically synthesized polypeptide ligand that produces ReFacto® of equivalent purity and potency. Finally, a virus-retaining filtration step has been introduced into the process, to provide additional assurance of viral safety of the product. No changes to the drug substance or drug product formulations were made, and the stability profile of the product is unchanged. The comparability of ReFacto® produced by the improved process to the ReFacto® available today has been established by evaluation of all release assays, a battery of biochemical characterization assays including peptide mapping and oligosaccharide fingerprinting, in vitro functional assays, and in vivo activity using a Hemophilia A dog model. No significant differences in the product are observed in all of these studies. Clinical drug product has been produced using the improved process, using full-scale bioreactors and purification steps, and clinical trials are underway at a number of international hemophilia centers.
ReFacto® AF (albumin-free), the so called 3rd generation, provides an improved viral safety profile for hemophilia A patients. The process mod-

ifications result in a superior safety profile with respect to the risk of transmission of blood-borne pathogens. Clinical trials have been initiated recently.

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***CANCER AND HEPARIN**

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Since more than a century, tumor-related alterations of the coagulation system are well known. In cancer patients, there is a high incidence of thrombembolic events as well as of systemic coagulation activation.
In 1930, Goerner was the first who raised the question, whether tumor-related coagulation activation might contribute to the neoplastic behavior of cells. He demonstrated that progression of subcutaneous tumors in rats could be prevented by anticoagulant treatment.
Meanwhile, the influence of heparin and other anticoagulants on cancer spread has been reported since the early 1960s. While results for oral anticoagulants, ASS and UFH were controversial, initial results concerning LMWH showed a potential beneficial effect of LMW heparin on the survival of cancer patients with DVT. Cancer patients suffering from thromboembolic events showed reduction of mortality up to 50% (3 months survival) when treated with LMWH in comparison to UFH. Effects on tumor spread as well as on metastasis were observed.
These beneficial effects of LMWH are caused not only by its inhibitory effect on the coagulation system, but also by several other mechanisms. These are p.e.: Inhibitory effects on cell adhesion, proliferation, migration and angiogenesis; influence on expression of heparanase, which correlates with the metastatic potential of tumor cells; inhibition of P-selectin-mediated interactions of platelets with carcinoma cells.
In conclusion, under LMWH treatment we may expect beneficial effects on the survival of cancer patients as well as reduction of thromboembolic events. Optimal dose regimen and optimal duration of heparin treatment in the cancer patient still have to be clarified.

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***ACTIONS OF HEPARIN IN CANCER PATIENTS**

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Activation of the coagulation system is observed in patients suffering from malignant diseases, resulting in increased thrombin generation. Thrombin acts as key enzyme of the coagulation cascade, but has several additional functions: It is known to be the most important platelet activating agent and influences expression of adhesion molecules: Thrombin induces selectin expression on the platelet surface as well as selectin-, ICAM-1- and VCAM-1-expression on endothelial cells. Due to expression of these adhesion molecules, “rolling” and “adhesion” of tumor cells on the endothelial surface is facilitated and migration through the endothelial barrier becomes possible. This is the first step for metastasis. Moreover, fibrin-deposition around tumor cells guarantees a stable stroma for tumor growth, stimulates tumor angiogenesis and provides a barrier against immunologic attacks and chemotherapy.
Heparin is able to influence all these mechanisms: In addition to its inhibitory effects on thrombin and factor Xa, cell adhesion, proliferation and migration as well as angiogenesis are suppressed by heparin. Expression of heparanase, which correlates with the metastatic potential of tumor cells, is decreased. Moreover, heparin treatment attenuates tumor metastasis by inhibiting P-selectin-mediated interactions of platelets with carcinoma cells.
Thus far, in cancer patients beneficial effects of heparin were mainly observed under LMWH treatment.This beneficial effect of LMWH in comparison to UFH in cancer patients is explained by different properties of LMWH and UFH: They express different affinity towards surface receptors and selectins. Increased inhibitory effects on angiogenesis, thrombin-induced platelet aggregation and on heparanase activity were observed under LMWH.
All these actions of heparin, which influence tumor spread and metastasis, offer a chance, that in the future accompanying LMWH therapy in cancer patients may reduce mortality. An additional benefit could be reduction of thromboembolic events.

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MANIFESTATION AGE OF THROMBOEMBOLIC EVENTS DOES NOT DEPEND UPON HOMOCYSTEINE LEVELS

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Introduction: Hyperhomocysteinaemia is thought to be a risk factor for thromboembolic events. Nevertheless, the pathophysiologic background for the increased thrombosis risk in hyperhomocysteinaemia is not yet clarified. In the present study, we tried to find out, whether manifestation age of thromboembolic events in patients with F. V Leiden mutation and in patients without any other thrombophilic defect is influenced by homocysteine level.
Patients: In total, 158 patients who had experienced thromboembolic events and 54 persons without thromboembolic complications were examined. A: 46 thrombosis patients proven for Factor V Leiden mutation, other thrombophilic defects were excluded. B: 112 thrombosis patients without thrombophilic defect. C: 24 patients with Factor V Leiden mutation without thromboembolic complications. D: 30 controls without thromboembolic complications and without thrombophilic defect.
Methods: In all persons examined, antithrombin, protein C and S, aPC ratio, plasminogen, lipoprotein (a) and homocysteine were measured. Moreover, Factor V Leiden mutation, prothrombin- and MTHFR-polymorphism was tested.
Results: A: 34/46 Factor V Leiden patients had normal (below 13 umol/l) homocysteine level, 12/37 had elevated homocysteine level (26%). Average manifestation age of thrombophilia in patients with normal homocysteine level was 33.0$\pm$12.7 years, in patients with elevated homocysteine level 37.0$\pm$14.2 years. B: 74/112 patients had normal, 38/112 had elevated homocysteine level (34%). Average manifestation age of thrombophilia in patients without trombophilic defect and normal homocysteine level was 33.7$\pm$13.1 years, in patients with elevated homocysteine level 40.7$\pm$14.1 years. C: 19/24 Factor V Leiden patients without thromboembolic events had normal, 5/24 had elevated homocysteine levels (20%). D: 29/30 control persons had normal, 1/30 had elevated homocysteine level (3%).
Conclusion: 1. Elevated homocysteine levels do not influence manifestation age of thromboembolic events. 2. Elevated homocystein levels are frequently found in patients with thromboembolic events - in Factor V Leiden patients as well as in patients without thrombophilia. Incidence of hyperhomocysteinaemia is significantly lower in healthy controls. Our results raise the question, whether elevation of homocystein is a consequence, but not a source of thromboembolism.

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TREATMENT WITH SOLVENT DETERGENT (SD) TREATED PLASMA IS EFFICACIOUS AND WELL TOLERATED IN THROMBOTIC THROMBOCYTOPENIC PURPURA (TTP) DUE TO CONGENITAL VON WILLEBRAND FACTOR-CLEAVING PROTEASE (VWF-CP)-DEFICIENCY

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A deficient activity of VWF-CP was found to be responsible for TTP. This protease cleaves ultralarge von Willebrand Factor multimers to normal size, hereby reducing the agglutinative properties of von Willebrand Factor. Deficient activity of VWF-CP either results from an autoantibody against the cleavage-site of the protease or from inherited absence of the protease.
Plasmapheresis or transfusion of fresh frozen plasma are the treatment of choice in TTP. Patients with an autoantibody need large volumes of FFP to remove and /or neutralize the autoantibody. A plasmaexchange should be performed to protect from volume overload. In patients with congenital VWF-CP-deficiency the activity is readily provided by infusion of small amounts of plasma.
Here we report on a girl who presented with recurrent thrombocytopenia and anaemia from birth on. Full pentad of characteristic TTP symptoms (severe thrombocytopenia, microangiopathic anaemia with erythrocyte fragmentation, neurological deficits, renal dysfunction, and fever) were observed at the age of 16 years. Congenital TTP was confirmed by severe VWF-CP deficiency in the absence of an acquired inhibitor, and diminished activities of VWF-CP in her father and brother. No inhibitor of VWF-CP was detected. After infusion of plasma the girl went into remission and remained asymptomatic under regular plasma therapy for 3 years. After 2 years of therapy with 1.2 l FFP every 3 weeks the patient exhibited allergic reactions to the transfusion, which forced the transfusions being stopped and administration of antiallergic therapy. In the patients plasma an soluble HLA-antibody was found. The source of VWF-CP was changed to SD-plasma. Since the introduction of SD-Plasma (Octaplas®) the patient is free of any side effects during transfusion. The transfusion intervalls have been shortened to 2 weeks with only 0,8 l plasma administered. The platelet count is stable over 200×10^9/l with no signs of haemolysis.
We conclude that therapy with SD-treated-plasma is as efficacious in the treatment of congenital VWF-CP-deficiency as FFP. Patients who are sensibilized against blood cell contaminated FFP may benefit from a change to SD treated plasma.

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QUANTITATION OF GLYCOPROTEIN IB AND IIB/IIIA-RECEPTORS DURING CORONARY ANGIOGRAPHY

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Introduction: It has been suggested that both heparin and contrast media alter platelet function during cardiac intervention (Grabowski et al. Thrombos Haemostas 1994). We used flow cytometry to quantitate GPIb and GPIIb/IIIa receptors in sequential blood samples from patients undergoing cardiac catherization (without PTCA). Methods: Blood samples were obtained before and during cardiac catherization and on the following day. Samples were immediately fixed. Platelet activation (CD62p-expression), microparticles, and microaggregate formation were measured with flow cytometry. From the same samples platelet surface receptor expression was determined with fluorescent mouse anti-CD41 (GPIIIa) and anti-CD42b (GPIbalpha) antibodies and with beads bearing defined numbers of anti-mouse antibodies (Quantum Simply Cellular Beads, Sigma). Results: The number of CD62p-positive platelets, microparticles, and platelet microaggregates before the start of catherization was not different in patients from normal controls. Mean GPIb expression was 43000 receptors/platelet and GPIIb/IIIa was 56000/platelet which was in the normal range. During cardiac catherization there was an increase in the mean number of platelet microaggregates (from 980 to 2500/µl) and a decrease in the mean number of CD62p-positive platelets (from 5000 to 3700/µl). There was a slight decrease in platelet microparticles which did not reach significance. There was no significant change in GPIb and GPIIb/IIIa expression on the platelet surface. Conclusions: The cardiac catherization procedure per se causes a small degree of platelet microaggregate formation. The number of CD62p-positive platelets decreases indicating that either CD62p is shed from the platelet surface or CD62p-positive platelets are removed into aggregates. Despite these changes there is no decrease or increase in GPIb and GPIIb/IIIa expression from the procedure. These findings may be relevant for the use of receptor blocking agents during cardiac intervention.

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***EFFECTS OF DESMOPRESSIN ON PLATELET FUNCTION IN AN ANIMAL MODEL AND IN PATIENTS WITH THROMBOZYTOPATHIA**

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Fawn-Hooded rats with storage pool disease were determined to be a good animal model for the investigation of the effectiveness of haemostasis active pharmaceuticals such as vasoactive desmopressin (Minirin®, DDAVP). During the studies with desmopressin a time- and dose-dependent reduction of the bleeding time was observed in all groups of Fawn-Hooded “bleeder” rats. The measured bleeding times under desmopressin treatment were comparable to those of healthy Wistar rats. As seen in investigations of factor VIII and von-Willebrand-Factor (vWF) in humans after injection of desmopressin, an increase of factor VIII in Fawn-Hooded rats was observed during the first half hour after infusion. The highest dosage showed the lowest FVIII increase. The vWF of the Fawn-Hooded rats showed a delayed increase, occuring one to two hours after infusion. Platelets were time- and dose-dependently activated with chape change after infusion of desmopressin. Clinical we treated different groups of patients with platelet function defects. In patients with Glanzmann Thrombasthenia we found no effect on platelet function and bleeding time. In the group with storage pool disease we found (except one patient with severe Hermansky-Pudlak-Syndrom) shortening of the bleeding time and no bleeding complications during surgical intervention. Also a large group of patients with unknown reason of prolonged bleeding time undergoing surgical intervention was treated with success.
In conclusion desmopressin causes an increase of FVIII- and vWF-level in rat and human. The alteration of these parameters seems to normalize the bleeding time in case of storage pool defect and in a group of surgical patients with prolonged bleeding time but has no effect in case of Glanzmann Thrombasthenia.

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PAR-INDUCED MAST CELL STIMULATION IN ACUTE INFLAMMATION

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Subject of the study: Activation of blood coagulation and thrombin generation occur in inflammation, tissue repair and other pathological processes. Thrombin activates a number of cell types (including mast cells) that plays a key role in these processes. The cellular effects of thrombin are mediated by G-protein coupled proteinase-activated receptors (PAR). Recently, the expression of mRNA for PAR-1 and PAR-2 on human and rodent mast cells was found (D'Andrea et al., 2000, Nishikawa et al., 2000). In previous experiments it was shown that thrombin activates mast cells, but the mechanism of thrombin signaling in mast cells is not yet clear. In the present studies the release of histamine and ß-hexosaminidase from rat peritoneal mast cells (RPMCs) was measured after addition of thrombin, trypsin and the PAR-1 activating peptide TRAP (SFLLRN) and PAR-2 activating peptide SLIGRL (PAR-2 AP) in control rats and rats with acute inflammation.
Methods: The RPMCs were isolated from lavage of the peritoneal cavity of rats. Histamine was measured spectrofluorometrically and the ß-hexosaminidase activity was determined using chromogenic substrate. Inflammation was induced by intraperitoneal injection of thioglycolate (40% (w/v) in 2 ml sterile water). Peritonitis was observed after 16 hours and at that time RPMCs were isolated and the effects of thrombin, trypsin, TRAP and PAR-2 AP on RPMCs and release of mediators were investigated.

Results: It was shown that thrombin (0.01-1 µM) induced release of histamine and ß-hexosaminidase from RPMCs. This response to thrombin is probably mediated by PAR-1, because TRAP (from 5 to 100 µM) caused also a significant release of histamine and ß-hexosaminidase from RPMCs and desensitization to thrombin could be demonstrated. Cathepsin G which is able to hydrolyze the Phe(55)-Trp(56) peptide bond of the N-terminus of PAR-1 reduced thrombin-induced mediator release. The PAR-2 AP (1-100 µM) activate also RPMCs. On the model of acute peritonitis an increase of mediators release by thrombin and TRAP as well as by trypsin (1 µM) and PAR-2 AP (100 µM) was shown in RPMCs. This may be due to additional expression of PAR-1 and PAR-2 on mast cells in the model of acute peritonitis.
Conclusions: These findings suggest that PAR-1 and PAR-2 might promote inflammatory responses to acute injury.

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THROMBOPHILIA – A RISK FACTOR IN CEREBRAL ISCHEMIA OF UNDETERMINED AETIOLOGY?

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Background: Ischemic stroke is a serious and life-threatening disease. Despite intensive diagnostic procedures aetiology of ischemia remains unexplained in about one third of cases. The role of thrombophilic disorders as a risk factor for stroke and TIA is still a matter of debate. In the present study patients younger than 55 years of age and with cerebral ischemia of undetermined aetiology were investigated.
Patients and methods: We studied 52 unrelated patients (pts) with an objectively confirmed ischemic stroke (39 pts) or TIA (13 pts). The medain age at onset was 40 years (range 19-55). Cardial or vascular causes of cerebral ischemia like atrial fibrillation, malformation, severe arteriosclerosis or small vessel occlusive disease were excluded by cardial and cranial vessel ultrasound and angiography examination. Concomittant risk factors like arterial hypertension, diabetes or smoking were present in 38/52 pts. The labaratory testing includes fibrinogen, antithrombin, protein C (PC), protein S, homocysteine, lupus anticoagulant, cardiolipin antibodies (IgG and IgM), lipoprotein (a) (Lpa), factor V G1691A, prothrombin G20210A and MTHFR C677T mutations and induced thrombocyte aggregation with different concentrations of ADP.
Results: Thrombophilia screening revealed abnormalities in 34/52 pts. 7/52 pts showed combined defects. Factor V mutation (1 homozygous) was present in 6 pts, heterozygous prothrombin mutation in 2 pts, hyperhomocysteinaemia in 10 pts (6 pts with homocygous MTHFR mutation), PC-deficiency in 1 pt and dysfibrinogenaemia in 1 pt. 7 pts had an elevated Lp(a) above 30 mg/dl, 8 pts antiphospholipid antibodies and 8 pts showed hyperreacive thrombocyte aggregation (sticky platelet syndrome).
Conclusions: In this highly selected group more than 50% of our investigated patients showed a thrombophilic disorder. According to this data thrombophilia seems to be an important risk factor in cerebral ischemia.

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LATE ONSET OF ANAPHYLAXIS AGAINST FACTOR IX IN A 19 MONTH-OLD BOY WITH SEVERE HEMOPHILIA DURING IMMUNE TOLERANCE THERAPY

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The development of an anti-factor IX inhibitor in patients with haemophilia B represents a rare but difficult problem because of the poor success rate, the risk for development of a nephrotic syndrome during the course of immune tolerance therapy and the possibility of anaphylaxis, mostly a the time or shortly after the detection of the inhibitor.
Case report: The 19 month-old boy was diagnosed to have severe haemophilia B at the age of 8 months because of repeated haematomas. He was started on a plasma derived factor IX product, but developed an inhibitor after 16 ED (5.2 BU/ml). Factor IX therapy was stopped until no factor IX inhibitor was detectable by Bethesda assay. For ITT the patient was given a dexamethasone pulse (2×0,5 mg/kg for 3 days) and IVIG (1 g/kg for 2 days), followed by high dose factor IX (2×150 mg/kg). The inhibitor was boostered to >90BU/ml for only a few days and immunmodulation was increased by adding mycophenolate-mofetil (2×300 mg/ m^2 po daily). The inhibitor was not detectable for the next months, but as factor IX half life had not normalised dexamethasone and IVIG were repeated after 8 weeks of ITT. After this second pulse half life of factor IX was 8 hours and ITT was continued. However, after 100 exposure days the patient suddenly developed generalised utricaria and moderate bronchospasm with coughing shortly after infusion of factor IX. The patient was admitted to the outpatient clinic and factor IX was given. The same symptoms of anaphylaxis developed, which resolved shortly after injection of corticosteroids. On a first attempt to eliminate the inhibitor the patient again showed signs of anaphylaxis after adding fresh frozen plasma (FFP) to the plasmapheresis circuit. Therefore the second course of plasmapheresis was started only after injection of corticosteroids and fresh frozen plasma was added only after 30 minutes when activated clotting time was above 400 s. However, the patient again reacted with coughing and wheezing, which resolved after injection of corticosteroids.
Conclusions: Physicians and parents should be aware that patients with haemophilia B may develop an anaphylaxis even several months and >100 exposure days after induction of ITT for an anti-factor IX inhibitor. Even fresh frozen plasma may be dangerous for these patients.

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CLOPIDOGREL REDUCES PLATELET-LEUKOCYTE AGGREGATES AND CD62-EXPRESSION IN PATIENTS WITH PERIPHERAL OCCLUSIVE ARTERY DISEASE (POAD)

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Formation of platelet-leukocyte-aggregates (PLA) via CD62-ligand and subsequent expression of MAC-1 on leukocytes is an important mechanism in thrombosis and inflammation. In a cross-sectional study, we assessed the formation of PLA in patients with POAD (stage IIa) under treatment with aspirin (group 1, N=15), clopidogrel (group 2, N=15), combined A+C (group 3, N=10), untreated patients (group 4, N=5) and age-matched healthy subjects (group 5, N=10). Assessment (flow-cytometry, either % cells with positive staining or mean flourescence intensitiy MFI) included PAC1 (the activated GPIIb/IIa-receptor), CD62, monocyte-PLA's and MAC-1.

Group	1	2	3	4	5
PAC1 (MFI)	50±23	42±20	37±18	46±6	50±24
CD62 (% +)	17±6*#	8±4	7±5	17±8*#	8±3
M-PLA(% +)	21±17*#	13±8	7±2	30±8*#	7±3
MAC-1(MFI)	757±236*#	461±242	403±229	840±64*#	536±285

* p<0.05 (vs. group 5) # p<0.05 (vs. group 2 and 3)

After stimulation with thrombin-receptor activating peptide, PLA formation and CD62 expression was enhanced by the 2-4 fold in untreated patients and those under aspirin, but not under C. The results indicate that (1) the activated GPIIb/IIa-receptor (PAC1) is similarly distributed in all groups, and not different from healthy controls, (2) CD62 and PLA are enhanced in POAD, and not inhibited by asprin treatment, but (3) are reduced under C and especially under combined C+A, to values similar to those obtained in healthy controls. The results point to a special role of C and related compounds in inflammatory reactions during thrombosis.

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INVESTIGATIONS ON THE EFFECT OF NATURALLY OCCURING HUMIC ACIDS AND SYNTHETIC HUMICACID-LIKE POLYMERS ON PLASMA PROTEIN C ACTIVITY

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Due to its ability to inactivate factor Va and VIIIa, activated protein C (PC) is a potent anticoagulant, which prevents further thrombin production. The impact of this anticoagulant pathway is demonstrated by the occurrence of a severe thrombotic disease in subjects affected by genetic PC deficiency. The attenuation of activated PC (APC) by fluorescent humic acids (FHA) from drinking water is suggested to cause thrombotic disorders in Blackfoot disease, an endemic peripheral vascular disease in Taiwan. The aim of this study was to determine the influence of naturally occurring HA and synthetic HA-like polymers on APC activity in both an isolated system and human plasma. As naturally occurring HA sodium humate isolated from a rainmoor peat in Mecklenburg-Vorpommern (Germany) and the commercially obtained Aldrich HA were used. As HA-like polymers, the oxidation product of caffeic acid (KOP) and the synthetic melanoidin M100A were selected. To determine the APC inhibition by the test substances, Protac-activated PC was either investigated in human plasma or in plasma-free medium. APC activity was measured using the chromogenic substrate method. NHA did not at all impair APC activity in plasma. KOP, Aldrich HA and melanoidin at the highest concentration used in this study (800 µg/mL) reduced APC activity by 60% (KOP), 52% (HA Aldrich)and 21% (melanodin), respectively. Only in the plasma-free system, the test substances were found to in-

hibit APC activity more efficiently. The strongest effect was observed with KOP. A concentration of 150 µg/mL reduced the APC activity about 50%. Nearly the same effect was achieved with 800 µg/mL of Aldrich HA and NaH. Like observed in the test system plasma the Melanoidin had a relatively weak influence on APC activity.
In conclusion, peat water HA in the concentration range tested do not impair the protein C anticoagulant pathway. Therefore, it seems unlikely that oral intake of HA from drinking water may induce a thrombophilic state.

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FACTOR XA INHIBITION BY NATURALLY OCCURING HUMIC ACIDS AND SYNTHETIC HUMIC ACID-LIKE POLYMERS

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Previous studies have shown that naturally occurring humic acids (NHA) as well as synthetic humic acid-like polymers (HALP) prolong the clotting time of blood in vitro and in vivo. This anticoagulant effect is mainly based on the inhibition of activated factor IIa (FIIa). The present study was designed to determine the influence of NHA and HALP on activated factor Xa activity (FXa). As HALP, the synthetic caffeic acid oxidation product (KOP) and the synthetic melanoidin M100A type M1 were selected. As NHA, sodium humate, isolated from a rainmoor peat situated in the coastal region of Mecklenburg-Vorpommern and the commercially obtained Aldrich HA were included in the experiments. We have investigated the direct inactivation and the inactivation of FXa in presence of ATIII. The protease activity was measured using a chromogenic substrate method in an isolated system. The test substances were found to inhibit dose-dependently free FXa activity. The strongest effect was observed with KOP, followed by NHA, HA Aldrich and Melanoidin M1. 50%-Inhibition concentrations ranged between 50 µg/mL and 300 µg/mL (Melanoidin M1). In the presence of ATIII, the inhibition of FXa by KOP (3,1-25 µg/mL9 was recognised as an additive synergistic effect.
In conclusion, peat water NHA from a rainmoor peat, Aldrich HA as well as the synthetic humic acid-like polymer KOP and the Melanoidin M1 inhibit FXa in an isolated system. In combination with ATIII, KOP has an additive synergistic effect.

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KINETIC OF INHIBITORS OF VON WILLEBRAND FACTOR CLEAVING METALLOPROTEASE IN THREE PATIENTS WITH SEVERE THROMBOTIC THROMBOCYTOPENIC PURPURA

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Acquired thrombotic thrombocytopenic purpura (TTP) is caused by inhibitors to von Willebrand factor cleaving metalloprotease (vWF-CP) and results in the presence of ultralarge multimers of von Willebrand factor (vWF), leading to platelet aggregation. Plasma exchange to remove the inhibitors and multimers and substitute vWF-CP is the treatment of choice. Here we present case reports on three female patients who were admitted with TTP (thrombocytopenia, hemolytic anemia with red cell fragmentation, renal and neurologic disturbance). Treatment consisted of daily plasma exchange (50ml/kg/day). One patient, with a history of lupus erythematosus, also received prednisone.
We serially monitored the patients for vWF-CP activity with a modified assay according to Gerritsen et al. [Thromb.Haemost. 82:1386-9, 1999] and inhibitor titers against vWF-CP using a newly developed assay based on mixing the patient's plasma with normal plasma. We also analysed the pattern of vWF multimers using SDS electrophoresis. In all patients ultralarge multimers of vWF were present, vWF-CP was too low to measure, and inhibitors were detectable (3.6/3.7/10.7 inhibitor units/ml). During plasma exchange, inhibitor titers decreased in all patients and platelet counts increased but after 6-37 days inhibitor titers increased again, accompanied by a fall in platelet counts. In one patient we initiated immunoadsorption while continuing plasma exchange. We found that after 14-45 days platelet counts returned to normal and the symptoms resolved completely but ultralarge multimers and inhibitor titers, between 0.6 and 6.2 inhibitor units/ml, were still detectable and vWF-CP absent. After 150-192 days the inhibitors disappeared and vWF-CP began to return to normal values.
These cases show that 1.) modified treatment protocols (i.e. immunoadsorption, immunosuppression etc.) can be more successful in treating patients with TTP who respond poorly; 2.) vWF-CP inhibitors can be present for months in asymptomatic patients, who could be at risk of relapse; 3.) measuring vWF-CP and inhibitors could be useful for diagnosing and monitoring TTP.

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SUCCESSFUL USE OF PROTEIN C CONCENTRATE (CEPROTIN®) IN TWO PATIENTS WITH SEVERE SEPSIS AND DISSEMINATED INTRAVASCULAR COAGULATION (DIC)

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We report 2 cases with septic shock and DIC successfully treated with a protein C concentrate (Ceprotin®, Baxter, Austria).
A 31-year-old woman had pneumococcal infection and severe DIC (fibrinogen 103 mg/dl, platelets 20 G/l, D-Dimer 119 µg/ml), cutaneous bleedings and ischemia of the leg. Treatment with antibiotics, FFP, platelet concentrates, heparin, and fibrinogen was unsuccessful. On the 2nd day Ceprotin® was initiated (14.000 U over 24 hours). Concomitantly, iv fibrinolysis (20 mg rtPA) was performed. Protein C activity increased from 0.38 to 1.47 U/ml, platelets and fibrinogen normalized. Sepsis and necrotic lesions resolved, but a toe had to be amputated.
A 48-year-old woman had meningococcal infection. Initially, no signs of DIC were present. The patient was included into a study with activated protein C (Drotregocin-alpha activated, Eli-Lilly, Austria). After 96 h platelets dropped to 23 G/l, fibrinogen to 70 mg/dl, D-dimer rose to 25.5 µg/ml, and necrosis of the toes on both feet occurred. Treatment with Ceprotin® was initiated to maintain protein C levels above 1.0 U/ml (total 40.000 U over 7 days). Protein C activity increased from 0.32 to 2.00 U/ml initially and remained stable at 1.00 U/ml thereafter. Platelets and fibrinogen normalized. A liver CT revealed a giant hemorrhagic necrosis. Sepsis resolved and the patient was discharged after 30 days.
None of the patients had signs of increased bleeding during treatment with Ceprotin®. We conclude, that Ceprotin® is an effective treatment of DIC during sepsis, without significant side effects. However, controlled studies are necessary to evaluate dosage, duration of administration, and the effects on outcome.

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A HISTOLOGICAL STUDY OF FVII-ACTIVATING PROTEASE (FSAP) DISTRIBUTION IN HUMAN TISSUE

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Aims: Structural and biological characteristics of a recently described plasma serine protease, which displays factor VII as well as prourokinase activating properties in vitro, indicated a potential dual role for this factor VII-activating protease (FSAP) in hemostasis. FSAP circulates in plasma as a proenzyme and can activate by an autocatalytic reaction or by urokinase. FSAP reveals high affinity to glycosaminoglycans rendering the interaction with extracellular matrix and cell surfaces probable. There is few information available as yetabout the distribution of FSAP in human cells and tissues and its potential cell physiological function.
Methods: Samples of different normal human tissues were immunostained employing two monoclonal antibodies (MAB) (Aventis Behring GmbH, Germany) to FSAP, recognizing the heavy- or light-chain domains of the activated protease respectively. A standard APPAP-protocol and semiquantitative evaluation was performed. Immunhistochemistry: pronase pre-digestion, primary antibody MAB 677 diluted 1:4000 and MAB 1189 diluted 1:350, APAAP technique.
Results: Almost all epithelial cell types showed a variable intracytoplasmatic immunoreactivity, in particular in different kinds of endocrine and neuroendocrine cell types like Leydig cells of the testis, throphoblastic und syncytotrophoblastic cells of the placenta and cells of the Langerhans islets of the pancreas. Furthermore, the endothelium of venes,arteries and plasma cells in the bone marrow as well as in different tissues were found positive with both monoclonal antibodies. In contrast, immunostaining for FSAP was negative or weak in mesenchymal derived cell types in a great variety of normal human tissues.
Conclusion: The results suggest that FSAP is a ubiquitious protein, because we detected it in a great variety of human tissues., in particular endodermal, mesodermal and ectodermal derived cell types. Beyond FSAP's potential role in hemostasis, further investigation is necessary to learn more about its interaction with cells and a potential function in cell physiology.

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RETHROMBOSIS UNDER LOW MOLECULAR WEIGHT HEPARIN IN THE YOUNG AGE – TWO CASE REPORTS

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Introduction: Low molecular weight heparins (LMWH) have been shown to be efficious in secondary prophylaxis of thrombosis in children and the recurrence rate of thrombosis seems to be less than 3% (Dix et al., J Pediatr 2000, 439-445). In contrast to adults, in children and in youths the dose of LMWH is adapted to the body weight and the anti-Xa activity. According to the literature for an effective prophylaxis in children an anti-Xa activity between 0.2 to 0.4 anti-Xa IU/ml is needed.
Case 1: A 15 years old boy suffered spontaneously from a left-sided sino-venous thrombosis. Treatment with unfractionated heparin (UFH) i.v. for 14 days led to a partial vessel recanalization. After that he received enoxaparin at 100 anti-Xa IU/kg s.c.o.d. corresponding to an anti-Xa activity of 0.3 IU/ml. One month later he suffered from a MRI-confirmed recurrent sino-venous thrombosis on the opposite side. An elevation of lipoprotein a (64.5 mg/dl; normal <30 mg/dl) was found as hereditary thrombophilic risk factor.
Case 2: During immobilization after surgical procedure a 16 years old boy suffered from an incomplete thrombosis of the left popliteal vein. Treatment was started with nadroparin at 95 anti-Xa IU/kg s.c.b.d.. After 8 days nadroparin dose was reduced to 55 anti-Xa IU/kg s.c.o.d. corresponding to an anti-Xa activity of 0.22 IU/ml. Three weeks later, the patient was completely mobilized, he developed a complete left-sided thrombosis in parts of femoral, popliteal, saphenous parva and soleus veins. A heterozygous state for factor V G1691 mutation was detected. In both cases a heparin-induced thrombocytopenia was excluded.
Conclusion: The two case reports demonstrate that under treatment with LMWH recurrence of thrombosis may occur even if the plasma anti-Xa activity is within the already established prophylactic range for children between 0.2 to 0.4 IU/ml. It could be speculated that for an effective secondary prophylaxis anti-Xa activities between 0.4 to 0.6 are needed. Patients with some hereditary thrombophilic risk factors might have a higher risk of recurrent thrombosis despite they are treated with LMWH.

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QUANTIFICATION OF SEROTONIN RELEASE FROM PLATELETS USING HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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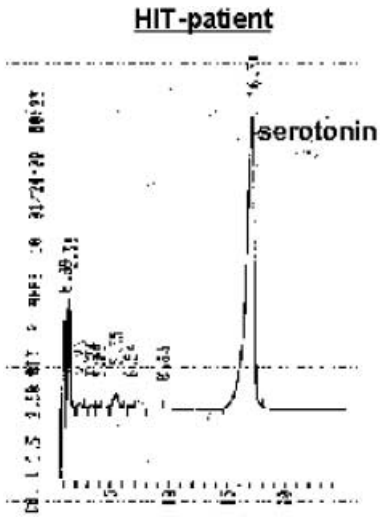
Background: So far, the release of serotonin can not be quantified from donor platelets in the presence of antibodies towards heparin/platelet factor 4 in serum of patients with heparin-induced thrombocytopenia (HIT). We have developed and validated a high pressure liquid chromatography (HPLC) for quantitative determination of serotonin.
Methods: The linear range of the dilution curve of serotonin was between 0 and 400 ng/ml. 99 serum samples were analysed from normal controls (n=60), HIT-patients (n=19) and non-HIT-patients (n=20) by serotonin release using HPLC (HPLC-SRA). We incubated serum of patients and donor platelets with 0 IU/ml, 0.2 IU/ml and 10 IU/ml heparin, low-molecular-weight heparin (LMWH) and 0,02 or 1 IU/ml danaparoid. The platelet count was set on 300.000 platelets/µl. With the aid of a serotonin standard-curve obtained by plotting the serotonin concentration versus the height of the eluted peak the concentration of the released serotonin could be measured.
Results: Serotonin eluted as a single peak from the column between 16-17 min (Fig.1). Sera from HIT-patients released 5.56-352.55 ng/ml and 6.67-1533.33 ng/ml serotonin in the presence of 0.2 IU/ml heparin or LMWH and donor platelets. In the presence of 0 IU/ml UFH or LMWH a maximum of 2.42 ng/ml serotonin was detected in these patients. After incubation with 10 IU/ml UFH or LMWH the highest concentration of serotonin was 20 ng/ml. Serotonin was not release in the presence of danaparoid. All healthy persons and non-HIT patients had negative results of serotonin release from donor platelets. The sensitivity and specificity of the HPLC-SRA were 94.7% and 100% compared to the EIA-SRA (Br J Haematol 2000, 109:182-6), respectively.
Conclusion: The HPLC-method permits the quantification of serotonin released from platelets and presents a reliable method for laboratory confirmation of heparin-induced thrombocytopenia.



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AN INTERLABORATORY STUDY ON THE VARIATIONS IN THE PLATELET COUNT AND IN PLATELET RENTENTION MEASURED IN A STANDARDIZED FILTER SYSTEM (RETENTION TEST HOMBURG: RTH)

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Background: An interlaboratory task force (Berlin: Kiesewetter, Grigorov, Monien; Frankfurt: Klinkhardt; Gießen: Matzdorff; Hamburg: Gutensohn; Ludwigshafen: Hellstern; Moskau: Bokarev; Wien: Dostal, Huber, Krenn; Homburg: Nemeh, Schenk) explored the variability of platelet retention based on the RTH test. The goal of he study was to compare in series precisions and to evaluate the current standard operation procedure.
Methods: 500 µl citrated whole blood was exposed to the "Eppendorf retention tubes" and centrifuged at 110 g for 5 minutes after incubation of 10 minutes. Platelet counts (PC) were calculated before and after the filter passage. The retention index (RI), and LCRI was calculated as "PC before" minus "PC after" divided through "PC before" (%). All laboratories used the same guideline procedures and their individual equipments (counter and centrifuges). Center comparisons with respect to precision of RTH as well as of platelet count was performed by applying Kruskal Wallis test to series mean values.
Results: 2 of the 8 laboratories didn't finish the complete program until now and the data of these laboratories are not included in this preliminary evaluation. 4 laboratories (29 serial measurements) evaluated 25 of 29 RI indices within the range given by the reference laboratory, 2 laboratories evaluated 9 of 11 values above the upper reference range (RI=45,4%). These 2 laboratories changed the standard conditions concerning centrifugation procedure. Therefore the series means of 6 laboratories were heterogeneous (means between 8% and 60%, p=0.005). Within series standard deviation computed for each center varied between 6% and 13%. Thus centers were not homogenous in this respect (p=0.03), too. The variability of the precision of platelet count in serial was found to be significantly different between the laboratories.
Conclusions: Most of the laboratories using first time the RTH test reproduced the reference values. In this sense the RTH meets the criteria for a simple screening test for platelet function. However all laboratories demonstrated high intra-essay and inter-laboratory imprecision in platelet count. The interlaboratory study gave several hints as to which parts of the RTH procedure need to be improved. At first special care is necessary in handling of material after centrifugation in order to obtain valid platelet counts. At second a careful standardization of the preanalytic procedures seems to be mandatory.

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LONGTERM-THROMBOPROPHYLAXIS WITH THE LOW MOLECULAR WEIGHT HEPARIN CERTOPARIN AFTER ENDOPROTHETIC JOINT REPLACEMENT OR OSTEOSYNTHESIS OF THE LOWER LIMB

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The peri- and postsurgical thromboembolic prophylaxis is a long established therapy standard. What remains controversial, however, is the duration of the prophylaxis. Universal and valid guidelines for this do not exist yet, neither nationally nor internationally. A few clinical studies have pointed, however, to the fact that the thromboembolic risk of high-risk patients persists longer than the station stay. Recently it was demonstrated that after surgery hypercoagulatory conditions sustain beyond hospital discharge with a respective risk of deep vein thrombosis (DVT) and pulmonary embolism (PE). The aim of the LP-1 study was to show that prolongation of thromboprophylaxis with Certoparin reduces the risk of venous thromboembolism (VTE) after endoprothetic joint replacement or osteosynthesis of the lower limb. The LP-1 was a multicentre, randomized, double-blind, placebo-controlled trial. 360 patients were initially enrolled, all of them received 3000 U aXa of Certoparin once daily for 14 days to prevent VTE. At day 14, 312 patients were randomized to prolong Certoparin application or to placebo up to day 42. During day 14 and day 42, 29 patients dropped out, mainly because of adverse events or withdrawal of consent; 10 patients could not be considered because of major protocol violations. Therefore, the per-protocol population consisted of 273 patients, 146 patients received Certoparin and 127 patients received placebo. Concerning demographic characteristics no significant differences were observed. 8 patients of the Certoparin group developed VTE (per-protocol population: 5.5%, 7 DVT, 1 PE) as compared to 18 patients of the placebo group (14.2%, 17 DVT, 1 combined DVT/PE). The analysis revealed a statistically significant difference in favor of Certoparin with a relative reduction rate of 61%. Two bleeding complications were obtained in the Certoparin group, additionally, two patients died due to bronchopneumonia. In conclusion, the LP-1 study clearly shows that patients after endoprothetic joint replacement or osteosynthesis of the lower limb have a persisting risk to develop VTE beyond the routine duration of thromboprophylaxis. Prolonged prophylaxis with Certoparin (3000 U aXa once daily) significantly reduced this risk and should be strongly recommended.

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***ROLE OF COAGULATION FACTOR XIII IN CARDIO- AND CEREBROVASCULAR DISEASES**

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After activation by thrombin, blood coagulation factor XIII (FXIII) plays an important role in clot stabilisation by cross-linking fibrin chains. Through this process soluble fibrin chains are transformed into a stable insoluble clot. Until recently, FXIII has not been studied in relation to cardio- and cerebrovascular diseases despite its important role in the final stage of the coagulation process. New insights regarding the role of FXIII in vascular diseases suggest a major role of this coagulation factor in vascular thrombotic disorders. A common G→T point mutation in codon 34, exon 2 of the A-subunit gene which codes for a Valin→Leucin change (FXIII-Val34Leu) only three amino acids from the thrombin activation site has been described. In Caucasians, the allele frequency has been shown to be around 23%. Because of the high allele frequency and the proximity of the amino acid change to the thrombin activation site, this polymorphism was a candidate for a role in the pathogenesis of thrombotic disorders. FXIIIVal34Leu has recently been shown to be protective against myocardial infarction (MI) and ischemic stroke, but this polymorphism is also associated with an increased risk for hemorrhagic stroke. Additionally, FXIII Val34Leu is supposed to be protective against pulmonary embolism and deep vein thrombosis, which could have been shown less clearly so far. As possible mechanisms for the antithrombotic effect, premature depletion of the mutant protein from circulation and altered fibrin structures of clots cross-linked by the mutant FXIIIa are under discussion. In addition, this common polymorphism possibly contributes to the contrasting cardiovascular risk in different ethnic groups. Another interesting observation is the interaction between FXIII and the insulin resistance syndrome, i.e. an interaction between a component of the coagulation cascade and therefore a thrombotic risk factor and classical atheromatous risk factors. Subjects possessing the protective Leu allele in whom there was still a history of MI were further investigated. These subjects had higher concentrations of plasminogen activator inhibitor-1 (PAI-1), insulin, proinsulin, and an increased body mass index (BMI), changes which were not observed in subjects possessing the Val/Val genotype. These findings indicated that inhibition of fibrinolysis through increased PAI-1 levels negates the protective effect of the Leu allele and suggest an interaction with insulin resistance. Therefore, FXIII must be considered as another coagulation factor contributing to the complex gene-environment interactions which are important for the pathogenesis of cardio- and cerebrovascular and thromboembolic diseases. Little is known about the plasma activity and antigen levels (A- and B-subunits) in subjects with vascular diseases but studies on FXIII measures in subjects with cardio- or cerebrovascular diseases are undergoing.

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ANALYTICAL VALIDATION OF THE NEW ROTEG® 05 WHOLE BLOOD HAEMOSTASIS SYSTEM

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Introduction: Rotation thrombelastography and ROTEG® analysis are an enhancement and extension of classical thrombelastography (TEG). ROTEG® 05 (“R5”) is an evolutionary modification of the previous analyser with enhancements, e.g. in the opto-mechanical detection system, in mechanics, ergonomy and in the electronic pipette. The scope of the study was the validation of R5 versus the ROTEG® 04 (“R4”).
Materials and methods: Assays: EXTEG, INTEG (ex-/intrinsically activated TEG, with reagents from Nobis or Pentapharm). Samples: Whole blood samples from various donors were tested untreated, diluted with saline (in order to simulate haemodilution), or spiked with different concentrations of heparin. Most samples were tested in duplicate in order to get data on the precision. Parameters: Coagulation time (CT), clot formation time (CFT), alpha- angle (a), clot firmness after 15 minutes (A 15).
Results: The precision within series (N=8 on two instruments each, mean values, in % CV) for a normal blood sample and INTEG was 7.0% (CT), 7.9%(CFT), 2.0% (a), and 3.3% (A15) for ROTEG 05, the respective data for ROTEG 04 were in a similar range. The correlation of the two instruments as tested with 39 normal, diluted or heparinised samples was r=0.949 (CT), 0.952 (CFT), 0.925 (a) and 0.964 for A15 for EXTEG and INTEG (pooled data). When the results of the two individual tests of a double determination on the individual instruments were plotted (regression 1st value/2nd value), the correlation coefficient was r=0.981 (CT), r=0.985 (CFT), 0.942 (A 15), r=0.97 (a) for R5 and r=0.926 (CT), r =0.936 (CFT), r=0.966 (A15) and r=0.91 (a) for R4.
Conclusion: The results demonstrate that the two ROTEG® versions generate substantially equivalent results. However, the precision of the new ROTEG® 05, as deducted form the regression of the duplicate analysis, was improved.

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LOCAL OVEREXPRESSION OF TISSUE FACTOR PATHWAY INHIBITOR INTERFERES WITH INTIMAL HYPERPLASIA AFTER ANGIOPLASTY IN HYPERCHOLESTEREMIC RABBITS

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Background: Tissue factor (TF), the major procoagulant of the atheroma and activated blood monocytes, is locally expressed at the site of balloon injury mediating a prolonged prothrombotic state. TF-pathway inhibitor (TFPI) regulates hemostasis and direct mitogenic effects of coagulation factor Xa and thrombin.
Aim: To interfere with the process of restenosis by percutaneous overexpression of TFPI.
Method and Results: Myc-tagged TFPI (n=10) or control (beta Galactosidase, n=10) adenoviral transfection (4.5×10^9 plaque forming units) was performed in iliac arteries of atherosclerotic NZW rabbits using a drug delivery catheter at 8 atm. Arterial expression of the transgene was confirmed by immunohistochemistry on day 2, 5 and 21 after percutaneous overexpression in media smooth muscle and intimal cells. Restenosis after 21 days as quantitated by intima to media (I/M) ratio was significantly reduced by 63% in AdV-TFPI treated arteries (0.9±0.4) when compared with AdV-beta Gal (2.7±0.7) treatment ($p<0.0001$).
Conclusion: Site directed percutaneous expression of TFPI in atherosclerotic arteries reduces intimal hyperplasia in response to balloon injury.

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PLATELET MONOCYTE CROSS TALK AND TISSUE FACTOR EXPRESSION IN STABLE AND UNSTABLE ANGINA

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Background: Tissue factor (TF), the major procoagulant in vivo, is usually absent from blood cells. However, monocyte (Mo) TF-expression and platelet activation are both features of unstable angina and may contribute to this prothrombotic state.
Methods: In 60 coronary artery disease patients with stable (SA, n=44) and unstable angina (UA, n=16) MoTF expression and total (CD42) and activated (CD62P+) platelet load was determined by flow cytometry on CD14+-cells. MoTF-dependent procoagulant activity (PCA) was measured by chromogenic assay. Prothrombin fragment F1.2 was determined by ELISA and expression of TF mRNA was confirmed by RT-PCR.
Results: MoTF-antigen level (16.4±3.3 MFI), MoTF-PCA (68.5±38.2 U/106 PBMC) and activated platelet load on Mo (CD62P+:198±130 MFI) were significantly higher in UA compared with SA (14.1±3.1 MFI, p=0.03; 47.9±18.1U/106 PBMC, p=0.04; 126±134 MFI, p=0.03). Both, total (r=0.69, p<0.001) and activated (r=0.47; p=0.002) platelet load positively correlated with MoTF Ag-expression, which further correlated with TF-dependent PCA and F1.2 levels. MoTF mRNA in was expressed in 26% of SA and in 37.5% of UA.
Conclusion: We show elevated MoTF-mRNA and MoTF-antigen expression paralleled by higher MoTF-dependent PCA in UA compared with SA patients and correlating with total and activated Mo-platelet load. This suggests increased platelet-leucocyte cross talk contributing to the procoagulant state in acute coronary syndrome.

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TISSUE FACTOR IN MORBIDLY OBESE PATIENTS AND IMPACT OF WEIGHT LOSS FOLLOWING GASTROPLASTY

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Background: Altered expression of proteins of the coagulation and the fibrinolytic cascade in obesity is associated with increased risk of cardiovascular morbidity and mortality. To which extent and by which mechanism obesity and weight loss affect tissue factor (TF) is not known.
Methods: Thirtyseven morbidly obese patients (4 men), median BMI 47 (range 42-53) undergoing vertical stapled gastroplasty with banded outlet to induce weight loss (postoperative BMI 33, range 29-36) were analyzed for metabolic, inflammatory and coagulation parameters before and after the operation. Mean intervall was 13 (range 11-17) months.
Results: In the patients studied plasma levels of TF correlate with TGF-β1 (r=0.48; p=0.027), known as a product of adipocytes and a potent inducer of TF in adipose tissue. TGF-b1 correlates with parameters of the metabolic syndrom such as elevated blood glucose (r=0.4, p=0.03, impaired glucose tolerance (IGT: r=0.46, p=0.01) and hypertriglyceremia (r=0.45, p=0.016). Weight loss was associated with significant reduction of plasma tissue factor (TF) (pre:314±181pg/mL; post: 235±113pg/mL, p=0.04), coagulation factor VII (pre: 128±21%, post: 112±18%, p=0.004) and correlated with decrease in prothrombin fragment F1.2 (pre: 2.4±3.4 nmol/L;post: 1.2±1.1 nmol/L, p=0.04; r=0,56, p=0.005). Chi-Square analysis showed 60% of obese patients with IGT in the high TF-group (>264pg/ml) and only 40% in the low TF-group (<264 pg/ml) wherase 100% of obese patients with normal glucose tolerance were in the low TF-group. Weight loss due to gastric banding was associated with higher loss of plasma TF (>56 pg/ml) in obese patients with IGT compared with NGT.
Conclusion: Elevated levels of plasma TF are significantly reduced with weight loss correlating with the improvement of the metabolic syndrome. No direct correlation was found with reduced proinflammatory parameters such as IL-6, CRP or TNFα.

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MTHFR GENOTYPE VERSUS FASTING HOMOCYSTEINE CONCENTRATIONS IN HEALTHY CHILDREN AND CHILDREN WITH THROMBOEMBOLISM

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The association of fasting homocysteine concentrations and the MTHFR genotypes in healthy Caucasian children and children with thromboembolic events was evaluated.

Methods: We measured fasting homocysteine concentrations and the MTHFR C677T genotypes in 291 children: 101 patients (3 to 6 months after the acute thromboembolic event), 121 healthy siblings, 63 healthy control children. Additionaly the MTHFR genotypes - (homocysteine concentrations not included) in 148 children with idiopathic stroke, and 345 children with venous thrombosis were measured.
Results: 1. In the healthy control population 10,4% the MTHFR TT genotype, 34.2% the CT genotype and 55.4% the CC variant was shown. MTHFR genotypes account for the following fasting homocysteine median (range) concentrations in healthy controls (CC: 6.0 µmol/l (2-15); CT: 7.0 µmol/l 3-22); TT: 6.0 µmol/l (3.8-10)) with upper age-specific 90th percentiles of 12.0 µmol/l (<12 months), 9,1 µmol/l (1-6 years), 9,5 µmol/l (7-10 years), and 10.6 µmol/l (11-18 years). The age-dependency was statistically significant (Kruskal-Wallis-test: p=0.029). The following frequencies of homocysteine concentrations >90th age – dependent percentile (patients versus controls), odds ratios (OR) and 95% confidence intervals (CI) were found: Patients 13.8%, Controls 3.2%, OR 4.8 (1.1-22.0), Fisher's exact test: p=0,03.
2. In children with stroke the MTHFR 677TT genotype was significantly increased (OR/95%-CI): 2.6/1.5-4.5), whereas this variant does not play a role in paediatric patients with venous thrombosis (OR/95%-CI: 1.2/0.63-2.4).
Conclusion: These data suggest that mildly elevated fasting homocysteine concentrations > the 90th age-dependent percentile are associated with vascular occlusion in children. The MTHFR 677TT genotype is a risk factor for ischaemic stroke in children of Caucasian origin, but not for venous thrombosis.

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THROMBOEMBOLISM IN CHILDREN: CLINICAL ASPECTS IN CARRIERS OF THE HETEROZYGOUS FV G1691A MUTATION IN COMPARISON WITH THE PT G20210A VARIANT

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The present study was designed to further investigate the circumstances of a first symptomatic thromboembolic onset (TE) in 848 consecutively recruited pediatric patients aged neonate to < 18 years (median 6 years; male 58%) carrying either the heterozygous FV G1691A mutation or the PT G20210A variant without additional established prothrombotic risk factors. The FV mutation, the PT G20210A variant, deficiencies of protein C, protein S, and antithrombin were evaluated concomitantly with potential triggering factors, i.e. immobilization, surgery, trauma, central lines, steroid administration, oral contraceptives (females) and smoking respectively. 158 of the 848 children carrying either the FV or the PT mutation, and not suffering from protein C-, protein S-, or antithrombin deficiency were enrolled in the study presented here. 121 of 848 patients carried the heterozygous FV mutation (14.3%; male n=67), and in 37 children the heterozygous PT mutation (4.4%; male n=23) was diagnosed. In patients carrying the FV mutation compared with carriers of the PT variant no statistical difference was found with respect to additional acquired triggering factors (Mann-Whitney p=0.87). With a median (range) age of 4.0(0.1-18) years a first thromboembolic event was diagnosed in children with the FV mutation, whereas pediatric patients with the PT mutation showed a first symptomatic TE with a median (range) age of 8.5(0.1-17.5) years respectively. The thrombosis-free survival was significantly reduced in male pediatric FV carriers compared with male PT carriers (logrank: p=0.015), however, no such difference was found in the female cohorts investigated (logrank: p=0.22). In conclusion, based on the data presented here the FV mutation as well as the PT variant are risk factors for symptomatic TE's in children, with a significantly earlier symptomatic onset in male carriers of the FV gene mutation.

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RELIABILITY OF PLATELET RETENTION MEASUREMENT USING THE PLATELET RETENTION TEST HOMBURG ("RT-H")

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Platelet adhesion was measured using a filter system developed in cooperation with "Eppendorf", the filter material used is polyurethane. In contrast to other test systems as described by O'Brien, Hellem, or in the <=PFA<=- the "Eppendorf retention filter" is athrombogenic. Pre-activa-

tion of platelets may therefore only be attributed to the shear stress induced by standardized centrifugal conditions. We aimed to evaluate the technical precision and biological variability of the <=RT-H<=assay in healthy blood donors and calculated the retention index (<=RI<=) of the total platelet population as well as the retention of large platelets (<=LCRI<=).
The interaction of the platelets with the filter system was characterized by electron microscopy. RT-H: Platelet rich plasma (<=PRP<=) was obtained by centrifugation of citrated blood at 138 g for 15 minutes. 500 µl <=PRP<= was exposed to the <=Eppendorf filter system<=30-180 minutes after the withdrawal of blood. The <=PRP<=- samples were centrifuged in the test system at 110 g for 5 minutes. Platelet count , and <=LCR<=(large cell ratio) were evaluated before and after the filter passage using a Sysmex counter (RI: 13.6% to 30.3%-95% range of confidence). Reference values for platelet retention was measured in two cohorts of healthy blood donors (group Ia: n=25; group Ib: n=50).
Transmission electron microscopical evaluation of the polyurethane filter systems shows that platelets adhere to the polyurethane surfaces, spread, form aggregates and degranulate. The coefficient of variation for the platelet count in multiple measurements in series is between 2.58% and 7.61% and from day to day 5.26% to 8.32% respectively.
After adding ADP, or Collagen the <=RI<= increases dose dependently. After adding platelet inhibitors (e.g. PGE1, ASS) the <=RI<= decreases dose-dependently. Platelet retention in healthy human beings ranged from 13.6% to 30.3%. (group Ia: mean ±SD: 21.9% ±4.2 %; group Ib: 20.99% ±4.01%). RT-H values did not differ significantly between both groups (<=LCRI<=: 25.9% ±6.8%).
The technical precision of the <=RT-H<= is acceptable and its biological variability in healthy blood donors is rather low (13.6% to 30.3.%-95% range of confidence). The retention of large platelets is significantly higher than the retention of the total platelet population.

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FACTOR VIII ONE-STAGE ASSAY VALIDATION
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Objective: Validation of the one-stage assay of FVIIIC in plasma according to the “Note of Guidance on Analytical Methods Q2B (CPMP/ICH/281/95).”
Background:The one-stage assay of FVIIIC in plasma is used since many years in coagulation laboratories using mostly commercial reagents.
A validation of the method has not yet been published.
Materials and methods: WHO-plasma standard 97/586 (NIBSC).Standard human plasma (three lots), FVIII deficient plasma (three lots) and Actin® (three lots) of Dade-Behring, D-Marburg. FVIII Control plasma and Coagulation Control A and N (Immuno-Progen, D-Heidelberg). Coagulation determination with a KC10 micro Coagulometer of Amelung, D-Lemgo.Statistical Analysis: Excel sheets of Klinker & Partner, Saarbrücken.
Results: The one-stage assay of FVIIIC was performed as described in DIN 58 909, 1995. The validation of the one-stage assay according to the “Note of Guidance on Analytical Methods Q2B (CPMP/ICH/281/95)” gave the following results: The method is linear over a range of 0.005 to at least 0.9 IU FVIIIC/ml (Mandeltest: 0.1244; Fkrit: 12.2463). The method is accurate over a range of 0.01 to at least 0.9 IU FVIIIC/ml (Variation: <6.5% from 0.92 to 0.05 and <19.0% below 0.01 IU FVIIIC/ml). The repeatability of FVIII controls is <6.3% for the range of 0.8 to 0.25 IU FVIIIC/ml. The intraassay pre-cision of the method is <5.0% over a range of 0.005 to 0.92 IU FVIIIC/ml. The confidence limit of the method is <9.1% over a range of 0.01 to 0.9 IU FVIIIC/ml. The detection limit of the method is 0.008 IU FVIIIC/ml.
Interpretation: A validation of the one-stage assay of FVIIIC according to the “Note of Guidance on Analytical Methods Q2B (CPMP/ICH/281/95)” has never been published. The data of our validation show for the first time the limits of the one-stage FVIII assay. Establishing the lower limit of detection is important in light of the recently published definition of severe Haemophilia as less than 0.01 IU/ml.

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FUNCTION-RELATED LOCALIZATION OF TISSUE FACTOR IN MYOCARDIUM
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Background: Tissue factor (TF, CD142) is well known as the principal initiatior of the extrinsic pathway of blood coagulation. However, several studies suggest that tissue factor is involved in non-hemostatic processes such as cell adhesion and migration via an interaction of its cytoplasmic domain with cytoskeletal proteins. TF expression is found in extravascular cells of many tissues, e.g. epidermis or bronchial epithelium. Interestingly, TF is abundantly expressed in the myocardium but not in skeletal muscle. To elucidate the possible role of TF in the myocardium, we examined the TF antigen content, TF mRNA and the microtopography of TF in the conducting and contractile parts of the heart muscle.
Methods: Human cardiac muscle samples were collected from autopsy material and stored frozen. Muscle fibres were prepared and stored separately. TF antigen content was assayed in cardiac muscle extracts obtained from frozen material using a laboratory ELISA. TF mRNA was analysed by RT-PCR using the Light Cycler. Immunohistochemistry was performed on paraffin tissue sections using anti-TF mAb and the Vectastain ELITE ABC kit for detection.
Results: Immunohistochemically TF was predominantly detectable in the transversal part of the intercalated discs, where it colocalized with structural important cytoskeletal proteins such as desmin and vinculin. In addition, much weaker TF staining was found in the conduction system of the heart as compared with the contractile myocardium. The TF antigen content in myocardial tissue extracts was lowest in right atrial tissue and highest in left ventricular myocardium ($p<0.01$), which correlated with the increasing number of contact sites of cardiomyocytes in these parts of the heart muscle. Furthermore, TF mRNA amounts as detected by quantitative RT-PCR were 4-fold higher in working myocardium compared to muscle fibres of the conducting system.
Conclusions: The predominant localization of TF at cardiomyocyte contact sites suggest a structural role of TF in the heart muscle besides its known hemostatic function. The higher TF mRNA and protein content in contractile compared to conducting muscle fibres are in favour of the hypothesis that TF might be associated with force-generating proteins in myocardium.

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THE ROLE OF FII G20210A AND FV G1691A IN JUVENILE STROKE PATIENTS
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Introduction: Various genetic defects of proteins regulating blood coagulation have been well established as risk factors of cerebrovascular disease in adults. However, the role of FII G20210A and FV G1691A in the pathogenesis of ischemic stroke remains controversial. Whereas some studies clearly indicate an increased risk for cerebral ischemia in carriers of FII G20210A or FV G1691A, others failed to demonstrate such an association. Since none of these studies focused on young patients (pts) suffering from idiopathic events in particular, the aim of the present study was to assess the role of FV G1691A and FII G20210A in juvenile spontaneous stroke or TIA, in which the etiology remains unclear in the majority of patients.
Methods and Patients: 121 unrelated pts (F 67/M 54) with an objectively confirmed juvenile ischemic stroke (n=97) or TIA (n=24) and 235 age- and sex-matched healthy subjects (F 118/M 117, aged 16-58 yrs) were studied. By assuming the theory, that a high proportion of genetic haemostatic abnormalities influences the thrombotic tendency already at young ages, we analyzed adult pts with early onset of cerebral ischemia only. At first onset, pts were aged 17-55 yrs (median age: 36 yrs). To focus on the role of FV G1691A and FII G20210A, pts with confirmed deficiencies of PS, PC, AT, or APA or HHCY were excluded from the current study. All study pts suffered from idiopathic events, meaning none of them had overt evidence of cardiovascular or systemic disorders or any other underlying condition known to be associated with an increased risk of stroke (excluding criterias). Furthermore, none of the pts had a history of venous thrombosis.
Results: The PT G20210A was significantly more prevalent in 13/121 pts as compared to controls [11% (stroke:11%/TIA:0%) vs. 2%; $p<0.001$; OR 5.04; 95% CI 1.92-13.22]. Furthermore, 19/121 pts were defined as FV G1691A carriers as compared to 8/235 among controls [16% (Stroke:13%/TIA:3%) vs. 3%; $p<0.001$; OR 4.61; 95% CI 2.08-10.19]. This results were not modified by sex. In 4/121 pts (3%) both defects were identified.

Conclusion: We observed a significant association between both FII G20210A mutation and FV G1691A and the development of ischemic stroke/TIA in young pts. Although less frequently as compared to the prevalence in venous thrombosis, the FV G1691A and PT G20210A mutations have been identified as the most common prothrombotic defects associated with juvenile ischemic stroke in the current study group.

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DYSFIBRINOGENEMIA FOLLOWING AFTER SNAKE BITE

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Introduction: The clinical characteristics of the Central and South American rattlesnake venoms may present neurotoxic, myotoxic and haemorrhagic effects based on many clinical reports. The venom of Crotalus snakes contains a thrombin-like component, which induced the consumption of fibrinogen, and can lead to mild or severe haemorrhagic events, which in each case varies. In our area, an 35-year old man, who was bitten in his left foot by a snake identified as a Crotalus durissus dryinas developed a coagulopathy. The aim of the investigation was to study the coagulation abnormalities in this case.
Results: Within seven hours the patient developed a coagulopathy which was characterized by acquired dysfibrinogenemia with prolongation of the thrombin time (max. 89 sec), Reptilase time (max.>120 sec), a deficiency of functional fibrinogen level- Clauss (<17 mg/dl) and normal level of immunological fibrinogen (215 mg/dl). Neither D-Dimers nor thrombocytopenia was identified in our patient. By replacement of fibrinogen, fresh frozen plasma, and aprotinin 6, 12, 22, 26, 33 and 38 hours after the bite normalization of all clotting values was achieved within 81 hours. The only haemorrhagic symptom was microhaematuria. The increased levels of the creatine kinase (CK, max. 997 U/l), lactic dehydrogenase (LDH, max. 268 U/l) and aspartate aminotransferase (AST, max. 41 U/l), and the detection of myoglobin in serum demonstrate the myotoxic activity of the venom.
Conclusion: Acquired fibrinogen disorders following the bite of Crotalus durissus dryinas are rare and affect the qualitiy of the circulating fibrinogen. Accurate and rapid diagnosis of acquired deficiencies of coagulation factors or the regulators of the fibrinolytic system is essential for the management of an appropriate therapeutic regime, and plays an important role in the clinical outcome of the patients after snake bite.

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The role of vWF:AgII in patients with acquired von Willebrand disease

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Introduction: Von Willebrand factor (vWF) is a multimeric glycoprotein, that originates from the vWF precursor pro vWF and results in mature vWF (vWF) and in the vWF propeptide (vWF:AgII). In contrast to normal subjects, vWF:AgII levels were increased in patients with acquired von Willebrand syndrome (AvWS). Published studies indicate, that the comparison between vWF:AgII and vWF:Ag and the presence of inhibitors might be important for the diagnosis of AvWS. The aim of the present study was to assess the role of vWF:AgII in our patients with AvWS.
Patients and Methods: 11 patients (F 5/M 6) with AvWS, associated with Valproat replacement, thrombotic thrombocytopenic purpura (TTP), essential thrombocythemia (ET), chronic lymphatic leukemia (CLL), chronic myeloid leukemia (CML), Paget von Schroetter syndrome and idiopathic form, were studied. At first onset, pts. were aged 8-71 years. None of the pts. enrolled had bleeding and family histories for the von Willebrand disease. We performed a quantitative analysis of plasma vWFAg and vWFAg:II by ELISA assays and the identification of autoantibodies to vWF (AbvWF).
Results: In our study group we found positive AbvWF in 3/11 pts., only one of them had vWFAg:II/vWFAg ratio higher than >22, but in all of the pts. the ratio was about 1. VWFAg:II levels (mean: 69%) were not significantly in 8/11 pts.higher as compared to vWFAg levels (mean: 51%). In these pts. vWFAg:II/vWFAg ratio was about 1, and in 3/11 pts. about 5. We found in 3/11 pts. vWFAgII/vWFAg ratio lower than 1, associated with vWF:RCo levels <35%, compatible with an inherited vWD, but lack of bleeding history inspite of surgery and deliveries. Non of the pts. with Valproat replacement had ratio about 5 or positive AbvWF.
Conclusion: In our study we found in 8/11 pts.laboratory features for the AvWS. The vWFAg:II/vWFAg ratio and the presence of antibodies to vWF appears to be helpful in the characterisation between congenital vWS and AvWS and should be performed in suspected AvWS.

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PLATELET AGGREGATE SIZE AS A PARAMETER OF PLATELET ACTIVATION IN VITRO: DETECTION WITH A LASER AGGREGOMETER

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Background: Platelet aggregometry is the most widely accepted assay for platelet function. However, this technique is of limited sensitivity to microaggregate formation, which appeared to be particularly important in identifying and differentiating thrombogenic states. A previously reported laser based technique enables to detect aggregate formation by analysis of relative dispersion (R) during in vitro platelet aggregation. The aim of the present study was to investigate the changes of aggregate size during platelet aggregation and to evaluate the effect of various glycoprotein IIb/IIIa antagonists on aggregate formation by aggregometry and by laser analysis.
Methods: The R value as a parameter of microaggregate formation was assessed in combination with measurements of light transmission (LT) by standard aggregometry. The microaggregate formation was verified by counting of single platelets, by a microscopic technique and by flow cytometry. To analyze the effect of Ca(2+) chelation platelet aggregation was studied in citrated (cPRP) or hirudinized platelet rich plasma (hPRP). The inhibition by the glycoprotein IIb/IIIa antagonists abciximab, EMD76334, eptifibatide on ADP- and collagen-induced aggregation was further investigated.
Results: ADP or collagen induce a dose-dependent increase in R values, which correlate significantly with the size of aggregates as observed by microscopy or flow cytometry. These findings confirm that the value of R determined by laser analysis can be used as a measure of the size of platelet aggregates. Dose response studies demonstrate that R increases more sensitively when compared to LT. With ADP in cPRP the ED50 for increase of R amounts to 0.7±0.2 µM and that for the increase of LT was 1.5±0.5 µM. In hPRP a tendency of lower LT values was observed but enhanced R values were measured when compared to cPRP. All tested glycoprotein IIb/IIIa antagonists inhibit both the LT and R, however higher concentrations are necessary for inhibition of microaggregate formation.
Conclusions: The combination of standard aggregation with laser analysis enables to investigate the formation of small aggregates and their transition into large aggregates. This allows to give additional information about the dynamics of platelet aggregation and may provide new insights into the platelet inhibition by glycoprotein IIb/IIIa antagonists.

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INTERACTION OF THE PLASMA HYALURONAN-BINDING SERINE PROTEASE WITH EXTRACELLULAR MATRIX PROTEINS

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Background: The plasma hyaluronan-binding serine protease (PHBSP) is a novel plasma protease which activates FVII in the absence of tissue factor and activates single chain plasminogen activators. It has also the ability to cleave HMW kininogen leaving behind activated kininogen and the vasoactive peptide bradykinin. PHBSP has a similar domain structure to plasminogen activators, FXII and hepathocyte growth factor activator, circulates in human plasma as a single-chain zymogen of 65 kDa and can undergo autocatalytic activation. Due to its high affinity to hyaluronic acid we were interested to see whether PHBSP also interacts with other extracellular matrix (ECM) molecules.
Methods: Ligand binding assays were performed by ELISA techniques. SDS-PAGE and Western Blot analysis was applied to test the potency of PHBSP to cleave ECM proteins.
Results: The binding studies showed that PHBSP binds tightly to several ECM proteins. Furthermore we found that at least two of these ECM molecules are cleaved by PHBSP and thus are potential physiological substrates of this protease.
Conclusion: The propensity to bind to several ECM proteins which are involved in controlled cell adhesion, spreading and migration further supports our hypothesis that PHBSP function is directed to the ECM/cell surface environment. The activation of pro-urokinase and HMW kininogen and the additional finding that PHBSP can cleave at least two ECM proteins indicates that this enzyme might participate in processes like cell adhesion, proliferation and matrix degradation.

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INHIBITOR INCIDENCE IN PREVIOUSLY UNTREATED PATIENTS (PUBS) WITH HAEMOPHILIA A AND B – A PROSPECTIVE MULTI-CENTER STUDY OF THE GTH

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In order to observe inhibitor development in PUPs with haemophilia A and B the prospective study was started in 1993. The protocol provides rigorous inhibitor testing und correlates inhibitor development with mutation type, type of concentrate, therapy regimen and further parameters. Until now 204 patients have been enrolled and 149 have been treated with factor VIII or IX concentrates (122 haemophilia A and 27 haemophilia B patients).
Out of 122 haemophilia A patients 30 developed inhibitors (14 high titer>5 BU, 14 low titer>0.6-5 BU, 2 transient inhibitors)after 12 exposure days (ED) in median (range 1-56) at the age of 0.9 years in median. The development of inhibitors was predomminantly observed in severe haemophilia A patients (33,8%). The percentage of inhibitors in recombinant treated patients (42%) was higher than in the plasma derived ones (26%) for those with F VIII <1% (p=0.06). In both groups the exposure status of the non-inhibitor patients shows no substantial differences. Out of 16 severely affected haemophilia B patients 2 developed inhibitors (10.5%) To confirm the data, more PUPs have to be included and followed up.

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HETEROGENEITY OF ANTIBODIES AGAINST THE PI(A1) ALLO-ANTIGEN ON PLATELET GLYCOPROTEIN IIIA AND ITS RELATION TO ALLOIMMUNE THROMBOCYTOPENIC SYNDROMES

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Alloimmunization against the PI(A1) epitope on platelet glycoprotein (GP) IIIa is the most frequent cause of fetal alloimmune thrombocytopenia (FAIT) and posttransfusion purpura (PTP). Due to unknown reasons the clinical severity varies considerably between patients ranging from isolated mild thrombocytopenia to life threatening intracranial bleeding. Recent evidence suggests that the region on GPIIIa recognized by anti-PI(A1) antibodies differs from one individual to another. Some anti-PI(A1) alloantibodies recognize an epitope comprised solely of the amino-terminal part of GPIIIa (Type I), whereas others seem to react with a complex epitope that requires a “long-range” disulfide bond formed by cysteine residues 5 and 435 (Type II). To study the clinical significance of this heterogeneity we compared the reactivity of 55 different anti-PI(A1) sera with wild type (Leu33Cys435) and a mutant (Leu33Ala435) GPIIIa isoforms.
Anti-PI(A1) form 41 mothers whose newborns suffered form FAIT and from 14 patients with PTP were characterized by GP-specific immunoassay. All sera were analysed by immunoprecipitation with lysates from biotin-labelled CHO cells expressing stable GPIIb/IIIa isoforms. The intensity of precipitated GP IIIa was quantified by densitometry. 27/41 (66%) of FAIT and 12/14 (86%) of PTP anti-PI(A1) could be assigned to Type I, whereas the remaining 14 and 2 sera, respectively, belonged to Type II. The relative reactivity was calculated as the quotient of mutant GPIIIa to wild type GPIIIa precipitates (mutant: wild type ratio). No correlation between relative reactivity and clinical severity (categorized to: isolated thrombocytopenia, cutaneous, organ, and intracranial bleeding) or the platelet count nadir in 35 FAIT patients (24 Type I, 11 Type II) was observed. When anti-PI(A1) from FAIT were compared to anti-PI(A1) from PTP, a significantly higher intensity of GPIIIa precipitates ($p<0.002$) as well as an increased relative reactivity ($p<0.02$) could be observed in PTP patients.
We conclude that PI(A1)-antibodies react with heterogenous epitopes on GPIIIa. About 2/3 react with the isolated N-terminus whereas 1/3 of the antibodies require the cysteine-rich region of GPIIIa. Clinical severity of FAIT was not correlated with anti-PI(A1) types. In anti-PI(A1) from PTP we observed stronger GPIIIa intensity and higher relative reactivities than in anti-PI(A1) from FAIT. This antibody heterogeneity might have an impact on the pathogenesis of PTP.

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UNEXPECTED CLINICAL RELEVANCE OF LOW-FREQUENCY POLYMORPHISMS IN THE CYSTEINE-RICH DOMAIN OF GLYCO-PROTEIN IIIA IN ALLOIMMUNE THROMBOCYTOPENIA

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Immunization against polymorphic structures on platelet glycoproteins (GPs) is responsible for fetal and neonatal alloimmune thrombocytopenia (FAIT). Besides the most frequently involved alloantigens PI(A1) and Br(a) a number of low-frequency polymorphisms has been observed. So far, immunization against a low-frequency alloantigen has been regarded a rare event.
We report on three mothers who presented with immunization against the GPIIb/IIIa complex of paternal platelets. In family 1 the father was known to be carrier of the Sr(a) alloantigen since the offspring of his brother suffered from FAIT due to anti-Sr(a). Antibody screening during pregnancies revealed evidence for immunization in the second of three pregnancies. The newborns in families 2 and 3 presented with the typical picture of FAIT with minimal platelet counts of 43/nl and 36/nl, respectively. Maternal sera were analyzed by GP-specific immunoassay with a panel of donor platelets carrying all known alloantigens including those with low frequency. Genotype analysis of paternal DNA was performed for all alloantigenic polymorphims on GPIIb/IIIa with reference to formerly established B-lymphoblastoid cell lines. Our studies revealed evidence for secondary immunization against the rare antigens Sr(a) (GPIIIa Cys636) and Gro(a) (GPIIIa His633). In the 3rd case, the new antigen, Oe(a), was responsible. By immunochemical studies the Oe(a) antigen could be assigned to platelet GPIIIa. DNA analysis of GPIIIa cDNA showed deletion of a lysine residue at position 611 (dLys611). Thus, all three polymorphisms are located in the cysteine-rich domain of GPIIIa. A mutation in the cysteine-rich domain responsible for gain of function of GPIIb/IIIa was recently reported. To study the functional relevance of the mutation underlying the Oe(a) alloantigen we established stable cell lines expressing GPIIIa Pro33Lys611 (wild type) and Pro33dLys611 (mutant). Analysis with RGD peptide and fibrinogen demonstrated that GPIIIa Pro33dLys611 could undergo conformational changes. No difference was found in the tyrosine phosphorylation of pp125FAK. These results suggest that deletion of amino acid 611 does not impair the function of GPIIb/IIIa. Analysis of the two alloantigens Sr(a) and Gro(a) is in progress. Studies of rare natural occurring polymorphisms are not only important due to their underestimated relevance in FAIT, but will also help to understand structure and function of GPIIb/IIIa.

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***AIR TRAVEL THROMBOSIS – SUMMARY OF AN EXPERT MEETING IN BERLIN**

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On June 9, 2001, an expert meeting was held in Berlin in order to accumulate present knowledge on the phenomenon of “air travel thrombosis” and to assess the available evidence.
Definition: The term “traveller´s thrombosis” comprises deep vein thrombosis (DVT), with or without pulmonary embolism (PE), that occurs during or within four weeks after travelling, predominantly in sitting posture. The sub-group of “air travel thrombosis” is defined as travel thrombosis that occurs when at least part of the travel was done by plane.
Current knowledge: In 1999 and 2000, three case control studies were published that focus on the association of travel with the incidence of deep vein thrombosis. The odd's ratios for travel thrombosis were between 1.0 (CI 0.3-3.0) (Kraaijenhagen et al, 2000) and 4.0 (CI 1.9-8.4) (Ferrari et al. 1999). A recently published study retrospectively analysed the incidence of severe PE in passengers arriving at Charles de Gaulle Airport Paris, France (Lapostolle et al. 2001). The frequency of PE was clearly related to the distance travelled, with the highest incidence of 4.77 per million arrivals for passengers who had travelled more than 10.000 km. The overall incidence of severe PE was 0.4 per million passengers.
Estimate of the absolute risk (see also: Bauersachs, R.: Critical estimation of absolute risk for travellers thrombosis): From the above mentioned-case-control studies an absolute incidence of 6 to 24 DVT per 100.000 travellers can be extrapolated for a time interval of three weeks after travelling. The incidence of PE without prophylaxis would range from 6 to 24 per million passengers, assuming a 10% rate of symptomatic PE in patients with symptomatic DVT. This incidence is about 30-fold higher than the one recently reported for severe symptomatic PE after arrival. Risk estimates for DVT associated with selected risk factors for DVT-histology were given. Odd´s ratios varied between 15 to 30 for DVT-history to 1.7 for more then three pregnancies.

Conclusions: Causality between air travel and thrombosis is currently not proven even though an association is considered probable.
There is a need for well-designed, strategic case-control studies, as prospective cohort studies are not feasible due to the assumed low incidence of DVT (estimated sample size >100.000 passengers). For travels with a duration of less than 3 hours no intervention is recommended. The risk for DVT associated with air travel of more than 3 hours will depend on the magnitude and the number of underlying risk factors, which are simultaneously present in the individual traveller, as well as on the distance travelled. Therefore, instead of risk stratification into different risk groups, cumulative assessment of risk assessment for the individual person is recommended. The spectrum of prophylactic measures covers non-pharmaceutical prevention of DVT with general procedures (for example compression therapy), and the administration of low molecular weight heparin in passengers with several and/or strong risk factors. Current evidence does not support the use of aspirin for prevention of venous thrombosis.

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COAGULATION FACTOR VII IS INFLUENCED BY POLYMORPHISMS, GENE-GENE INTERACTION AND ENVIRONMENTAL FACTORS IN HEALTHY INDIVIDUALS – RESULTS FROM THE LUGEN STUDY
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Background: Coronary heart disease and acute coronary syndroms lead to a high number of deaths in the industrialized countries. High levels of coagulation factor VII (FVII) are known to increase the risk of ischemic vascular disease. However, there is a lack of data about genetic and environmental factors particular gene-gene interaction influencing plasma levels of FVII in healthy individuals. LUGEN is an ongoing prospective cohort study dealing with these issues.
Materials and Methods: We examined 488 healthy individuals (267 men, 221 women) from Southwest Germany. All subjects were well-characterized in terms of common cardiovascular risk factors. We examined FVII R353Q, FVII HVR4 by PCR, FVII activity (FVII:C) by chromogenic assay, FVIIa by two-stage clotting assay, prothrombin fragments 1+2 (F1+2) and beta-Thromboglobulin (beta-TG) by ELISA.
Results: There were significant differences in FVII:C and FVIIa levels between R353Q genotypes (FVII:C: R353R, 128 U/dl; R353Q, 109 U/dl; Q353Q, 94 U/dl; FVIIa: R353R, 126 mU/ml; R353Q, 86 mU/ml; Q353Q, 36 mU/ml; $p<0.001$). In contrast, only in H5/H6 genotype FVII:C and FVIIa levels were found to be significantly higher than in the remaining genotypes ($p<0,05$; $p<0,001$). We could observe an additional effect of R353R combined with H5 allel on FVIIa levels ($p<0,05$), but not on FVII:C. FVII:C increased with age and BMI, after menopause and under oral contraceptives (OC). Postmenopausal hormone replacement decreased FVII:C. However, FVIIa was also increased under OC use but was not influenced by age, BMI and menopause. Neither FVII:C nor FVIIa were influenced by smoking habits. FVII polymorphisms did not alter F1+2 or beta-TG levels.
Conclusions: Our results concerning prevalences of FVII polymorphisms were similar to those observed in healthy controls of earlier case control studies. Therefore they may serve as additional controls in forthcoming studies. We found FVII:C and FVIIa to be markedly influenced by R353Q and HVR4 polymorphisms. The combination of both polymorphisms was associated with a greater effect on FVIIa than a single polymorphism. However, none of the polymorphisms studied caused significant alterations of F1+2 or beta-TG levels. Further we could identify different endogenous and exogenous factors influencing FVII:C and FVIIa levels.

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ECARIN CHROMOGENIC ASSAY (ECA) – A NEW CHROMOGENIC ASSAY USEFUL FOR CLINICAL MONITORING OF DIRECT THROMBIN INHIBITORS LIKE HIRUDIN
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Recombinant hirudin, a direct antithrombin has been increasingly used in therapeutic and prophylactic anticoagulation. Clinical monitoring of hirudin is necessary to define the therapeutic range and to avoid over- or underdosage. By now different methods both clotting and chromogenic assays are in use but there is no standardized method of choice for routine hirudin determination in plasma.
In the present study a new chromogenic assay for rapid and specific determination of recombinant hirudin in plasma, the Ecarin Chromogenic Assay (ECA), is described. The assay is based on the cleavage of a chromogenic substrate by generated prothrombin activation products (mainly meizothrombin) and their concentration-dependent inhibition by hirudin. Prothrombin activation is induced by ecarin, a snake venom metalloprotease from Echis carinatus. Change in optical density is recorded at 405 nm.
In ECA there is a linear correlation between plasma hirudin concentration of about 0.2-2.0 µg/ml and change in optical density. The wide measuring range allows hirudin determination in subtherapeutic, therapeutic and toxic range using a calibration curve or a calibration standard. With ECA also synthetic direct thrombin inhibitors can be determined.
Because ECA is no coagulation assay, it is not influenced by plasma fibrinogen level. It could be demonstrated that hirudin can be monitored in the presence of heparin, in prothrombin-deficient plasma, or in plasma of orally anticoagulated patients. ECA is independent of ATIII and heparin since the generated prothrombin activation products are not inhibited by ATIII/heparin. The results of ECA do not depend on patients prothrombin level due to external addition of prothrombin.
ECA is particularly suitable as routine diagnostic test because it can be easily carried out as fully mechanised chromogenic substrate assay for hirudin determination. It can be performed in citrated plasma on clinical chemistry analyzers provided with an option for optical measurements. For the automated assay only two different reagents are necessary and there is no need for predilution of plasma samples.

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SUCCESSFUL MANAGEMENT OF COMPLICATED ANTIPHOSPHOLIPID ANTIBODY SYNDROME (APAS) WITH LONG-TERM IMMUNOSUPPRESSION AND FRACTIONATED HEPARIN
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Introduction: Standardized guidelines for diagnosis and therapy of APAS are difficult to establish due to its poorly understood pathophysiology and variability of clinical phenotype.
Patient and methods: A 51-year-old woman with recurrent syncopes and seizures experienced an ischemic infarction of the cerebellum in 1993 and two infarctions of the cerebrum in 1997. In 2/00 a mamma carcinoma was treated with local surgery and adjuvant chemotherapy. In 3/00 an extended ischemic infarction of the left middle cerebral artery was diagnosed and the patient started on oral anticoagulation (OA) with phenprocoumon (PT INR 2-3). However, hospitalisation for recurrent thromboembolism (TE) was necessary in the following eight months. On admission in 1/01 fine reticular livedo racemosa of the extremities, swelling of the left leg and a painful necrotic-ulcerative lesion at the left lateral ankle were found. Platelet count, PT INR (Innovin) and APTT were 87/nl, 2.7 and 88 s (normal, 25-38 s), respectively. Lupus anticoagulant, IgG-anti-CL, IgG-anti-beta2GPI and anti-ds-DNA antibodies were excessively elevated. ANA screen and test for factor V (FV) Leiden mutation were negative.
Treatment and course: APAS with iliac vein thrombosis and cutaneous arteriolar vasculitis was diagnosed. OA was stopped and full-dose anticoagulation with Fraxiparin initiated. After four days the PT INR and APTT increased to 3.6 and 165 s, respectively, with a selective prothrombin (FII) of 81% indicating increased in vitro binding of immune complexes to phospholipids. This observation was followed by transient aggravation of cutaneous vasculitis and development of a blue toe syndrome supporting recent evidence of cellular activation by OA-dependent immune complexes. After three months of immunosuppression (IS) with i.v.-cyclophosphamide and oral prednisone the PT INR normalized and the APTT decreased to 45 s along with complete resolution of cutaneous symptoms. The current medication is Innohep, prednisone and azathioprine. No further TE was recognized during ten months of follow-up.
Conclusions: I) APAS may resemble Sneddon's syndrome and SLE II) APAS aggravation may be triggered by malignoma and/or malignoma specific therapy III) In case of uncontrollable OA fractionated heparin and long-term IS may reliably prevent TE IV) Coumarin-dependent coagulation factors (e.g., FII) may form membrane-associated immune complexes causing platelet activation and/or arteriolar vasculitis.

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VON WILLEBRAND FACTOR-CLEAVING PROTEASE (VWF-CP) AND VWF MULTIMERS AFTER SPLENECTOMY IN A PATIENT WITH ACUTE THROMBOTIC-THROMBOCYTOPENIC PURPURA (TTP)
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Introduction: In acute TTP unusually large (UL-) VWF multimers, which are not degraded by the deficient VWF-cp, are thought to play a major role in the formation of microthrombi by aggregating platelets under con-

ditions of high vessel shear stress. Recent data have suggested that the pathophysiology of splenectomy in plasma refractory patients with acquired TTP consists of the removal of B lymphocytes producing an inhibitor of VWF-cp.
Patient and methods: A 22-year-old female with acute TTP experienced two early relapses despite intensive plasmapheresis and steroid therapy. Laparoscopic splenectomy was carried out 85 days after the initial presentation and was followed by consistent normalization of platelet counts and haemolytic parameters (e.g., LDH, bilirubin, reticulocytes). Activity of VWF-cp and presence of a circulating inhibitor were determined using a modified collagen-binding assay essentially as described by Gerritsen et al (1999). UL-VWF multimers were detected by an immunoblot assay with subsequent densitometric gel analysis. VWF antigen (VWF:Ag) and VWF collagen-binding activity (VWF:CBA) were assayed by an in-house ELISA technique. RESULTS: On the first TTP relapse we found severe protease deficiency (<1%) and high levels of an inhibitor (8 U/ml) without evidence of UL-VWF multimers (VWF:CBA/Ag ratio 1,09) in the patient plasma (PP). Severe protease deficiency persisted up to six months after splenectomy (<3%) and resulted in the transient appearance of UL-VWF multimers as reflected by a maximum VWF:CBA/Ag ratio of 2,16. Over this time period the inhibitor level decreased to 2 U/ml. Low but significant protease activity (10%) and disappearance of the inhibitor were first detectable as late as nine months after splenectomy and were concomitant with disappearance of “ultralarge” multimers from the PP (VWF:CBA/Ag ratio 1,28). CONCLUSION: We describe the first patient with acute and plasma refractory TTP who showed sustained inhibition of the VWF-cp up to six months after splenectomy despite full clinical haematological TTP remission. Therefore, we conclude tentatively that at least in a subgroup of patients with acute, nonfamilial TTP the beneficial effect of splenectomy is not exclusively due to the removal of splenic B lymphocytes producing an inhibitor of VWF-cp, and that protease inhibition seems to be less cause of than predisposition to TTP requiring additional, hitherto unknown factors, to cause disease manifestation.

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***ANTI-THROMBOTISCHE EFFEKTE VON STATINEN**

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Nach heutigem Verständniss liegt den meisten akuten Komplikationen der Arteriosklerose eine lokale Thrombose auf dem Boden einer Erosion oder Ruptur der fibrösen Kappe eines arteriosklerotischen Plaques zugrunde. Insbesondere gilt dies für die instabile Angina pectoris und den akuten Myokardinfarkt. Im Falle einer lokalen Kontrolle der Thrombose kommt es im weiteren Verlauf zur bindegewebigen Organisation des Thrombus und zur Entwicklung einer fibrösen, kalzifizierten Plaque, d.h. rezidivierende kleine lokale Thrombosen spielen eine wichtige Rolle für die Entwicklung hochgradiger Gefässstenosen.
Grosse prospektive Studien zeigen, dass Statine die Progression der Arteriosklerose hemmen und das Risiko von Herzinfarkten und Schlaganfällen senken. Neue Erkenntnisse weisen dabei auf eine antithrombotische Wirkung der Statine hin. Dabei werden verschiedene komplimentäre Mechanismen diskutiert: Die Senkung der Serumlipidspiegels durch Statine führt zu einer Stabilisierung von arteriosklerotischen Plaques und dadurch zur Reduktion der lokalen Exposition des prothrombogenen Tissue Factor. Statine steigern die Zahl zirkulierender endothelialer Progenitorzellen und verbessern dadurch die Reparatur potentiell thrombogener endothelialer Läsionen. Ein wichtiger Cholesterin-unabhängiger Effekt von Statinen ist die Freisetzung von endothelialem NO. Neben der eNO vermittelten Vasodilatation und der Verminderung der Leukozytenadhäsion ist die Hemmung der Thrombozytenaktivität eine der wichtigsten Gefäss-protektiven Wirkungen von eNO. Statine führen im Tierversuch nicht nur in Endothelzellen, sondern auch in Thrombozyten zu einer Hochregulation der eNOS. Dies führt in Wildtyp-, nicht jedoch in eNOS -/- Mäusen, zu einer Herabregulation der Thrombozytenaktivitätsmarker ß-Thromboglobulin und Plättchenfaktor-4. Darüber hinaus wirken Statine durch Hochregulation des Tissue Plasminogen Activators (tPA) und Herabregulation seines Inhibitors (PAI-1) profibrinolytisch.
In den letzten Jahren ist die Erkenntiss gewachsen, dass der Inflammation eine entscheidende Rolle in der Pathophysiologie thrombotischer Ereignisse zukommt. Daher tragen die anti-inflammatorischen und antioxidaiven Wirkungen der Statine wesentlich zu einer Hemmung der Thrombusentstehung bei.
Durch die aktuellen Erkenntnisse über die Bedeutung lokaler Thrombosen für die Pathogenese der Arteriosklerose und ihrer akuten Komplikationen erfährt die bisher für die Lipidsenkung etablierte Statin-Familie eine neue Bewertung, z.B. bei der Behandlung von akuten Koronarsyndromen und dem Schlaganfall.

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***EFFECTS OF FACTOR XIII AND CL-ESTERASE INHIBITOR ADMINISTRATION ON INTESTINAL MICROCIRCULATION DURING EXPERIMENTAL ENDOTOXEMIA – A STUDY USING INTRAVITAL MICROSCOPY**

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Endotoxemia leads to excessive activation of coagulation and the complement system. Most important pathophysiological consequences are vasodilation, increased vascular permeability, disseminated intravascular coagulation and derangement of microcirculation. Factor XIII seems to protect endothelial barrier function. Complement fragments activate leukocytes which cause further endothelial damage.
Hypothesis: Factor XIII and C1-esterase inhibitor (C1-INH) administration may attenuate endothelial injury as well as leukocyte-endothelial interactions. Both substances can improve microvascular blood flow.
Methods: 56 Wistar rats under general anesthesia were divided into 8 groups: In group 1-4 intestinal functional capillary density (FCD) and leukocyte adherence on venular endothelium were studied by intravital fluorescence microscopy (IVM). In group 5-8 mesenteric plasma extravasation (FITC-albumin) were determined by IVM. Groups 1 and 5 served as controls. Groups 2 and 6 received endotoxin infusion (5 mg/kg b.w. / 2 hrs). In group 3 and 7 we administered 100 U/kg b.w. C1-INH during endotoxemia. In group 4 and 8 we administered 50 U/kg b.w. F XIII. Statistical analysis: 2-way-ANOVA followed by Scheffé test.
Results: Endotoxemia resulted in a significant decrease of mucosal FCD (-18.5% vs. control group; $p<0.05$) which could be significantly attenuated by administration of factor XIII (-3.7% vs. control) and C1-INH (-9.5% vs. control). The C1-INH treatment also attenuated significantly intestinal leukocyte adherence in submucosal venules (-35% vs. endotoxin group; $p<0.05$). After F XIII therapy a tendency to attenuation of leukocyte adherence became apparent. We found an reduction of mesenteric plasma extravasation in C1-INH group (-44% vs. endotoxin group; $p<0.05$), and a trend to lower plasma extravasation in F XIII group.
Conclusions: Both substances have antiinflammatory effects, can reduce capillary leakage and improve microvascular blood flow and may be thus of clinical importance in the treatment of sepsis.

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PRODUCTION OF B-DOMAIN-DELETED CLOTTING FACTOR VIII IN A STABLE HUMAN HIGH-EXPRESSION CELL LINE

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A strategy for the production of a novel recombinant FVIII has been set up using a stable human cell line. The goal of this approach is to combine high expression level with features of the product allowing to meet prior unsatisfied needs in treatment of haemophilia A such as low adverse reactions and long systemic half-life.
HEK 293T cells were stably transfected with a FVIII cDNA construct in which the B-domain was replaced by a unique hinge region. Several variants encoding defined single amino acid substitutions were selected for expression level and in vitro activity. The most promising cell banks were adapted to serum-free culturing and showed stable high-level FVIII expression for at least 3 months. Purified FVIII from these cultures showed satisfactory properties in vitro. Absence of components derived from animal material and a generally low protein background allow new strategies for the preparative purification procedure.

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***PROPHYLAXIS OF CATHETER-INDUCED THROMBOSES WITH SPECIAL RESPECT TO ARMPORTS**

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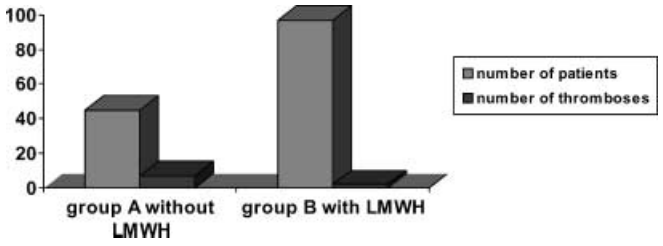
Central venous access devices are indispensable for chemotherapy, parenteral nutrition and analgesia in patients suffering from advanced malignancies. Armports are easily and quickly subcutaneously (sc) implanted at the patients’ forearm. But patients with cancer have a much higher risk of thromboses which even is increased by the implantation of catheters.
Patients with advanced gastrointestinal malignancies (n=142) received armports (Healthport miniMax, n=119, or Bard Titan Low Profile Ports, n=23). Patients were weekly monitored by painstaking physical examination until death. In case of suspected thromboses additional diagnostic procedures i.e. angiography, CT or MR were initiated. The first 45 pa-



tients (group A) were not given prophylactic anticoagulation. Low molecular weight heparins (LMWH), i.e. dalteparin 5000 IU or certoparin 3,000 IU sc once daily, were indefinetely given to the following 97 patients of the study population (group B).
Symptomatic deep vein thromboses of the upper extremities were detected in 7 patients in group A (16%) and in 2 in group B (2%) from 2 to 290 days after port implantation. A total of 28064 catheter placement days can be overviewed so far. Taken both groups together 0,004 thromboses and 0,004 systemic infections/1,000 catheter placement days were counted.
Rate of thromboses can be lowered by prophylactic sc LMWH in patients with armports and gastrointestinal malignancies. LMWH most probably not only affects the rate of thromboses induced by peripheral armports but also prevents thromboses induced by malignancies themselves. A further aspect for a general recommandation of prophylactic sc LMWH in those patients might be the inhibiting influence on metastases.

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COMBINED INTRAVENOUS AND SUBCUTANEOUS ADMINISTRATION STRATEGY OF RECOMBINANT HIRUDIN PREOPERATIVELY IN A PATIENT WITH ARTIFICIAL AORTIC VALVE, HIT TYPE II AND TERMINAL RENAL FAILURE

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Background: Recombinant hirudin (r-hirudin; Lepirudin, Refludan®) is currently approved in both the European Union and the United States for the treatment of patients suffering from heparin-induced thrombocytopenia (HIT) as alternative anticoagulant. Generally, r-hirudin is administered intravenously (i.v.), though subcutaneous (s.c.) administration has also previously been shown to be safe and effective.
Objectives: To demonstrate a safe and effective administration strategy of r-hirudin prior to surgery in a patient with an artificial aortic valve, who is suffering from HIT type II and terminal renal failure.
Methods and clinical findings: Renal insufficiency (Cockgroftclearance <10 ml/min) required the application of a cimino shunt. Having been treated with oral anticoagulation, medication had to be changed to r-hirudin prior to surgery. Continuous intravenous infusion was undesirable due to the increased risk of endocarditis in a patient with artificial aortic valve. Thus, one hour after administration of an i.v. bolus of 0.05 mg/kg r-hirudin, r-hirudin was administered intravenously at a medium infusion rate of 0.04 mg/kg/h over 8 hours. Thereafter 10 to 15 mg r-hirudin were administered subcutaneously twice a day for five days. aPTT, the ecarin clotting time (ECT) and INR were monitored continuously. Subcutaneous administration of r-hirudin was adjusted according to aPTT and ECT (aPTT target range >65 s). Two days before surgery, r-hirudin administration was discontinued.
Results and Conclusion: Anticoagulation by an initial intravenous loading dose followed by subcutaneous administration of r-hirudin was effective (mean aPTT: 68 s, mean [r-hirudin] as measured by ECT: 1.1 µg/ml) and safe: No bleeding complications occurred, neither were thromboembolic events diagnosed. Surgery was performed without complication. In conclusion, this report presents a first hint that safe and effective anticoagulation can be achieved in patients with renal insufficiency using a combined i.v./s.c. r-hirudin administration strategy.

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PLASMASEPARATION, NOT HEMOFILTRATION ALLOWS FOR SUFFICIENT ELIMINATION OF R-HIRUDIN ANTI-R-HIRUDIN ANTIBODY COMPLEXES

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Background: Recent studies reveal that a substantial percentage of patients treated with r-hirudin for more than five days develop anti-r-hirudin antibodies (AHAb). It has hardly been investigated how AHAb may influence filtration characteristics of r-hirudin.
Objective: To identify an effective strategy for r-hirudin elimination in the presence of AHAb.
Methods: Using a closed in vitro filtration circuit, filtration of r-hirudin (lepirudin) by two high-flux hemodialyser membranes (polysulfone and AN69) and a plasmapheresis membrane (Microporous device TF10) was investigated in the absence as well as in the presence of monoclonal AHAb. Experiments were performed in albumin solution and whole blood each spiked with 500 ng/mL r-hirudin. Final concentration of the AHAb was 20 µg/mL. Filtration was performed for one hour. Samples for r-hirudin measurements (ecarin clotting time or S2238 chromogenic assay) were simultaneously drawn every 15 min at a pre-filter, post-filter, and filtrate draw point, respectively. R-hirudin sieving coefficients (SC) were calculated according to the standard equation.
Results: In the absence of AHAb both hemodialyser membranes and the plasmapheresis membrane allowed for significant r-hirudin filtration (SC >0.7). AHAb almost abolished r-hirudin filtration when hemodialyser membranes were used (SC <0.05). In contrast, the plasmapheresis membrane allowed for significant elimination of r-hirudin (SC >0.9) when AHAb where present.
Conclusion: AHAb markedly reduce r-hirudin elimination by hemofiltration. In the presence of AHAb hemofiltration may not constitute a measure to rapidly reduce r-hirudin plasma level. In this case, plasmaseparation is a suitable means to effectively remove r-hirudin – AHAb complexes. This may be particularly important in case of r-hirudin overdosage in AHAb-positive patients.

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FIRST SUCCESSFUL INHIBITOR ELIMINATION WITH A NEW PROTOCOL IN A HIGH RESPONDING HAEMOPHILIA A PATIENT AFTER FAILURE OF VARIOUS IMMUNE TOLERANCE INDUCTION REGIMENS

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We report on a 14-year-old boy who developed a high titre inhibitor against F VIII (maximum inhibitor titre 1562 Bethesda units, BU) at the age of 1 year. Immune tolerance induction according to the Bonn protocol and the Malmö regimen failed.The patient imposed with severe bleeding episodes.
Then immune tolerance induction was intended again using a monoclonal anti-CD20 antibody (MabThera, Roche, 375 mg/m² body surface) as a single intravenous infusion followed by the administration of F VIII concentrate (100 IU/kg bw twice daily). The cell-surface antigen CD-20 dropped to 0 and the inhibitor titre decreased continuously to 0.14 BU. The admistration of MabThera was repeated when the inhibitor titre rose slightly. Additional immunesuppressive therapy was given (Cyclosporin A 3 mg/kg bw po q2). In the meantime type I inhibitor changed to type II. Thereafter the inhibitor titre dropped to 0 BU and recovery turned towards normal ranges.
No bleeding episodes nor severe side effects in particular immunological failure occured during the whole treatment period (17 months). However, the time of follow up since last administration of MabThera is rather short (2 months).

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USE OF A LOW MOLECULAR WEIGHT HEPARINOID (DANAPAROID SODIUM) FOR CONTINUOUS RENAL REPLACEMENT THERAPY IN INTENSIVE CARE PATIENTS

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Purpose: To evaluate the efficacy and safety of danaparoid in the treatment of critically ill patients with acute renal failure and suspected heparin-induced thrombocytopenia (HIT), requiring renal replacement therapy (RRT).

Patients and methods: Retrospective analysis of 13 consecutive intensive care patients with acute renal failure and suspected HIT, who were treated with danaparoid for at least 3 days during RRT.
Results: In 8 patients continuous veno-venous hemofiltration was performed. The mean infusion rate of danaparoid was 140±86 U/h. Filter-exchange was necessary every 37.5 hours. In 5 patients continuous veno-venous hemodialysis was used. A bolus injection of 750 U danaparoid was followed by a mean infusion rate of 138±122 U/h. Filters were exchanged every 24 hours. In 7/13 patients even a low mean infusion rate of 88±35 U/h was efficient. Mean anti Xa-levels were approximately 0.4±0.2 aXaU/ml. Persistent thrombocytopenia despite discontinuation of heparin-treatment was observed in 9/13 patients, due to disseminated intravascular coagulation (DIC). HIT was confirmed by increase in platelet count and positive heparin-induced antibodies in 2/13 patients. While no thromboembolic complications occurred, major bleeding was observed in 6/13 patients, which could be explained by consumption of coagulation factors and platelets due to DIC in 5/6 patients. 9/13 patients died due to multiorgane failure and/or sepsis. In none of these patients the fatal outcome was related to danaparoid-treatment.
Conclusions: In critically ill patients with renal impairment and suspected HIT a bolus injection of 750 U danaparoid followed by a mean infusion rate of 50-150 U/h appears to be a safe and efficient treatment option, when alternative anticoagulation is necessary.

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ARE ELEVATED FACTOR VIII LEVELS IN CHILDHOOD A RISK FACTOR FOR CEREBRAL THROMBOSIS?
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Introduction: Several risk factors for cerebral thrombosis in childhood have been identified. However, there are only few reports on elevated factor VIII levels in children with cerebral infarction. We report on 3 children with cerebral infarction who had factor VIII levels above 150%.
Patients: Patient 1 is a 8-month old child with a congenital heart defect (AVSD and insufficiency of the mitral valve). 6 weeks after closure of the AVSD and mitralcleft an acute hemiparesis of his right arm and leg was developed. On MRI an cerebral infarction was detected. Patient 2 is a 17-month old child with chronic hemiparesis. As neither CT or MRI were performed no definite diagnosis was obtained. Patient 3 is a 9-year old child with acute hemiparesis simultaneously with an acute diarrhea. On MRI an cerebral infarction was detected. In all three patients all investigated prothrombotic risk factors were negative. However, factor VIII levels, investigated 3 months after the acute onset of symptoms were 194, 243 and 272%, respectively.
Conclusions: Elevated factor VIII levels may represent an independent risk factor in arterial cerebral thrombosis in childhood. However, larger studies are needed to confirm this hypothesis.

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***ONGOING CLINICAL TRIALS WITH RFVIIA**
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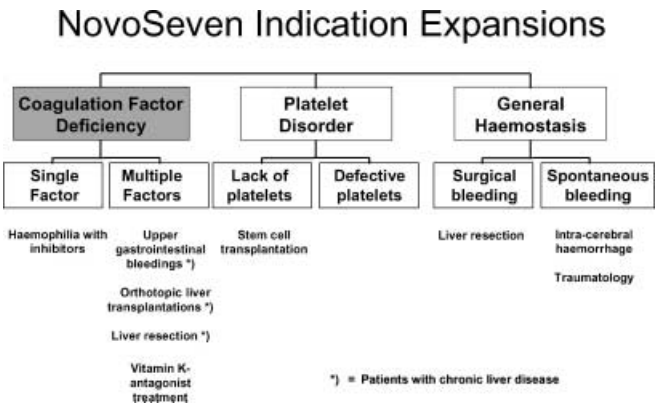
Recombinant activated factor VII (rFVIIa, NovoSeven®) is registered in most regions of the world for the treatment of bleeding episodes in haemophilia patients with inhibitors. Since the product has become available, many case stories have reported on the use of rFVIIa as a haemostatic agent in non-haemophilia patients. This has led to a development programme of clinical trials investigating the effects of rFVIIa in patients with bleeding episodes of many different aetiologies (see figure).
Most of these phase II trials are designed for the use of rFVIIa as rescue treatment in episodes with severe life-threatening bleeding (upper gastrointestinal bleeding, vitamin K-antagonist treatment, stem cell transplantation, intra-cerebral haemorrhage, and traumatology). Some of the phase II trials are focusing on prophylactic use of rFVIIa in order to improve haemostasis during surgery (orthotopic liver transplantation and liver resection), with the intention to subsequently avoid or reduce the need for transfusions and achieve other pharmacoeconomical benefits. To demonstrate the use of rFVIIa in a prophylactic and rescue setting, respectively, the liver resection protocol and the stem cell transplantation protocol will be used to exemplify trial designs.



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THE SCREENING POWER OF METHYLENETETRAHYDROFOLATE REDUCTASE (MTHFR) POLYMORPHISM VERSUS PLASMA HOMOCYSTEINE CONCENTRATION IN PATIENTS WITH SYMPTOMATIC STENOSIS OF THE INTERNAL CAROTID ARTERY (ICA)
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Hyperhomocysteinemia is an important and independent risk factor for vascular disease even in the mild form. About 35% of patients with stroke and up to 47% of patients with peripheral arterial disease have elevated plasma levels of homocysteine (HCY). HCY can be significantly increased as a result of interactions of genetic and acquired factors. Related to genetic factors, the most common causes are deficiencies of cystathionine ß-synthase and MTHFR. The relationship of plasma homocysteine and MTHFR polymorphism, specifically their screening power, is still unknown. This study was performed in 96 patients (68.9±8.9 years) with symptomatic stenosis of ICA and 96 healthy control subjects (66.8±9.9 years). The percentage of women in both groups was 23%. The plasma HCY concentration was determined using a commercial kit for fully automated analysis (AxSYM, Abbott). MTHFR 677 T polymorphism (TT and CT genotype) was assessed by PCR technique. The mean plasma HCY concentration was significantly higher in group with stenosis of ICA compared to the controls, i.e. 12.43±6.06 µM vs. 10.16±3.16 µM, ($p<0.05$). A HCY plasma concentration of 1.5 SD above the mean value of the control group was taken as cut-off for a pathological versus physiological plasma concentration. The sensitivity and specificity of HCY was 0.27 and 0.94, respectively. The positive predictive value was 0.82. Between patients with stenosis of the ICA and controls there was no significant difference in the frequency of the MTHFR 677 CT and TT genotype (47% vs. 47% and 8.3% vs. 11.4%, respectively). Patients with CT genotype had significantly higher plasma HCY concentrations than CC patients, 14.19±7.8 µM vs. 10.98±5.2 µM, $p<0.05$. Sensitivity and specificity of MTHFR polymorphism (both genotypes) was 0.56 and 0.40. The positive predictive value was 0.48. Conclusions: In our study population with a given pretest disease probability of 50%, the determination of plasma HCY concentration with a positive predictive value of 0.82 is more suitable for screening and monitoring of patients at risk than analysis of the MTHFR polymorphism.

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PHARMACOKINETIC CHARACTERIZATION OF DIPETARUDIN, A NEW DIRECT THROMBIN INHIBITOR
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Background: Dipetarudin is a genetically engineered fusion protein with a molecular mass of 7.5 kDa, composed of the N-terminal head structure of dipetalogastin II and a fragment of the exosite 1 blocking segment of hirudin, connected through a five glycine linker. Kinetic analysis revealed that it is a slow, tight-binding inhibitor of thrombin, with a Ki of 446±85 fM. The aim of this investigation is to study the pharmacokinetic behavior of dipetarudin in rats.
Methods: Wistar rats were anaesthetized by a parenteral injection of 1.5 g/kg ethylurethane. A catheter was placed in the left jugular vein for blood sampling and another one was placed in the right jugular vein for a continuous infusion to ensure diuresis. Dipetarudin was applied intra-

venously or subcutaneously as a single bolus injection of 1 mg/kg body weight. Blood and urine samples were collected before dosing and at definite time intervals after dosing. Antithrombin activity was determined by ecarin clotting time.
Results: The pharmacokinetic behavior of dipetarudin can be best described by an open two-compartment model with first-order elimination. After an intravenous bolus injection of dipetarudin, values of 4 min for the distribution and 35 min for the elimination half lifes were obtained. After subcutaneous administration, the maximum blood level was reached after 30 min, with a bioavailability of 80%. In bilaterally nephrectomized rats, the dipetarudin blood level dropped during the distribution phase, but after 60 minutes remained nearly constant at about 20% of the administered dose, which demonstrated the exclusive renal elimination of this inhibitor. The excretion of dipetarudin followed a linear kinetic. The cumulative excretion showed that approximately 0.7% of the administered amount was excreted per hour in the urine in active form. Thus, 80% of the applied dose was recovered over 120 hours.
Conclusions: Dipetarudin is a very potent thrombin inhibitor with a molecular mass and a Ki comparable to hirudin but with a different pharmacokinetic behaviour, thereby dipetarudin could be useful as anticoagulant or antithrombotic drug.

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GPIIB/IIIA ANTAGONISTS MARKEDLY POTENTIATE PLATELET-MONOCYTE INTERACTIONS AND TISSUE FACTOR EXPRESSION FOLLOWING PLATELET ACTIVATION IN WHOLE BLOOD IN VITRO

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Activated platelets are known to contribute to tissue factor (TF) expression on monocytes. GPIIb/IIIa antagonists, which inhibit the final common pathway of platelet aggregation, have not proved to be effective antithrombotic agents after oral administration. Since inhibition of aggregation by such agents does not inhibit platelet activation we investigated the effects of the GPIIb/IIIa antagonists integrilin, abciximab and MK-852 on the formation of platelet-monocyte (P-M) and platelet-neutrophil (P-N) conjugates in whole blood (WB) following platelet activation with collagen. We also quantitated TF expression on leukocytes.
Conjugate formation and TF expression were measured by flow cytometry, i.e. leukocytes were analysed for the platelet-specific antigen CD42a as well as for TF (mean fluorescence intensity; mean±sd).
Overall, platelet activation resulted in P-M as well as P-N formation irrespective of the anticoagulant (citrate or hirudin) or the duration of platelet stimulation (10 to 30 min). For example, incubating citrated WB with 5µg/ml collagen for 30 min increased P-M (i.e. CD42a on monocytes) from 63±30 to 228±66, and this was potentiated by integrilin (0.06-1.5 µg/ml) in a dose-dependent manner. A maximum value of 366±76 was observed at 0.5 µg/ml integrilin. Formation of P-M was associated with an increase in monocyte TF expression from 9±4 to 21±6, and this was also potentiated by integrilin with a maximum of 45±18 at 0.5 µg/ml integrilin. Platelet activation also resulted in a marked increase of P-N formation from 13±5 to 80±28 and a small increase in neutrophil TF expression from 9±3 to 10±4. Both were slightly enhanced by integrilin (1.5 µg/ml) to 109±19 and 11±4, resp. In WB the collagen-induced fall in the number of single platelets was inhibited by 1.5 µg/ml integrilin by only 53±19%, but there was a complete inhibition in platelet-rich plasma. This may indicate that in WB the inhibition of platelet aggregation by integrilin was associated with an increased adhesion of the non-aggregated platelets to leukocytes.
Increased tissue factor expression as a consequence of platelet-leukocyte conjugate formation could contribute to thrombus formation and could explain the poor antithrombotic effectiveness of GPIIb/IIIa antagonists after administration to man.

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COMPARATIVE PHARMACOLOGICAL PROFILE OF HEPARIN AND AN ULTRA-LOW MOLECULAR WEIGHT OLIGOSACCHARIDE MIXTURE DERIVED FROM THE GAMMA-IRRADIATION CLEAVAGE OF HEPARIN

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C3 is a heparin-derived oligosaccharide mixture, which is being developed for the management of Alzheimer's disease (AD) and vascular dementia. Unfractionated heparin (UFH) is known to exhibit strong anticoagulant and antiprotease effects through binding with antithrombin (AT), heparin cofactor II (HCII), and the release of tissue factor pathway inhibitor (TFPI). Since molecular weight influences the functions of heparins, the comparative effects of UFH and C3 on the coagulation processes and their anticoagulant activity were evaluated in in vitro tests, including prothrombin time (PT), activated partial thromboplastin time (aPTT), thrombin time (TT), Heptest, anti-thrombin (IIa), anti-Xa, and protease generation assays. In addition, the anti-IIa and anti-Xa activities were also measured in biochemically-defined systems containing AT and HCII. C3 exhibits a lower molecular weight (2.10±0.12 kDa) compared with that of UFH (14±3 kDa). It has a relatively low anticoagulant activity (20 USP U/mg). In the anti-Xa assay and clot-based Heptest assay, C3 exhibited lower potency, with IC50 5.2±1.6 and 12.5±2.2 mcg/ml, respectively, which were markedly higher than those of heparin. Similar results were also noted in the aPTT assay. In the thrombin-associated anti-IIa, PT, and TT assays, C3 did not show any activity, whereas UFH exhibited strong effects. In the purified AT and HCII systems, C3 only exhibited affinity to ATIII, which was significantly lower than that of UFH ($p<0.05$). Unlike UFH, C3 did not produce any augmentation of agonist-induced aggregation of platelets and was resistant to the neutralization effects of protamine sulfate. Despite its low molecular weight, C3 produced a sustained increase in the TFPI release in primates after i.v. and s.c. administration. Studies on the radiolabelled C3 revealed that the oligosaccharide components of C3 are capable of passing through the blood-brain barrier. These results indicate that in contrast to UFH, C3 possesses much lower anticoagulant and antiprotease effects, which may contribute to its lower bleeding index. These observations suggest that despite a lowered anticoagulant potency, C3 represents a unique antithrombotic agent with distinct bioavailability profiles for the management of CNS associated ischemic and thrombotic disorders.

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FLOW CYTOMETRIC MONITORING OF ORAL GPIIB/IIIA ANTAGONIST THERAPY

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For monitoring anti-aggregatory therapy with glycoprotein IIb/IIIa receptor antibodies (Reopro®) measurement of fibrinogen binding by flowcytometry is beside to turbidimetric aggregometry the most common method. New oral platelet GP IIb/IIIa receptor-antagonists are proven in clinical trials. For forthcoming indications, such as long time anti-aggregatory therapy in primary and secondary prophylaxis after cardiovascular events a small and lower therapeutic range must be ensured, for optimal effectiveness and concomitant limited haemorrhage complications. It is not known whether the existing monitoring methods can be transferred to peptidomimetic antagonists with their different pharmakokinetic characteristics. For monitoring oral GPIIb/IIIa antagonists we used flow cytometry with an monoclonal FITC conjugated antibody against fibrinogen. First we determined influences of various erythrocyte and thrombocyte cell counts and the effect of lysed erythrocytes on fibrinogen binding. Under the given conditions thrombocytopenia had no influence on the flow cytometric measurement on fibrinogen binding, same as anaemia. However increased concentrations of lysed erythrocytes showed a platelet activation in a linear relationship. To determine dilution effects under petidomimetic therapy, dilution series with SR121566a (a peptidomimetic prodrug) spiked blood were measured. Dilution of the spiked samples showed a reduced inhibitory effect of the peptidomimeticum on fibrinogen binding up to nearly 12% (1:100 dilution). In a clinical trial turbidimetric aggregometry was compared with flow cytometric fibrinogen binding using 1:1 diluted whole blood. Patients were pretreated with ASS and/or clopidogrel and blood was taken before and after drug administration and spiked in vitro with SR121566a. The results of the clinical trial showed an increasing inhibition of fibrinogen binding in the flow cytometrc analyse depending on the concentration of antagonist from 0% to 95% (ReoPro®, ASS, Clopidogrel and SR121655a). In contrast aggregometry was not significantly inhibited in the range lower than 50% receptor blockade. In particular the therapeutic range of forthcoming oral GPIIb/IIIa inhibitor therapy for secondary prevention after cardiovascular events need a standardized and sensitive method for monitoring this longterm therapy. The results of this study demonstrate, that whole blood flow cytometry seems to be the only item which fulfills this requirements.

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INTERLEUKIN 1 RECEPTOR ANTAGONIST GENOTYPE IS ASSOCIATED WITH CORONARY ATHEROSCLEROSIS IN PATIENTS WITH TYPE 2 DIABETES

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The proinflammatory cytokine IL-1 is thought to play a key role in atherogenesis. The interleukin 1 receptor antagonist (IL-1 ra) is a major modulator of IL-1 activity. A variable number tandem repeat (VNTR) polymorphism in the IL-1 ra gene has been described of which allele 2 (IL-1 ra*2) is associated with single vessel coronary artery disease (CAD) and diabetic nephropathy. We studied the relationship between the IL-1 ra genotype and CAD in patients with type 2 diabetes. 787 consecutive patients admitted for suspected CAD were included in the study. According to the current criteria of the American Diabetes Association (ADA) 250 patients suffered from type 2 diabetes mellitus. Among these, the IL-1 ra*2 carriers (n=108) had a significantly higher prevalence of CAD (85.2%) compared to the non-carriers (73.2%). The difference was statistically significant in a multivariate logistic regression model (odds ratio [OR] 2.2, 95% CI 1.1-4.3, p=0.02). In patients without diabetes the prevalence of CAD was similar in IL-1 ra*2 carriers and non carriers (55.7% versus 55.6%). Our results suggest that IL-1 ra*2 is associated with CAD in patients with type 2 diabetes.

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***PATHOLOGICAL ANGIOGENESIS: MOLECULAR ASPECTS AND THERAPEUTIC APPROACHES**

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Growth and metastasis of solid tumors depend on the formation of new blood vessels which originate from the existing vascular system. These blood vessels grow into the tumor and thus provide the necessary nutrients and growth factors for tumor progression. At the same time, the newly formed blood vessels allow tumor cells to disseminate and form metastases in distant organs. Normally, vascular homeostasis is regulated by a balance of angiogenic and anti-angiogenic mechanisms. Tumor-induced angiogenesis is mainly sustained by the production and secretion of angiogenic factors originating from tumor and stroma cells. The most prominent angiogenic factor is the vascular endothelial growth factor VEGF. Recently, additional angiogenic factors and their respective receptors have been identified and related to tumor angiogenesis. Among these, the angiopoietins and their receptor TIE-2 have been investigated to some detail. Angiopoietin-1, which binds to and activates TIE-2, is obviously responsible for the stabilization of vessels under homeostatic conditions. Angiopoietin-2 binds to the same receptor as angiopoietin-1 but is antagonistic with respect to angiopoietin-1. It destabilizes blood vessels and under appropriate conditions induces complete regression. In the similar situation angiopoietin-2 induces the destabilization of blood vessels, and the angiogenic factor VEGF produced by the tumor induces the massive formation of new vessels. When human melanoma cells A375 are stably transfected to produce the soluble variant of the angiopoietin receptor TIE-2 (sTIE-2) they show a substantial inhibition of tumor growth on nude mice. Similar effects have been seen with the soluble variant of the VEGF-receptor FLT-1 (sFLT-1). In both cases, the vessel density of the tumors is significanty reduced. These experiments show that the inhibition of the angiopoietin/TIE-2-system similar to the inhibition of the VEGF/VEGF-receptor-system has an anti-tumoral effect, most probably due to the inhibition of tumor angiogenesis. Thus, inhibition of both signalling systems seem to be a valid strategy for the development of novel anti-angiogenic therapies. Recently, the inhibition of the VEGF-receptor tyrosine kinase by the compound PTK787/ZK222584 has been shown to substantially inhibit tumor growth and metastases formation.

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A PROSPECTIVE STUDY ON PHARMACOKINETIC PARAMETERS OF C1-INHIBITOR CONCENTRATE (BERINERT) IN 40 PATIENTS WITH HEREDITARY ANGIOEDEMA (HAE)

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Hereditary angioedema (HAE), an autosomal-dominant disorder with deficiency of functional C1-Esterase Inhibitor (C1-INH) is clinically characterized by recurrent ubiquitary swelling. Particularly laryngeal edema is associated with a high mortality reported up to 30%. The prophylactic use of attenuated androgens or antifibrinolytics revealed severe drug-related side effects (mental illness, amenorrhea, hirsutismus, virilisation, hepatocellular adenoma, severe headache) as well as failure during acute attacks. In addition this therapeutic option is not recommendable during childhood and in reproductive women.
Safety and efficacy of C1-INH concentrate (Berinert TM) in the treatment of acute attacks in HAE patients has been shown in several studies. Half-life is determined with 64 hours in a pilot study. By contrast clinical course of C1-INH activities indicate higher consumption and individual pharmacokinetics.
To clarify these data, a prospective study on pharmacokinetic parameters (recovery and half-life) of Berinert TM in 40 patients with HAE was initiated.
Group I (n=20) patients, aged 6,7-68,3 years with mild clinical course receiving Berinert TM on-demand treatment. Group II (n=20) patients, aged 22,8-61,7 years with severe manifestation of HAE receive prophylactic C1-inhibitor concentrate (Berinert TM) twice times weekly. After 500-1000 IU Berinert as an intravenous bolus injection blood samples were taken before and after substitution by determined time points (0; 15; 20; 30; 45 minutes, 1; 2; 4; 6; 8; 12; 16; 24; 28; 32; 36; 48; 52; 56; 60; 72 hours). Recovery of functional C1-inhibitor was in group I in median 2,25% IU kg bw (range 1,66-3,75) and half-life in median 46,50 hours (range 25,5-92,5). In group II recovery of functional C1-inhibitor was in median 2,79% IU kg bw (range 1,53-4,23) and half-life in median 31,75 hours (range 14,50-68,00). The half-life of functional C1-Inhibitor was very significant higher (46,50 vs 31,75 hrs) in group I, patients with mild manifestation of HAE and on demand therapy, as in group II.
In regard to virus safety, neither seroconversion of hepatitis A-, B-, C-, G-nor HI viruses was observed.

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***THE MANAGEMENT OF CATASTROPHIC BLEEDS IN TRAUMA AND SURGERY BY AN INJURY SPECIFIC SITE SPECIFIC AGENT (RECOMBINANT ACTIVATED FACTOR VII)**

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Uncontrolled hemorrhage is a major cause of death in trauma patients, accounts for about 40-50% of the mortality. Most critically ill trauma patients develop profound multifactoria coaguloplthy due to: Activation of coagulation and fibrinolysis (with hyperfibrinolysis in massive trauma) with consumption and degradation of coagulation factors and platelets, hemodilution, massive transfusion with toxic effects of stored blood, hypothermia, acidosis Etc. Introduction of a site specific hemostatic agent enhancing hemostasis only at the site of injury may decrease hemorrhagic mortality in trauma. Such agent – rFVIIa ,has been used successfully 11 years in patients with hemophilia developing inhibitors to factor VIII or IX . FVIIa only activates the coagulation system upon complex formation with tissue factor, exposed at the site of injury. The use of rFVIIa in trauma and surgical patients was avoided due to the theoretical risk of thromboembolic complications. Our recent in a pig trauma model supports the compartmentalized action of rFVIIa only at the site of injury.The ethical committee approved the use of rFVIIa in trauma and surgical patients suffering uncontrolled bleeding.
Patients: Twenty-six patients (12 trauma-5 penetrating and 7 blunt and 14 surgical) were treated with rFVIIa after all conventional hemostatic measures had failed. Patients were critically ill, multi-transfused [mean packed red blood cells 30.2±18.3 in trauma ,19.2±16.8 in surgery, as well as multiple units of FFP, Platelets and cryoprecipitate) and most had severe “DIC”-like coagulopathy).Trauma patients were younger than the surgical patients(age 25±17 vs. 47±24)
Results: The hemorrhage ceased within minutes after 1-3 doses of rFVIIa in all patients, except one trauma patient who exsanguinated. Total dose was 195±112 and 126±42 mcg/kg in trauma and surgical patients respectively. Significant shortening of PT(from 22±7.9 to 10.4±2.6 sec. and from 19.9±12.1 to 11.7±9.9 sec $p<0.001$ In trauma and surgical patients respectively) and aPTT(from 71±38.9 to 42.2.±24 sec and from 45±20.7 to 34.3±5.5 $p<0.05$ In trauma and surgical patients respectively) was observed within 15 minutes. Blood requirement decreased significantly after rFVIIa from 30±18.3 to 2.8±2.5 in the trauma patients and from 19.2±16.8 to 2.2±4.4 in the surgical patients $p<0.005$. One patient in each group developed clinical DVT .No systemic activation of coagulation was observed. Survival: 8/12 (66%) and 11/14 (78.5%) trauma and surgical patients respectively survived and recovered.
Suggestions: rFVIIa seems to be a promising adjunct hemostatic agent in trauma and surgical patients suffering massive hemorrhage. Controlled studies are required.

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UPREGULATION OF GP IIB/IIIA-RECEPTORS DURING PLATELET ACTIVATION AND EFFICACY OF RECEPTOR BLOCKADE

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Introduction: During platelet activation additional GPIIb/IIIa-receptors move from internal pools to the platelet surface. The GPIIb/IIIA inhibitor abciximab is primarily platelet bound with low free plasma levels while other inhibitors (e.g. tirofiban) have much higher plasma concentrations. It is possible that abciximab might not translocate to newly externalized receptors fast enough to prevent platelet microaggregate formation. We used flow cytometry to quantify GPIIb/IIIa receptors during platelet activation. Methods: Citrated whole blood with and without abciximab or tirofiban was incubated with TRAP (5 µM) or ADP (2 µM) at 37°C under stirring conditions. Aliquots were removed at sequential time points and fixed. Microaggregate formation was determined with flow cytometry. In addition platelets were labeled with primary mouse-anti-CD41 (GPIIIa) antibodies and secondary FITC-anti-mouse antibodies. FITC-labeling was quantified with beads bearing defined numbers of mouse antibodies.
Results: Within few seconds after activation with TRAP the platelet count dropped from 265000/µl to 19000/µl and the number of microaggregates increased to 10000/µl. The mean number of GP IIb/IIIa receptors increased from 52000/platelet to 65000/platelet (+25%). With abciximab (5 µg/ml) the mean number of GP IIb/IIIa receptors increased from 56000/platelet to 77000/platelet (+38%), the platelet count dropped to 112000/µl, and platelet microaggregates increased to 9200/µl. Platelet microaggregate formation was reversible. With tirofiban (50 ng/ml) receptors increased from 53000/platelet to 66000/platelet (+25%), the platelet count dropped to only 200000/µl and there was no increase in platelet microaggregates. Platelet activation with ADP gave similar results. Conclusions: These data show that during the early phase of activation additional GP IIb/IIIa receptors externalize to the platelet surface and abciximab does not bind to these new receptors quickly enough to prevent platelet microaggregate formation. Microaggregate formation with abciximab is still reversible, most likely because the number of unblocked receptors is not high enough to maintain a stable aggregate. With tirofiban there was no microaggregate formation because there is enough free inhibitor to bind to newly externalized receptors. Despite absence of microaggregate formation with tirofiban there was a mild drop in platelet count. It is possible that some platelets bound to other cells e.g. leukocytes.

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QUANTITATION OF GLYCOPROTEIN IB DURING PLATELET ACTIVATION AND PLATELET AGGREGATE FORMATION

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Previous studies suggest downregulation and internalization of GPIb during platelet activation (Michelson Blood 1991, Hourdille Blood 1991). However, changes in GPIb surface expression have not been correlated with aggregate formation. We used flow cytometry to quantify GPIb receptors during platelet activation in whole blood in vitro. Samples were prepared with and without tirofiban to see how much incorporation of activated platelets into aggregates affects GPIb counts on the remaining single platelets.
Methods: Citrated whole blood with and without tirofiban (50 ng/ml) was incubated with TRAP (5 µM) or ADP (2 µM) at 37°C under stirring conditions. Aliquots were removed at sequential time points and fixed. Platelet activation (CD62p-expression) and microaggregate formation was determined with flow cytometry. For quantitation of GPIb platelets were labeled with primary mouse-anti-CD42b (GPIb) antibodies and secondary FITC-anti-mouse antibodies. Labeling was quantified with beads bearing defined numbers of mouse antibodies.
Results: Within 60 seconds after activation with TRAP the platelet count dropped from 265000/µl to 19000/ml and CD62p-positive platelets and platelet microaggregates increased. At the same time the number of CD42b binding sites decreased only minimally from 31000/platelet to 28000/platelet, this change was not significant. Tirofiban prevented the formation of platelet aggregates. With tirofiban there was no change in CD42b binding sites at all. Platelet activation with ADP gave similar results.
Conclusions: This study finds no significant change in GPIb-expression during the early phase of platelet activation with agonist doses high enough to aggregate >90% of platelets. With the agonist concentrations used activated platelets do not downregulate GPIb even when they are stopped from disappearing into aggregates. This does not rule out GPIb downregulation with higher agonist concentrations and longer activation periods. However, it is unlikely that platelets will still circulate in vivo when they have been exposed to such high concentrations for long periods. A decrease of GPIb expression has been described in patients (Wahba Thromb Research 2000). An alternative explanation would be that this is not from internalization of receptors but due to the preferential loss of platelets with high GPIb expression. These results might be relevant for the use of anti-GPIb antibodies as anticoagulants in the future.

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COMPARATIVE EFFECTS OF UNFRACTIONATED HEPARIN AND LOW MOLECULAR WEIGHT HEPARIN ON ANTITHROMBIN ACTIVITY IN THE TREATMENT OF DEEP VEIN THROMBOSIS

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Antithrombin (AT) is an important inhibitor of blood coagulation. It has been shown that functional AT levels are reduced during unfractionated heparin (UFH) therapy. Despite several clinical trials, the effects of low molecular weight heparins (LMWHs) on the functional AT levels have not been fully elucidated. Functional AT levels were measured in an open, randomized study with blind assessment, that was performed in 104 centers (CORTES trial). This study compared a LMWH, Clivarin (Knoll AG, Ludwigshafen, Germany/Abbott, Abbott Park, IL) given subcutaneously b.i.d. for 5-7 days (n=288) or o.d. for 28 days (n=313) versus UFH (n=305) given intravenously for 5-7 days for the treatment of deep vein thrombosis (DVT). Citrated blood samples were drawn on days 1, 5-7, and 21 after treatment with UFH (5000 U IV bolus followed by infusion of same dose) or Clivarin (7,000–12,600 IU aXa, adjusted to patients weight). Functional AT levels were measured using an amidolytic method from Stago (Parsippany, NJ) on the STA Compact, automated coagulation analyzer (Stago, Parsippany, NJ). AT levels were found to be lower in the UFH group in comparison to the Clivarin treated patients (<75% AT levels in 10% of the clivarin group and 30% of the UFH group). In addition, the group of patients who received Clivarin b.i.d. had a higher prevalence of low AT levels (33%) in comparison to the patients who received Clivarin o.d. (24%). Quartile analysis showed that approximately 30% of the patients in the UFH group had AT levels that decreased to levels between 50-75%. In comparison, in the Clivarin o.d. group only 18% and in the b.i.d. group 20% decreased to this level. The immunologic levels of AT did not change in the different groups of patients. These results show that UFH causes a more pronounced decrease in AT levels compared to LMWH. A o.d.regimen of LMWH results in less of a drop in AT then b.i.d. In addition, the o.d. regimen showed a trend towards better efficacy in the clinical endpoints. In contrast to functional AT, the functional heparin cofactor II did not differ significantly in the various groups included in this study. In conclusion, an o.d. dose of LMWH may be a better regimen to treat patients with DVT and to maintain desirable levels of antithrombin. These studies further underscore the increased efficacy of LMWHs.

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PARADOXICAL PLATELET ACTIVATION BY GLUCOPROTEIN IIB/IIIA INHIBITORS – A POSSIBLE ROLE OF THE HUMAN PLATELET ANTIGEN-1 POLYMORPHISM?

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The human platelet antigen-1 (HPA-1) polymorphism has been shown to influence the inhibitory actions of abciximab. Thus, we hypothesized that this polymorphism might also be the cause for the paradoxical activation of platelets by GPIIb/IIIa inhibitors. The effects of abciximab (1-10 µg/ml), tirofiban (3-30 nM), or eptifibatide (0.3-3 µg/ml) on basal and ADP (3 µM)-induced CD62P externalization were measured in 62 healthy blood donors and 177 patients with stable coronary artery disease. All subjects were genotyped by GALIOS and automated fluorescence correlation spectroscopy. Although a significant platelet hyperreactivity was observed in the patients, the HPA-1 genotype did not influence basal or ADP-induced CD62P expression. A moderate (2-fold) stimulation of CD62P expression by abciximab (10 µg/ml) but not by tirofiban or eptifibatide was observed in one patient carrying the HPA-1 b/b allele. In no other subject any activation of platelets by GP IIb/IIIa inhibitors was observed. It is concluded that paradoxical platelet activation by GP IIb/IIIa inhibitors is a rare (<2%) phenomenon. HPA-1 b/b genotype might be a contributing determinant but does not predict platelet activation by GP IIb/IIIa inhibitors.

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BIOCHEMICAL AND COAGULO-PHYSIOLOGICAL CHARACTERIZATION OF PEG-COUPLED SYNTHETIC LOW MOLECULAR THROMBIN INHIBITORS

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Background: In recent years, synthetic low molecular thrombin inhibitors have been gaining in importance in therapy of thromboembolic diseases. However, additional comprehensive pharmacologic investigations are necessary because of some negative pharmacokinetic properties of these substances (e.g. the poor oral absorption or their metabolism in liver). Synthetic low molecular thrombin inhibitors coupled to high molecular inert vehicles, like polyethylene glycols, could represent a possibility to improve such negative characteristics.
Methods: The selected PEG-coupled synthetic thrombin inhibitors Mtr-Asn(PEG2000-OMe)Adf-Pip, Mtr-Asn(PEG10000-OMe)Adf-Pip as well as Mtr-Lys(PEG10000-OMe)Adf-Pip provided by VitaResc were investigated by analytical HPLC and mass spectroscopy. The dissociation constant Ki was determined for each substance using an amidolytic assay. Several standardised coagulation assays (ECT, PT, aPTT, TT) were used for determination of the coagulo-physiological characteristics.
Results: The PEG-coupled substances Mtr-Asn(PEG2000-OMe)Adf-Pip, Mtr-Asn(PEG10000-OMe)Adf-Pip as well as Mtr-Lys(PEG10000-OMe)Adf-Pip were investigated in comparison with their basic substances. The Ki-values of the PEG-coupled inhibitors are lower than the values of the basic substances. The Ecarin Cloting Time is the best of the tested coagulation assays for determination of the antithrombotic activity. All tested substances have only a very low effect on prothrombin time and are not quantificable using thrombin time.
Conclusions: The results of Ki-determination showed that the derivatisation of basic substances using PEG2000 and PEG10000, respectively, leads to partly remarkably stronger inhibitors. But, the investigations of the influence of the inhibitors in different coagulation assays reveal that the PEG-coupled substances show lower effects in comparison with the basic inhibitors. Now an in vivo characterization is necessary to determine their activity in natural systems.

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PHARMACOKINETICS OF THE PEG-COUPLED SYNTHETIC LOW MOLECULAR THROMBIN INHIBITOR MTR-ASN (PEG10000-OME) ADF-PIP

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Background: Besides the in vitro characterization of the PEG-coupled synthetic thrombin inhibitors the investigation in vivo is very important and inevitable for their use in therapy of thromboembolic diseases. So, the PEG10000 derivative Mtr-Asn(PEG10000-OMe)Adf-Pip was investigated pharmacokinetically in pigs to get exact data in greater mammals.
Methods: The PEG10000 substance was administered in anaesthetised pigs as intravenous bolus and subcutaneously in several doses. At definite time intervals blood was taken and urine samples were collected. The content of the inhibitor was determined in blood, plasma and urine using Ecarin Clotting Time. Furthermore the plasma was tested in different standardised in vitro coagulation assays (PT, aPTT, TT).
Results: After intravenous bolus administration of lower doses (1mg/kg, 2.5 mg/kg) of PEG10000 derivative typical blood levels were found. The substance was eliminated rapidly and nearly the complete amount of substance given was found in urine. After subcutaneous administration of different doses of inhibitor comparable results were found. First the blood levels of inhibitor increased until tmax, then they decreased slightly and a long lasting blood level of more than 20-24 hours was detected. At lower amounts of inhibitor given tmax was 1-2 hours and 80-100% of the inhibitor was found in urine. At higher doses (10 mg/kg, 20 mg/kg) tmax was 6-8 hours and only 40-50% was detected in urine.
Conclusions: The results of these studies showed that high amounts of the PEG10000 derivative are available after administration and after the distribution phase there is a long elimination phase, especially after subcutaneous injection, where the inhibitor is available in small amounts. Higher amounts of inhibitor given increase the blood level just a little bit in comparison to lower amounts given, but the long lasting blood level is maintained for more than 30 h. The pharmacokinetic investigations are not terminated. More details will be estimated in near future.

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ANTITHROMBIN III TREATMENT FOR SEVERE HEPATIC VENO-OCCLUSIVE DISEASE IN CHILDREN WITH WILMS' TUMOR AND ACUTE LYMPHOBLASTIC LEUKAEMIA

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Veno-occlusive disease (VOD) of the liver is a well known complication in patients undergoing high-dose chemotherapy and bone marrow transplantation. It has also been reported following conventional anticancer chemotherapy, particularly in children treated for nephroblastoma and other malignancies (e.g. rhadomyosarcoma, AML, Hodgkin's disease). Although in most patients this complication resolves uneventful, fatal cases have been reported. Severe VOD after transplantation has a high mortality rate from 45% to 98%. New strategies of hemostatic therapies significantly improved the prognosis of VOD. Chemotherapy related VOD in Wilms`tumor usually has a good prognosis. We report about two patients with Wilms`tumor and two with acute lymphoblastic leukaemia, who developed severe veno-occlusive disease of the liver according to the Baltimore criteria with hepatomegaly, ascites, hyperbilirubinemia, weight gain and in one patient short time lethargy, while receiving chemotherapy.
Elevated LDH levels ranged from 872 to 12000 U/l in our patients. All patients had thrombocytopenia between 29000-40000/µl, Antithrombin III (AT III) and protein C levels were decreased, and two patients had gastrointestinal bleeding. All patients developed a coagulopathy because of severe hepatic dysfunction. Two patients received low doses heparin at the onset of VOD. The therapy was changed to AT III (20-80 IU/kg body weight 2 times a day) supplementation without heparin when thrombocytes were very low or gastrointestinal bleeding occured. Because of severe thrombocytopenia two patients received AT III immediately without heparin. Resolution of VOD was observed in all patients with the administration of AT III. All patients survived without any sequelae with a median follow up of 28 months (range 8-48 month). High dose AT III infusion in patients with severe chemotherapy related hepatic veno-occlusive disease is successful and can also be applied effectively in case of bleeding.

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ASEPTIC MENINGITIS INDUCED BY HIGH-DOSE GAMMA-GLOBULIN IN CHILDREN WITH IDIOPATHIC THROMBOCYTOPENIC PURPURA

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High dose intravenous immunoglobulin (ivIG) is used for many conditions in Childhood. Common side effects include headache, fever, chills, and nausea; these usually resolve within an hour after stopping or slowing the infusion and respond to symptomatic treatment. More serious effects are anaphylaxis, haemolysis, hepatitis, thrombosis and aseptic meningitis.
Aseptic meningitis after high dose immunoglobulin therapy has been reported in several conditions, including idiopathic thrombocytopenic purpura (ITP). Symptoms headache, fever, neck stiffness often develop after several courses, beginning six to 48 hours after infusion and clearing within two to four days.
We report 6 episodes of aseptic meningitis diagnosed in 4 patients on the basis of clinical signs and cerebrospinal fluid (CSF) pleocytosis during treatment with high dose (IgG). The patients received different immunoglobulin preparations in doses between 400 mg/kg to 1 g/kg daily. Interestingly we observed an increase of thrombocytes counts in 1 of 6 episodes of (ivIG) treatments (in these patients with meningitis). In all these cases, aseptic meningitis has appeared on the second or third day of IVIG therapy and treatment has always been interrupted. Examination of CSF revealed an increased leukocyte count (350-3800/mL neutrophils and lymphocytes). No pathogens were detected in the CSF and bacterial culture was negative. In all cases the symptoms resolved within 2 days.
The pathogenesis of aseptic meningitis following the administration of ivIG is unknown. Several immunoglobulin preparations may induce aseptic meningitis. Aseptic meningitis may cause severe clinical problems, which resolve rapidly without therapy and unnecessary explorations e.g. CT Scan or MRI should be avoided. Neurologic complications caused by the IgG preparations used in the treatment of childhood ITP occur more frequently than has previously been suggested and may substantially increase the costs of treatment. Because of the low risk of life-threatening bleeding and several complications, the indication for high dose IgG should be restricted in patients with ITP.

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NEW MOLECULAR DEFECTS IN CASES OF AFIBRINOGENEMIA AND HYPOFIBRINOGENEMIA

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Genetic diseases characterized by quantitative defects of plasma fibrinogen have been reported in less than 200 families. Afibrinogenemia was found in about 120 cases and the number of hypofibrinogenemic patiens reported is about 40. Molecular defects have been elucidated in about 50 cases of afibrinogenemia and 12 hypofibrinogenemic patients. Mutations responsible for the quantitative fibrinogen defects are found in all three fibrinogen genes with a clustering in FGA.
We have studied two Venzuelan and one German case of afibrinogenemia and two German patients with hypofibrinogenemia. Genomic DNA was isolated and all exons and exon-intron boundaries of the three fibrinogen genes were amplified by PCR. All fragments were subjected to direct DNA sequencing.
In the two Venezuelan cases frameshift mutations in the FGA gene were detected: del 1216T and del 4378G changing Aalpha chain sequence starting at position 21,Val and 349,Gly, respectively. Premature stop codons presumably occur at codons 42 and 401 thus producing defective shortened Aalpha chains which are unable to support assembly and/or secretion of fibrinogen molecules from hepatocytes. In the German case of afibrinogenemia a nucleotide exchange C3282T in the FGB gene was detected. This nonsense mutation creates a stop codon in position 17, Arg of the Bß chain. The two Venezuelan mutations have not been described before whereas the latter defect was also reported for an Iranian and an Italian family. All three afibrinogenemic patients are homozygous for the indicated mutations.
In contrast, in the hypofibrinogenemic patients two different missense mutations were found in heterozygous state: FGB C3441T causing amino acid exchange 70, Pro®Ser in the Bbeta chain (variant Hamburg I) and in the second case FGG A7685G with protein defect 345, Asn®Asp in the gamma chain. Both defects have not been reported before. These molecular abnormalities probably produce gross sterical changes molecule affecting assembly, secretion or stability of the fibrinogen molecule. The analysis of these new cases of afibrinogenemia and hypofibrinogenemia may support understanding of normal production of the major coagulation factor and the pathological aspects of this process.

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DYSFIBRINOGENEMIA DUE TO NEW MOLECULAR DEFECTS IN FIBRINOPEPTIDE A

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Hereditary dysfibrinogenemia is a rare disorder caused by structural defects of the fibrinogen molecule. Clinically, both bleeding and thromboembolism is observed in dysfibrinogenemic patients. However, about half of the cases are asymptomatic. Molecular defects have been elucidated in about 200 cases. The most frequent abnormalities are mutations causing changes in the amino acid Aalpha 16, Arg, thus impairing the cleavage of fibrinopeptide A by thrombin. These account for about 30% of all dysfibrinogenemias. However, other mutations in the fibrinopeptide A sequence are very rare.We report here on four new molecular defects with amino acid exchanges in positions 9, 13, 15 and 16 of the Aalpha chain.
Patients from three families are asymptomatic. In one case there is a bleeding tendency. Plasma fibrinogen concentration is in the normal range when measured immunochemically and decreased (2 families) or (sub)normal (2 families) when measured by the Clauss method. Thrombin time is prolonged to a variable extent.
In order to elucidate the underlying mutations, genomic DNA was isolated and all exons and exon-intron boundaries of the three fibrinogen genes were amplified and the resulting PCR products subjected to direct DNA sequencing. Functional tests of isolated fibrinogen samples included thrombin- and batroxobin-induced fibrin gelation and fibrinopeptide release. Isolated fibrinopeptide samples were subjected to HPLC analysis in order to detect abnormal peptides and to quantify released peptides.
Mutation screening revealed four new defects in the FGA gene in heterozygous state: T1182C, G1193A, T1200A, and C1202G causing the amino acid exchanges in the Aalpha chain 9, Leu>Pro, 13, Gly>Arg, 15, Val>Glu and 16, Arg>Gly, respectively. In the latter case, impairment of fibrinogen function is similar to the frequent mutation 16, Arg>Cys with a severely decreased fibrin gelation and reduction of released fibrinopeptidesA to about 50%. In the other three cases, fibrinopeptide release and fibrin gelation are only slightly or moderately affected. In the case of the Aalpha 13, Gly>Arg defect an abnormal fibrinopeptide could be detected by HPLC.
Further studies of these fibrinogen variants should provide new information on the participation of certain amino acid residues of the fibrinopeptide A sequence in the interaction with thrombin and thrombin-like snake venom enzymes.

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PREVENTION OF VENOUS THROMBOEMBOLISM AFTER KNEE ARTHROSCOPY. COMBINED DATA OF A RANDOMISED CONTROLLED TRIAL AND A LARGE OUTPATIENT COHORT TREATED WITH REVIPARIN

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Venous thromboembolism (VTE) is a common, important complication after orthopedic surgery. However, only few trials investigate the incidence of VTE in outpatients undergoing knee arthroscopy. The present trial and the large cohort study address the risk of VTE in those patients and determine the efficacy and safety of a low molecular weight heparin in preventing venous thromboembolism. This is the largest safety cohort to date and the first controlled randomised trial using objective diagnostic methods with blinded outcome assessment to reveal the incidence of VTE in outpatient arthroscopy and determine efficacy and safety of a low-molecular-weight heparin (LMWH - reviparin) in preventing VTE in these patients.
262 patients undergoing elective knee arthroscopy were prospectively randomized to receive either no treatment or reviparin sodium once daily sc. for 7-10 days. Additional 620 patients were included in an uncontrolled cohort receiving the same active treatment. The incidence of deep vein thrombosis detected by compression colour-coded sonography and the rate of symptomatic pulmonary embolism were the composite outcome measure. Both groups were comparable with regard to demographics and baseline characteristics. Among the 737 LMWH-treated patients only 5 DVT (0.68%) were detected. This overall result was well in line with the 239 evaluable patients included in the controlled, randomized trial (122 no treatment, 117 receiving LMWH). 6 DVT were detected - 5 in the control group (5/117-4.1%) and only one in the active treatment group (1/116-0.85%). The odds ratio of 4.95 approximates a relative risk reduction of about 80%. Treatment with reviparin was safe, no major bleeding occured. One patient had a transitory fall in platelet count below 100 Gpt/L without any clinical symptoms.
Patients undergoing knee arthroscopy have a moderate risk of venous thromboembolism and effective prophylaxis can be achieved with low molecular weight heparin (reviparin).

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LOW MOLECULAR-WEIGHT HEPARIN (REVIPARIN) DIMINISHES TUMOR CELL ADHESION AND INVASION IN VITRO, AND DECREASES INTRAPERITONEAL GROWTH OF COLOADENOCARCINOMA CELLS IN RAT AFTER LAPAROSCOPY

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Metastasis, adhesion and invasion of tumor cells involve a cascade of complex phenomena, which potentially can be affected by glycosaminoglycans. We studied the influence of a low-molecular-weight heparin, reviparin, on the intraabdominal tumor growth in rats undergoing laparoscopy. We used adenocarcinoma cells CC531 to study cytotoxicity, anti-adhesive, and anti-invasive effects of reviparin in vitro, and tumor growth in vivo.
In vitro assays: Adhesion of 1×10(5) CC531 adenocarcinoma cells onto microtiter plates coated with 10 mikrogram / ml collagen type I or 10 mikrogram/ml Matrigel was significantly reduced by 5.52; 11.04; 27.6 mg/ml reviparin versus 0.9% saline (P<0.001). The cytotoxicity of reviparin on 1×10(4) adenocarcinoma cells was studied in a similar assay, showing no specific effect of reviparin on the viability of these cells. Transwell dual chambers with polycarbonate filters coated with 100 mikrogram/cm² Matrigel were used to investigate the effect of 0.27; 0.55; 1.10; 2.76 mg per well on the invasion of 1×10(5) adenocarcinoma cells. We found a highly significant inhibition of tumor cell invasion (P<0.001) by all reviparin concentrations used in our assay.

In vivo experiments: CC531 adenocarcinoma cells (5×10(6) cells/ml) were intraperitoneally applied to Wistar Albino Glaxo rats (n=150, Harlan, Germany) with a median weight of 278 grams. Rats were divided into 15 groups with 10 animals each, underwent laparoscopy, and 1 ml saline alone, or containing 0.5; 2.0; 4.0; and 10 mg reviparin per kg bodyweight was introduced for intraperitoneal lavage or s.c. After 21 days the animals were sacrified, and tumor weight was determined. We found that application of 4.0 and 10 mg/kg bodyweight, but not 0.5 or 2.0 mg/kg bodyweight significantly (P<0.01) decreased tumor weight compared to the control group, which received saline alone. This effect was most pronounced after the combined i.p. and s.c. application, whereas after i.p. application alone, only the highest dose of 10 mg/kg BW caused a significant inhibition of tumor growth.
Low-molecular-weight heparin, reviparin, given in combination of i.p. lavage and s.c. injections, significantly diminishes intraabdominal tumor growth of CC531 adenocarcinoma cells in rats undergoing laparoscopy. This may offer additional therapeutic options for patients undergoing laparscopic cancer surgery.

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4G/5G PROMOTOR POLYMORPHISM IN THE PLASMINOGEN ACTIVATOR INHIBITOR 1 GENE IN WOMEN WITH A HISTORY OF PREECLAMPSIA OR HELLP SYNDROME – PRELIMINARY RESULTS

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Background: The aetiology of preeclampsia (PE) and the HELLP syndrome (hemolysis, elevated liver enzymes, low platelets) is still unknown. Recent studies suggested an association between these severe complications of pregnancy and hereditary risk factors for thrombophilia including factor V Leiden, prothrombin 20210A, and others. The 4G allele of the 4G/5G polymorphism in the gene for plasminogen activator inhibitor 1 (PAI-4G/5G) has been reported to be associated with thrombosis and also with pregnancy complications. In an ongoing retrospective study we are investigating hypothetic genetic risk factors for PE/HELLP.
Aim of the study: To determine whether the PAI-4G/5G polymorphism is associated with PE/HELLP.
Patients: So far, 109 women with a history of PE or HELLP have been examined. 112 healthy medical students (females and males) of similar age to the patients served as a control group.
Methods: Genotyping for PAI-4G/5G was performed using genomic DNA form whole blood, PCR amplification, and melting temperature analysis with fluorescent hybridization probes using the LightCycler instrument.
Results: The PAI-4G/5G genotype distrubutions and allele frequencies were as follows:

	total	4G/4G	4G/5G	5G/5G	4G allele	5G allele
Patients (PE/HELLP):	109	35 (32%)	46 (42%)	28 (26%)	0.53	0.47
Controls	112	27 (28%)	44 (45%)	26 (27%)	0.51	0.49

The differences between patients and controls are statistically not significant (p>0.05). The genotype distribution and allele frequencies in the control group are in agreement with published data for Caucasian populations. There is no deviation from Hardy-Weinberg equilibrium.
Conclusion: The PAI-4G/5G genotype of a pregnant woman does not appear to be strongly related to her risk of developing preeclampsia or the HELLP syndrome. This is in agreement with our recent finding in the same study group that common thrombosis risk factors such as factor V Leiden and the prothrombin 20210A variant also were only slightly more prevalent in the PE/HELLP patients. However, due to the small sample size weak interactions cannot be ruled out such as a small predisposing effect of the 4G allele. PE and HELLP may well be the result of complex interactions of several weak aetiopathogenetic factors in the haemostatic and other systems.

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COMPREHENSIVE LONG-TERM CARE FOR 17 PATIENTS WITH THROMBOTIC THROMBOCYTOPENIC PURPURA (TTP) WITH AND WITHOUT RELAPSE

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Introduction: We report the long-term, comprehensive care for 17 TTP-patients (F:M=10:7) observed between 1982 and 2001 in respect of potential triggers, clinical symptoms, recurrence and treatment. The age of the pts. at initial episode ranged from 13-52 years for F (mean=29) and from 36-54 years for M (mean=39).
Results: We could identify for 12/17 pts. the following potential triggers for initial TTP-episode: oral contraceptives (n=4), infection (n=4), pregnancy (n=1), SLE (n=1), Ticlopidin (n=1) and Norfloxacin (n=1). Clinical symptoms at first episode were neurological disorders (n=13), petechias (n=8), headache (n=7), seizures (n=5), coma (n=4), abdominal pain (n=3), hematoma (n=3) and hematuria (n=2). 8/17 pts. (M:F=3:5) experienced a total number of 21 relapses. The clinical symptoms during relapse were less severe: petechias (n=3), headache (n=3) and only occasionally neurological disorders, hematuria and hematoma. 16/17 pts. were treated successfully by plasmapheresis (pp) against FFP and corticosteroids. We choose pp-therapy, because in our initial experience FFP alone was less effective. The number of pp ranged from 5-86 (mean=28) at initial episode and from 4-50 (mean=11) at relapse. Pts. with severe neurological disorders were additionally treated by vincristine. We performed successfully a laparascopic splenectomy for a male patient with 7 relapses within 44 months and severe shunt problems. This pt. had before splenectomy severe deficiency of vWF-cleaving protease activity secondary to inhibitory activity. One female pt. died with 37 years during initial episode from myocardial infarction (autopsy of coronary arteries showed hyaline thrombi). In this pt. the pp-treatment was discontinued before normalisation of LDH-level and platelet count. The time between first symptoms and start of treatment amounted for the initial episode from 4 up to 30 days (mean=9d), whereas relapse was diagnosed and treated within 1 to 5 days (mean=1,5d).
Conclusions: Various potential triggers for onset of TTP could be identified. During initial episode the pts. presented many facets of the disease, mostly neurological disorders. Our results of long-term, comprehensive care for 17 TTP pts. demonstrate, that early diagnosis thus rapid treatment by plasmapheresis against FFP, corticosteroids and possibly vincristine is essential for prognosis and outcome.

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INHIBITION OF THE AKT, P38 MAPK AND IKK/NF-KAPPA B SIGNAL TRANSDUCTION PATHWAY BY STATINS IN HUMAN MONOCYTES

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Background: Statins are known to exert anti-inflammatory and antithrombotic vascular effects. The molecular mechanisms underlying these non-lipid pleiotrophic effects are poorly understood. In this study we examined the effect of statins on the LPS-induced Akt-, p38 MAPK- and IKK/NF-kappaB signaling in cultered monocytes (Mo).
Methods: Mo isolated from healthy blood donors were incubated with LPS (10 µg/ml) in the presence or absence of Cerivastatin or Simvastatin (0.01-5 µM). The activation of Akt, p38 MAPK and NF-kappa B, as well as the IkB-alpha degradation were analysed by Western blot and electrophoretic mobility shift assay.
Results: Cerivastatin and Simvastatin inhibited in a dose-dependent manner the LPS-induced phosphorylation of Akt and p38 MAPK. Additionally these statins prevented phosphorylation and degradation of IkB-alpha and the following NF-kappaB activation in response to LPS. These inhibitory effects were reversed when cultures were incubated with the metabolite L-mevalonate.
Conclusions: Statins were shown to prevent LPS-induced activation of Akt, p38 MAPK and IKK/NF-kappaB due to the inhibition of the production of mevalonate. This effect appears to be in contrast to the PI3-kinase stimulating effects of statins in endothelial cells.

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CELLULAR EFFECTORS ENHANCE THE (AUTO-)ACTIVATION OF FACTOR VII ACTIVATING PROTEASE (FSAP)

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Factor VII activating protease (FSAP), a serine protease present in plasma, is a single chain proenzyme and has the ability to activate both, factor VII and pro-urokinase. In addition, (auto-)activation of FSAP can occur, which is significantly enhanced in the presence of heparin, while calcium ions stabilize both the active and inactive forms of FSAP. It remains poorly understood, however, how the activity of FSAP is controlled in vivo. In this study, we investigated the influence of cells on the (auto-)activation of FSAP. Activation of the FSAP zymogen was determined by employing a chromogenic substrate assay and was detected by Westernblot-analysis. On cultures of human umbilical vein endothelial cells (HUVEC), (auto-)activation of FSAP was enhanced 4-fold within 10 min as compared to the absence of cells. Phosphatidylethanolamine and other typical membrane lipid could not significantly enhance the FSAP (auto-)activation. Conditioned medium of HUVEC was also found to have the same effect in provoking (auto-)activation. Stimulation of the endo-

thelial monolayer by cytokines had no influence. This activity was not changed by treatment of the supernatant with protease inhibitors such as aprotinin, leupeptin or phenyl-methyl-sulfonyl-fluoride or by heat denaturation. However, (auto-)activation was inhibited by physiological concentrations of zinc ions. These results indicate that (auto-)activation of FSAP is accelerated by soluble heat-stable factor derived from HUVEC but not by proteolytic cleavage involving other proteases. Hence, the human vascular endothelium might provide a physiological entity to activate FSAP, which leads to subsequent events on blood coagulation and fibrinolysis.

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***ANTIANGIOGENIC EFFECTS OF SEROTONIN ANTAGONISTS IN CHICK AREA VASCULOSA MODEL**

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Chick area vasculosa proved an useful model in screening for angiogenic or antiangiogenic effects. Briefly, White Leghorn eggs are incubated for 65 h at 37°C and 85% humidity and then transferred to shell-less culture. Substances of interest are dissolved, thickened with tragacanth (3%) and applicated onto the area vasculosa in droplets of 1.4 µL. After 9 h, the reaction is assessed semi-quantitatively using a score. Imaging of application sites is done using a colour video camera inside the incubator. First investigations using this model indicated a possible role of serotoninergic mechanisms in early angiogenesis in area vasculosa. Therefore we tested several serotonin antagonists. Pindobind ($5HT_{1A}$) was the most sensitive antagonist showing a dose dependent inhibition in the range of 0.3–14.5 nmol. Cyproheptadine, a $5HT_2$ antagonist, was effective in the dose range of 0.86–43 nmol. Methiothepin mesylate ($5HT_1$) and Isamoltan hemifumarate ($5HT_{1B}$) were nearly equally effective in the range of 4–155 nmol and 8.2–123 nmol, respectively. Surprisingly, Buspiron-HCl, a $5HT_{1A}$ agonist, did not cause stimulation, but inhibition of angiogenesis in the range of 33-332 nmol. S-WAY ($5HT_{1A}$) inhibited angiogenesis in a dose range of 60–427 nmol. All substances tested showed a dose dependent effect with maximum efficiency of 76-97%. For some substances, a decreased inhibitory effect was induced by preceding or simultaneous application of 57 nmol serotonin. Due to structural similarities of area vasculosa vessels to tumour vessels, early formation of capillaries in area vasculosa is of great importance as a screening model. 5HT antagonists, especially $5HT_1$ receptor antagonists represent promising candidates for further investigations using animal models where new drug profiles can be expected.

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QUALITY OF LIFE IN ORAL ANTICOAGULATION – COMPARISON OF SELF-MANAGEMENT VERSUS STANDARD CARE

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Introduction: Several studies showed the advantages of self-management of oral anticoagulation concerning INR results, therapy safety and cost-effectiveness compared with standard care by the general practitioner or special anticoagulation clinics. The assessment of quality of life is not reflected by cost-effectiveness analysis and is therefore quantified increasingly in health care.
Methods: A questionnaire was developed to assess the quality of life in patients after 6 months on therapy with oral anticoagulants. The 18 items included the description of the feeling of general independence, changes of planning and conducting vacations, changes of the communication with the treating physician and other situations, which were anticipated to be or not to be modified by an oral anticoagulant therapy. A total of 224 patients were educated to use the self monitoring system and 92 patients on routine anticoagulant care served as control. The level of significance between the groups was set at $p<0.01$ using the Mann-Withney-Test and a correction for multiple testing.
Results: The following items gave higher scores after 6 months of therapy for patients on the self-management control: general independence during daily life, self-efficacy, general mood, family life, self-confidence and vitality, ecc. 92,7% and 63,2% of INR values were within the therapeutic range of 2.0 to 4.0 for patients using the self-management and the general care system, respectively. Statistical significant differences in the number of thromboembolic and major bleeding complications were not observed between both groups.
Conclusion: The quality of life is significantly improved if patients use the self-monitoring device to control the treatment with oral anticoagulants. Not only therapy safety and cost-effectiveness underline the recommendation to enable patients under oral anticoagulation to determine their INR results and to learn dose adjustment on their own.

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TOXIC BLOOD LEVELS OF PEG-HIRUDIN CAN BE ANTAGONIZED BY PMMA DIALYZERS IN VIVO

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Hirudin is able to both antagonize prothrombotic processes and neutralise clot-bound thrombin. By this, hirudin is an ATIII-saving anticoagulant and antithrombotic agent of special clinical importance. By creating molecular weight-enlarged hirudins by coupling to polyethylene glycol, an even better pharmacokinetic quality results. A specific antidote is required to prevent iatrogenic intoxication of PEG-hirudin, characterised by increasing severe bleeding tendency. In comprehensive in vitro investigations we have found that polymethyl methacrylate (PMMA) capillary dialyzers can bind PEG-Hirudin and can therefore be used as effective antidote principles for PEG-Hirudin. The aim of the preclinical study presented here was to investigate if this binding mechanism will also apply to an experimental haemodialysis (HD) in a nephrectomised dog and if PMMA dialyzers will actually be suited as functional antidote for PEG-Hirudin. For the investigations, male beagle dogs were used. The dogs were narcotised using 25 mg/kg Pentobarbital i.v. Two hours after bilateral nephrectomy a bolus injection of 1 mg/kg PEG-Hirudin was given. The experiment was run for 180 min after start of HD. PEG-Hirudin blood/plasma concentration was measured using Ecarin Clotting Time. The aPTT was performed according to manufacturer's instruction. In all seven PMMA experiments, the PEG-Hirudin blood level decreased from 12-15 µg/ml (cp0) to 6.1-7.4 µg/ml 120 min after PEG-Hirudin bolus. At the beginning of HD using PMMA dialyzer, a significant steep decrease of PEG-Hirudin blood level occurs. At the end of experiment, a mean PEG-Hirudin blood level of 2.0 µg/ml was measured. For comparison, one animal got a HD using a cellulose acetate high flux filter of comparable size. Not any change in PEG-Hirudin blood level occurred compared to control experiments without HD. 180 min after start of HD, a residual blood level of 4.2 µg/ml was measured. From the detoxification experiments presented here it is concluded that more than 50% of the PEG-Hirudin administered into the dog is bound by the PMMA dialyzer and that this binding capacity is not saturated at this time. Thereby, PMMA dialyzers represent an effective antidote mechanism for PEG-Hirudin. By specific binding of this molecular weight-enlarged Hirudin to a polymer surface, a fast detoxification of extracellular space of the patient becomes possible.

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INCIDENCE OF HIT II ANTIBODIES IN PATIENTS ON RENAL REPLACEMENT THERAPY

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Renal replacement therapy (RRT) of patients with renal insufficiency using chronic intermittent haemodialysis can be performed only when a relatively high dose of heparin is administered to the patient before each haemodialysis. During long-term RRT, a patient gets approximately 1.5-1.7 million IU heparin per year supposing a high risk for heparin induced thrombocytopenia (HIT II). However, relatively few severe cases of HIT II are known in these patients. An incidence of HIT II in 0.3-1.8% of patients on RRT is reported. Furthermore, a number of case reports can be found on severe HIT II reactions in patients on RRT, but more often in patients having acute renal insufficiency syndromes making a long lasting acute haemodialysis treatment necessary.
In a comprehensive diagnostic study the incidence of HIT II antibodies in haemodialysis patients being treated with heparin for a long time, was to be investigated. As diagnostic test, C14 serotonin release assay (SRA) was used. 260 patients from three German dialysis centres were involved in the study. Blood was taken before and at the end of dialysis. Serum was gained by centrifugation, 6 hours after blood withdrawal at the latest, and was then frozen at -80°C and tested for antibodies within 6 weeks. Surprisingly, 45-48% of the patients showed antibodies in SRA. More than 80% of these patients had a positive result before haemodialysis, but were negative at the end of haemodialysis. 11% of the patients showed a positive antibody-test both before and after haemodialysis. Before haemodialysis, the titer was more than twice the titer at the end of haemodialysis. 9% of the patients were positive only at the end of haemodialysis.
From our investigations the following conclusions can be drawn: 1. An astonishing high number of patients have a positive test for HIT II-antibodies, although only few patients show typical clinical signs. 2. Together with activated or aggregated platelets, HIT II antibodies are removed from the circulation by the polymer surface of dialyzer. In in vitro studies we confirmed that HIT II antibodies-containing high molecular platelet aggregates (white clots) adhere to the polymer surface of dialyzers if

they were pre-treated with blood or plasma. 3. Haemodialysis using capillary dialyzers causes a regulation of HIT II antibodies thereby representing a preventive principle that protects patients against generalised severe thrombocytopenia with thrombotic reactions.

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THROMBOTIC EVENTS IN CHILDREN WITH ACUTE LYMPHOBLASTIC LEUKEMIA (BFM-PROTOCOLS): PREDNISONE VERSUS DEXAMETHASONE ADMINISTRATION

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Purpose: Recently published data suggest that the prothrombin G20210A variant, the TT677 methylenetetrahydrofolate reductase genotype, the factor V G1691A mutation, deficiencies of protein C, protein S, antithrombin and elevated lipoprotein (a) concentrations are associated with venous thromboembolism in paediatric patients treated according to the BFM-protocol using prednisone combined with E. coli asparaginase (CASP) during induction therapy. To assess whether the risk of vascular occlusions associated with established prothrombotic risk factors are additionally modified by the specific treatment modalities administered, e.g. the interaction between prednisone (P)/CASP or dexamthasone (D)/CASP the present matched-pair analysis in prospectively enrolled children with leukemia was performed.
Patients and Methods: 336 consecutively recruited leukemic children treated according to different BFM-protocols (P: 60 mg/m^2; n=280; D: 10 mg/m^2; n=56) were studied in this matched-pair analysis (5:1). Study endpoint was the onset of a symptomatic vascular accident.
Results: No significant difference was found in the prevalence rates of prothrombotic risk factors in the Caucasian populations studied (chi-square p=0.72). Symptomatic venous thromboembolism occurred in 31 P-treated patients (11.1%) compared with one stroke-like episode in the D-treatment group (1.8%; Fisher's exact p=0.02). In addition, the cumulative thrombosis-free survival was significantly reduced in children treated with P/CASP compared with children in the D/CASP arm (Figure: log-rank p=0.033).
Conclusions: The data presented here give evidence, that the use of prednisone concomitant with E. coli asparaginase administered in a paediatric leukemic patient suffering at least one of the aforementioned prothrombotic risk factors is clearly responsible for the symptomatic onset of venous thrombosis in the majority of cases.

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ANALYSIS OF PROTHROMBOTIC RISK FACTORS AND CLINICAL CONDITIONS IN CHILDREN SUFFERING A SECOND STROKE EVENT

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The present analysis was designed prospectively to evaluate the risk of recurrent ischaemic stroke (IS) in a cohort of 454 children with a first symptomatic stroke onset (neonates n=149; children >6 months of age n=305). The patients were followed for a median(range) of 44 (20-56) months to determine the frequency of recurrent IS. The estimated relative risk (RR) and 95% confidence interval (CI) to suffer from a second stroke event associated with prothrombotic risk factors (factor V (FV) G1691A mutation, the prothrombin (PT) G20210A variant, deficiencies of protein C, protein S, antithrombin, and lipoprotein (Lp) (a) >30 mg/dl or (kringle 4 repeats <28)) was determined concomitantly with potential underlying circumstances, i.e. cardiac diseases, cerebral vascular malformations, trauma, infectious or metabolic disorders (logistic regression model). Whereas in the neonatal group one infant (0.7%) with hyperhomocysteinemia suffered two early restroke episodes, in 19 of 301 children >6 months of age (6.3%) recurrent IS confirmed by MRT imaging was diagnosed with a median (range) of 4 (1-15) months following the first stroke onset (RR/95% CI: 11.0/1.5-81.8: patients >6 months). In addition, the RR/95% CI was significantly increased for Lp(a) carriers (4.4/1.9-10.5) and children with confirmed protein C deficiency (3.5/1.14-10.9). In contrast, the RR/95% CI was not significantly increased in carriers of the FV mutation (0.4/0.05-2.8), the PT variant (1.3/0.2-9.6), or protein S deficiency (2.9/0.8-11.3). No recurrent IS was observed in patients with antithrombin deficiency or antiphospholipid antibodies. Multivariate analysis confirmed increased Lp(a) and protein C deficiency as independent risk factors for recurrent IS. In addition, IS of vascular origin contributed significantly to the adverse outcome (OR/95%CI: 3.7/1.1-13.3), whereas the mode of anticoagulation used (none, aspirin, LMWH) did not influence the risk of a recurrent event (0.3/0.07-1.3). In conclusion, data shown here give evidence that re-stroke in neonates is rare, and that carriers of increased Lp(a) or protein C deficiency, as well as patients with stroke of vascular origin have an increased risk of recurrence if the first stroke onset have occurred later than six months of age.

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SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS) CATALOGUE OF THE FXIII GENE ESTABLISHED BY THE ANALYSIS OF 400 ALLELES WITH DHPLC TECHNOLOGY

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Introduction: Factor XIII (FXIII) is a plasma transglutaminase that circulates in blood as a tetramer (A2B2). It catalyzes the formation of covalent bounds between fibrin monomers, thus stabilizing the fibrin clot and increasing its resistence to fibrinolysis. FXIII A-subunit gene is located on chromosome 6p24-25 (2193bp cDNA) while B-subunit gene is situated on chromosome 1q31-32 (1923bp cDNA). The FXIII gene is known to be highly polymorphic. Some of these polymorphisms have shown some relevance for clinical practice. The Val34Leu variant within the FXIIIA might have a protective effect against myocardial and brain infarction, Tyr204Phe may be associated with recurrent miscarriage.
Patients, materials and methods: Two hundred german blood donors were subjected to molecular genetic analysis. All exons including the promotor region were amplified. Heteroduplexes of two donors were formed and loaded on the WAVETM DNA Fragment Analysis System (Transgenomics, San Jose, USA). The melting temperature of specific DNA sequence was choosen as recommended by Stanford Genome Technology Center (http://insertion. stanford.edu/ dhplc.html). Exons with abnormal patterns in the dHPLC analysis were sequenced using an automated sequencer system.
Results and discussion: A total of twenty-one SNPs could be found by our screening technique: seventeen in the coding region and four in the non-coding region. Eight of twenty-one SNPs are novel thus reported for the first time: three of them in FXIIIA (5' flanking region nt -246 G>A, Ala527Ala, Gly592Ser) and five in FXIIIB (Glu248Lys, Ile288Ile, Ile322Thr, Met503Ile, untranslated region TTTC A/G TTATT). The novel SNPs within the coding region were found at low allele frequencies of <5%. SNPs in non-codig region showed a high frequency (>15-50%). In addition we detected all common SNPs within the FXIIIA e.g. Val34Leu, Tyr204Phe, Pro564Leu, Glu567Glu, Val650Ile, Glu651Gln. Novel SNPs within the coding region could be of essential importance for clinical studies within patients cohorts suffering from cardio-, cerebrovascular and thromboembolic disorders which will be a subject of future studies.

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LARGE SCALE GENOTYPING IN PATIENTS WITH HAEMOPHILIA A – AN UPDATE ON 1200 PATIENTS

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The phenotype of haemophilia A is due to the deficiency or absence of coagulation factor VIII:C caused by a great number of heterogenous mutations within the large F8 gene (7.2 kb cDNA in 26 exons). The present project aims to determine the genotype in a substantial proportion of the about 6000 German haemophiliacs by means of high throughput mutation screening methods as DGGE or dHPLC. Based on this high number of patients various aspects on the pathogenesis of haemophilia A can be investigated.
So far about 1200 patients with severe and non-severe haemophilia A have been analysed. 34% of the patients showed an intron 22 inversion, 43% a point mutation (34% missense, 9% nonsense), 10% a small insertion/deletion, 3% a large deletion and 3% a splice site mutation. The causative mutation could not be identified in about 5% of the patients, of which half can even not be detected when sequencing the whole F8 cDNA (Graw et. al, pers. communication). Notably, more than 250 of the mutations found have not been described before, thus underlining the great variety of mutations in the F8 gene. Three mutation hot spots could be verified: i) the prevalent intron 22 inversion, ii) CpG sites which were the source for 35% of the point mutations and iii) two series of adenines in exon 14 that accounted for 25% of all small deletions/insertion. Missense mutations were distributed equally throughout the F8 gene with

the exception of exon 14. Especially in the middle part of exon 14 that comprises 30% of the F8 gene missense mutations were almost absent. We found a substantial proportion of de novo mutation arising as somatic mosaicisms which contradicts the current understanding of mutation development in haemophilia A and has great impact for genetic couseling. The type of the mutation has been shown to be a decisive influence factor for inhibitor formation in haemophilia treatment. So far, 10 different classes of mutations types could be defined with inhibitor prevalences ranging from 0% in splice site mutations to 88% in multi domain large deletions.

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***ALTERNATIVE ANTIKOAGULATION MIT NIEDERMOLEKULAREN HEPARINEN – ERGEBNISSE DER EASE-STUDIE**

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Hintergrund: Bei Patienten mit Herzklappenersatz und/oder Vorhofflimmern wird eine Unterbrechung der oralen Antikoagulation vor interventionellen Maßnahmen oder Operationen mit erhöhtem Blutungsrisiko empfohlen. Da in diesem Zeitraum das Thromboembolierisiko steigt, wird üblicherweise eine alternative kurzfristig steuerbare Antikoagulation mit unfraktioniertem Heparin empfohlen. Im Gegensatz zu unfraktioniertem Heparin haben niedermolekulare Heparine eine bessere Bioverfügbarkeit, eine längere Halbwertszeit, und der Gerinnungseffekt lässt sich besser vorhersagen. Ziel der prospektiven randomisierten Studie war es, die Effektivität der Antikoagulation unter unfraktioniertem und niedermolekularem Heparin zu vergleichen.
Methoden: Von Januar bis November 2001 wurden konsekutive Patienten mit oraler Antikoagulation bei Vorhofflimmern und/oder Klappenersatz und Indikation zu einer Herzkatheteruntersuchung in die Studie eingeschlossen. Nach stationärer Aufnahme wurde die orale Antikoagulation ausgesetzt. Die Patienten erhielten ab einem INR<2 randomisiert entweder gewichtsadaptiert Enoxaparin 2× täglich s.c. oder gewichtsadaptiert unfraktioniertes Heparin i.v.. Die Intervention erfolgte bei einem INR <1,5. Postinterventionell wurde die orale Antikoagulation wieder aufgenommen. Die aktivierte PTT (Ziel: 1,5–2,0× des Ausgangswertes) und die anti-Faktor-Xa–Aktivität (Ziel: 0,5–1,0 IU/ml) wurde täglich bestimmt. Primärer Endpunkt war die Zeit (Tage) bis zum Erreichen einer effektiven Antikoagulation unter dem jeweiligen Therapieregime. Sekundärer Endpunkt war der Prozentsatz der Tage der gesamten Behandlungsdauer unter ineffektiver Antikoagulation.
Statistik: Unter der Annahme, dass die Dauer bis zum Erreichen einer effektiven Antikoagulation im Mittel unter unfraktioniertem Heparin bei 3 Tagen, unter niedermolekularem Heparin dagegen bei 1,3 Tagen liegt, hat die Studie beim Einschluss von insgesamt 62 randomisierten Patienten eine statistische Power von 90% einen signifikanten Unterschied auf dem Niveau von α=0,05 mit dem Logrank-Test zu finden.
Ergebnisse: 32 Ptn wurden mit niedermolekularem und 36 mit unfraktioniertem Heparin behandelt. Der Zeitraum bis zum Erreichen einer effektiven Antikoagulation war in der Enoxaparin-Gruppe signifikant kürzer als in der unfraktionierten Heparin-Gruppe (1,2±0,5, gegen 2,9±2,4 Tage, P<0,0001). Der Prozentsatz der Tage unter ineffektiver Antikoagulation war in der Enoxaparin-Gruppe signifikant höher als in der unfraktionierten Heparin-Gruppe (6,6±9,6 gegen 45,4±27,4%, P<0,0001). Relevante Blutungen traten werden in der Enoxaparin behandelten noch in der unfraktionierten Heparin Gruppe auf.
Schlussfolgerung: Unsere Studiendaten zeigen, dass niedermolekulare unfraktionierten Heparinen in Bezug auf die Effektivität der Antikoagulation in der Umstellungsphase einer oralen Antikoagulation überlegen sind. Im Vergleich zu unfraktionierten kann mit niedermolekularen Heparinen schneller eine effektive Antikoagulation erreicht und auch eine anhaltend effektive Antikoagulation gewährleistet werden.

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***FAKTOR XIII – STRUCTURE AND FUNCTION AND IST ROLE IN INFLAMMATORY BOWEL DISEASE**

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Factor XIII is a transglutaminase circulating in blood as an inactive tetramer consisiting of two a and two b subunits. Factor XIII becomes activated by thrombin and is then able to form peptide bonds between glutamin and lysin residues. Its main substrate is fibrin. Through the action of Factor XIII the initially weak fibrin clot is transfomed into a stable three-dimensional network by the introduction of bonds between alpha and gamma chains of fibrin respectively. Furthermore via the incorporation of antiplasmin, which serves also as a substrate for FXIII the clot acquires resistance to fibrinolysis. Besides its role in coagulation it has been proposed that factor XIII may have other functions as well. A wide range of substrates are known to exist, among them are fibronectin and collagen. Their interaction with factor XIII may be of importance for wound healing and tissue remodelling. Based on these considerations it has been proposed that factor XIII may be useful in treating patients with chronic inflammatory bowel disease (CIBD). In an acute bleeding episode factor XIII may be helpful in stopping the bleeding by establishing firm fibrin clots and by facilitating the healing process of the inflamed mucosa. While data from phase II trials on the treatment of patients with CIBD with factor XIII were promising, a phase III trial had to be terminated prematurely because of insufficient patient accrual, a substantial effect of factor XIII in those patients treated so far could not be detected. Recently we observed decreased plasma levels of factor XIII in patients with graft versus host disease (GvHD) of the bowel following allogeneic stem cell transplantation. The decrease was related to the degree of GvHD and was readily reversed in those patients in whom GvHD could be succesfully treated by steroids. Thus a role for factor XIII in GvHD of the gut is proposed and the investigation of factor XIII as part of the treatment of these severely ill patients seems to be justified.

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***TISSUE FACTOR IN ACUTE MYOCARDIAL INFARCTION**

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Thrombosis on disrupted atherosclerotic lesions is a pivotal event in the pathogenesis of acute myocardial infarction (AMI). Systemic factors contribute to alterations in the coagulation pathway and may play a role in the initiation and propagation of thrombosis.
In AMI monocyte procoagulant activity is increased by up-regulation of Tissue Factor (TF). Elevated TF expression was associated with an increase in systemic inflammatory mediators interleukin-6 or C-reactive Protein, that constitute a strong stimuli for TF expression in monocytes. As a functional correlate mononuclear cells of patients with AMI generated significantly higher amounts of FXa compared to patients with stable angina. These in vitro findings corresponded to an increased thrombin formation in patients with AMI that was demonstrated by elevated plasma concentrations of prothrombin fragments F_{1+2}. Contrary to our findings on TF, we did not observe upregulation of the endogenous inhibitor Tissue Factor Pathway Inhibitor-1 (TFPI-1) on circulating monocytes during reperfusion in AMI. However, the constitutive expression of TFPI-1 on the monocytic surface was sufficient to partially inhibit induced TF activity after AMI. In endothelial cells, we recently described quaternary complex formation of GPI-anchored TFPI-1 with the TF-FVIIa-FXa-assembly and subsequent translocation in caveolae as a major mechanism of TF-inhibition by TFPI-1. The regulation of monocytic TF by TFPI-1 found in AMI exhibited central characteristics of this mechanism. Removal of TFPI-1 from the monocyte surface by Phospholipase C confirmed, that large amounts of surface-bound TFPI-1 were GPI-anchored. The importance of GPI-anchored TFPI-1 was corroborated by the inability of exogenously added recombinant TFPI-1 to further inhibit TF activity.
The increased procoagulant activity in AMI is a result of elevated monocytic TF activity which is only partially inhibited by endogenous, monocytic GPI-anchored TFPI-1. Anticoagulant therapy by direct inhibition of TF activity may, thus, be particularly effective in AMI.

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PROTEOLYSIS OF TISSUE FACTOR PATHWAY INHIBITOR-1 BY THROMBOLYSIS IN ACUTE MYOCARDIAL INFARCTION

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In acute myocardial infarction (AMI), surface-bound TFPI-1 inhibits an increased monocyte procoagulant activity. In addition, TFPI-1 is released from microvascular endothelial cells after treatment with heparin and thereby contributes to its antithrombotic properties.
We examined 19 patients of a randomized study comparing intravenous fibrinolysis with alteplase (n=9) and revascularization by stent placement (n=10). Before and after therapy we obtained blood samples for analysis of monocytic TFPI-1 surface expression by flow cytometry and plasma TFPI-1 concentrations by immunoassay.
We found a significant decrease in surface TFPI-1 on circulating monocytes 24 hours after thrombolysis (P=0.006) that was not observed after stenting. Systemic plasma TFPI-1 concentrations increased immediately after stenting by 71±14% (P=0.008) whereas after thrombolysis, a decrease in TFPI-1 plasma concentrations by 21±11% was observed (P=0.075). In vitro experiments confirmed that plasmin decreased TFPI-1 surface expression dose-dependently. Activation of the fibrinolytic system by alteplase in AMI decreases surface-associated TFPI-1 on circulating monocytes and plasma TFPI-1. Reduced TFPI-1 may contribute to thrombotic complications after fibrinolysis in AMI.

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OVEREXPRESSION OF GLYCOSYL PHOSPHATIDYLINOSITOL-ANCHORED TISSUE FACTOR PATHWAY INHIBITOR-1 INHIBITS TISSUE FACTOR ACTIVITY IN ECV304 CELLS

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Cellular initiation of coagulation by the tissue factor (TF)-Factor VIIa (FVIIa) complex is inhibited by endogenous TFPI-1, whereas exogenously added TFPI-1 is targeted to a degradation pathway. This study investigates the relevance of glycosyl phosphatidylinositol (GPI)-anchoring to the anticoagulant properties of TFPI-1. For GPI-anchoring of TFPI-1 we used the GPI-attachment sequence of decay-accelerating factor (GPI-TFPI-1), and compared it with wildtype TFPI-1. After transfection of GPI-TFPI-1 surface expression of TFPI-1 increased to 134±9% of mock transfected cells (mean±SEM, P=0.004) and TF activity was reduced by 18±9% compared with mock transfections (P=0.004). No changes in TFPI-1 surface expression or TF activity were observed after transfection with TFPI-1 wildtype. The effect of GPI-TFPI-1 was not due to altered TF expression. GPI-anchoring is an essential prerequisite for surface expression of TFPI-1 and inhibition of TF activity. Gene transfer of GPI-anchored TFPI, therefore, may be an efficient tool to inhibit local TF-induced coagulation.

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HEPSIN EXPRESSION IS NOT INFLUENCED BY PROINFLAMMATORY CYTOKINES

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Background: Blood coagulation is initiated by complex formation between factor VIIa (FVIIa) and its cellular cofactor tissue factor (TF). Activation of this pathway requires minute amounts of FVIIa. At present the mechanisms which are involved in the generation of basal levels of FVIIa are still unclear. One possibility is the activation of FVII by cell-bound proteases. Hepsin is a possible candidate enzyme, since it has been shown that cells expressing hepsin demonstrate a TF-independent procoagulant activity. In the present study the influence of proinflammatoric cytokines on hepsin expression levels has been investigated in different target cells.
Methods: Hepsin expression was analyzed by Northern-blotting and RT-PCR-technique in endothelial cells (HUVEC), in the monocytic cell line THP-1, in primary human monocytes and in the hepatoma cell line HepG2. Total RNA was isolated at time points 0, 2, 4 , 8, 24 hours. Cells were stimulated with 40 ng/ml TNF alpha, 20 ng/ml IL-6, and 100 ng/ml IL-8. For Northern-blotting a full-length hepsin cDNA labelled with 32P-dCTP was used. The house keeping enzymes GAPDH and ß-actin were used for normalization. In addition, TF-expression was studied for all cell types and time points tested.
Results: Hepsin expression was detectable exclusively in HepG2 cells. There were no transcripts detectable in endothelial cells and monocytes. Expression levels of hepsin were found constant in HepG2 cells over a time period of 24 hours. Stimulation with cytokines has no influence on the hepsin-mRNA levels. In contrast, TF-expression was significantly upregulated in all cell types.
Conclusion: Our data indicate that hepsin is constitutively expressed in hepatic cells. Hepsin expression is not up- or downregulated by cytokines. These findings are in accordance with the hypothesis that hepsin is involved in low grade generation of FVIIa.

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EXPRESSION OF PROTEASE-ACTIVATED RECEPTORS IN HUMAN SAPHENOUS VEINS

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Aim of the study: The receptors of human saphenous veins for endogenous agonists are of special interest because saphenous veins are used for bypass grafts. The frequency of venous thrombosis appears to be increased in patients with varicosis. In patients with primary varicose veins of the lower legs this may lead to changing in the thrombin receptor sensitivity and expression. The aim of the present study was to evaluate the expression of thrombin- and other protease-activated receptors (PAR-1, -2, -3, -4) in human varicose saphenous veins.
Methods: The varicose saphenous veins were obtained from patients 21 to 65 years of age after saphenectomy. Total RNA was isolated from the veins by using TRIzol reagent. cDNA was produced from these RNA samples by RT-PCR of 2.5 µg total RNA. The cDNA encoding the four PARs were amplified by using PCR. Control PCR was performed without RT or with the primers for the housekeeping gene GAPDH. For each cDNA sample the CT values of GADPH and target were determined. Immunohistology was performed using the strepavidin-biotin alkaline phosphatase method.
Results: Varying degree of intimal thickening was seen in the varicose veins. PAR-1 immunoreactivity was weak in the endothelium and in the part of the intima underneath as well as moderate in the media. PAR-2 staining was rather strong in the intima and media. No PAR-1 and PAR-2 staining was found in the adventitia. Western blots confirmed the presence of PAR-2. The veins showed a weak staining for PAR-3 in the endothelium/intima and media. For PAR-4, no staining in the veins was detected. We examined the PARs in human varicose saphenous veins by RT-PCR and were able to detect the RNAs corresponding to PAR-1, PAR-2, and PAR-3. All products were of expected length and no interfering bands were visible.
Conclusion: The protease-activated receptors (1-3) are present on endothelium and in the media in varicose veins. PAR-2, the trypsin and tryptase-sensitive receptor displays the strongest expression.

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DIFFERENT EFFECTS OF UNFRACTIONATED HEPARIN, LOW MOLECULAR WEIGHT HEPARIN AND PENTASACCHARID (FONDAPARINUX) ON FACTOR XA- AND THROMBIN-INDUCED ERK-1/2 ACTIVATION AND MITOGENESIS IN HUMAN VASCULAR SMOOTH MUSCLE CELLS

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Background: The synthetic pentasaccharide Fondaparinux (FPA) is the first selective inhibitor of factor Xa (FXa). FPA, unfractionated (UFH) and low molecular weight (LMWH) heparin all mediate their anticoagulant actions via binding and activation of antithrombin. UFH and LMWH have also been shown to suppress the proliferation of vascular smooth muscle cells (SMC). In most in vitro experiments, the inhibitory potency of heparin was examined after stimulation of these cells by serum or growth factors, like PDGF or bFGF. The present study investigates the influence of UFH (Liquemin(TM)), LMWH (Enoxaparin) and FPA on the activation of extracellular signal-regulated kinases (ERK-1/2) and DNA synthesis in human SMC after stimulation by FXa or thrombin.
Methods: Arterial and venous SMC were isolated by the explant technique from the media of human saphenous vein and internal mammary artery, respectively, from patients undergoing aortocoronary bypass surgery. Activation of ERK-1/2 was detected in cell lysates by Western blotting. Mitogenic effects were assessed by measuring [3H]-thymidine incorporation into cellular DNA.
Results: After pretreatment of the SMC with Liquemin(TM) (1-10 U/ml) or Enoxaparin (3-100 µg/ml) for 30 min., FXa- and thrombin-induced ERK-1/2 phosphorylation was inhibited concentration-dependently. Incubation of the SMC with FXa (100 nM) or thrombin (1 U/ml) for 24 hours resulted in a 3-5 fold increase in DNA synthesis. In the presence of Liquemin(TM) or Enoxaparin, both thrombin- and FXa-induced mitogenesis were significantly reduced. In contrast to the heparins, FPA (1-10 Anti-XaU/ml) appeared not to affect DNA synthesis and phosphorylation of ERK-1/2 significantly.
Conclusions: Inhibition of ERK-1/2 activation and mitogenesis by UFH and LMWH demonstrates their interference with the mitogenic signaling and confirms a correlation between ERK-1/2 activation and mitogenesis. The failure of FPA to interfere with these mechanisms is novel and requires further studies to elucidate the mechanisms behind.

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DETERMINATION OF PLATELET AGGREGATION AFTER IN VITRO TREATMENT WITH ASA OR REOPRO® BY USING AN AGGREGOMETRIC (BCT®) AND A CENTRIFUGAL (BCS®) ANALYZER

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Background: Generally platelet aggregation measurements are performed on so called aggregometers. Since these specialized instruments are not fully automated and not always available in the clinical laboratory we developed assay protocols on the coagulation analyzers BCT® and BCS®. The effects of platelet function inhibitors (ASA, ReoPro®) on the aggregation response to different agonists were investigated to test the suitability of these instruments for platelet aggregation.

Methods: Both BCT® and BCS® (Dade Behring) are turbidimetric, fully automated coagulation analyzers. The BCT allows permanent stirring during the measurement and therefore is very similar to an aggregometer. The BCS® is a centrifugal-type analyzer rotating a cuvette-containing rotor while measuring the absorbance in the reaction mixture. To start the reaction 15 µl of activator containing solution are added to 135 µl of platelet rich sample plasma.
Results: The platelet aggregation patterns obtained on the BCT are very similar to those known from typical aggregometric measurements. In contrast the reaction kinetics on the BCS® are much more linear due to sedimentation of platelets and non-stirring conditions. As a result phase II of the aggregation response to e.g. Epinephrine cannot be distinguished from phase I. Nevertheless, evaluated as Vmax (maximum absorbance change per minute) the aggregation responses of ASA and ReoPro® treated platelets are very similar both on the BCT® and the BCS®. The inhibitory effect of ASA on platelet aggregation is most pronounced using Epinephrine and Arachidonic Acid as agonists whereas the maximum effect of ReoPro® is shown utilising ADP and Epinephrine.
Conclusions: The performance of platelet aggregation measurements employing the coagulation instruments BCT® and BCS® is practicable and might be an alternative to the use of conventional aggregometers. Comparative usage of the two platelet activators ADP and Arachidonic acid may help to distinguish inhibitory effects caused by ASA or ReoPro®.

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***GENETIC AND ENVIRONMENTAL INFLUENCES ON LEVELS OF THE PROTHROMBOTIC PLASMA PROTEIN THROMBIN-ACTIVATABLE FIBRINOLYSIS INHIBITOR (TAFI) IN MONO- AND DIZYGOTIC TWINS**

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TAFI is a recently described plasma zymogen (Procarboxypeptidase U), which potently attenuates fibrinolysis. The active form of TAFI (TAFIa) is generated by the thrombin-thrombomodulin complex on the endothelial surface. TAFIa acts by removing carboxyterminal lysine residues from partially degraded fibrin, resulting in decreased binding of plasmin to fibrin and an impaired fibrinolytic activity shown by a prolonged clot lysis time.
Elevated TAFI levels are a mild risk factor for venous thrombosis (odds ratio: 1.7, 95%-CI: 1.1-2.5) and decreased TAFI levels are reported as a protective factor (OR: 0.1; 95%-CI: 0.02-0.9). In contrast, study results for arterial thrombotic events are not that sound. In one study patients with stable angina showed elevated TAFI levels compared to controls. In another study patients with acute myocardial infarction (AMI) showed decreased TAFI levels, which can be also confirmed by our own data in patients with unstable angina and AMI.
Several single nucleotide polymorphisms (SNPs) of the 5′-regulatory region of the TAFI gene are correlated to TAFI levels. However, little is known about the influence of environmental factors on TAFI levels. We studied the influence of both factors, genetic and environmental, by investigating the plasma levels of TAFI and other fibrinolytic plasma proteins (PAI-1, tPA, APP, D-Dimer) in 91 pairs of mono- and 84 pairs of dizygotic twins. Plasma concentration of the proteins did not differ significantly between both groups. However, variation within the individual twin pairs showed significant differences for TAFI, tPA and D-Dimer and a trend for PAI-1 between mono- and dizygotic pairs. Lowest variation was observed for TAFI in monozygotic twin pairs and highest also for TAFI in dizygotic twin pairs (median CV: 8.1% (MZ) vs. 42.3% (DZ), p=0,002) followed by D-Dimer (23% (MZ) vs. 31.4% (DZ), p=0,01), tPA (33.1% (MZ) vs. 42.3% (DZ), p=0.01) and PAI-1 (12.6% (MZ) vs. 17.4% (DZ), p=0.18), APP results did not differ significantly (14.1% (MZ) vs. 15.8% (DZ), p=0.42). In 26 subjects (mono- and dizygotic) a 6-month follow-up sample was available. Intraindividual variation of plasma concentration was again lowest for TAFI (median CV: 12.4%) comparable to the interindividual variation in the monozygotic twin pairs.
TAFI levels seem to be strongly genetically controlled. An influence of environmental factors could not be observed because variations in monozygotic twins were only within the range of the assay variation.

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A NOVEL NO-INDEPENDENT GUANYLYL CYCLASE STIMULATOR, BAY 41-8543, AND ITS ACTION ON PLATELET FUNCTION

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Soluble guanylyl cyclase (sGC) catalyses the biosynthesis of cGMP from GTP. NO is a potent activator of sGC. Activation of the enzyme facilitates conversion of GTP to the intracellular second messenger cGMP. By formation of cGMP as a second messenger, sGC plays an important role in the cardiovascular system, e.g. in regulating vascular tone and platelet function. BAY 41-8543 is a novel, highly specific and potent NO-independent sGC stimulator. This study investigates the in vitro effects of BAY 41-8543 in human platelets and its antithrombotic effects in rats. BAY 41-8543 concentration-dependently increased (0.1-30 µM) 1.7-fold - 26-fold cGMP levels. This activation resulted in a potent inhibition of collagen-induced platelet aggregation (IC_{50}=0.095 µM) and aggregation mediated by the thromboxane A2 mimic U 46619 (IC_{50}=0.76 µM) in washed platelets. Thrombin or TRAP-6 (thrombin receptor agonist)-mediated aggregation was only weakly effected with an IC_{50}=17 µM or 7 µM, respectively. In platelet rich plasma the effect was less pronounced. BAY 41-8543 inhibited platelet aggregation induced by collagen (IC_{50}=5.7 µM) and ADP (IC50=12 µM) but did not effect aggregation induced by TRAP-6 (IC_{50}>24 µM). To determine whether the antiplatelet effects observed in vitro also occur in vivo, we examined the effects of BAY 41-8543 on the tail bleeding time and thrombus development in the $FeCl_3$ thrombosis model in rats. A doubling of bleeding time was observed after 1.0 mg/kg p.o. (p<0.001). BAY 41-8543 displayed an antithrombotic effect after 3 mg/kg p.o.. Thrombus mass was reduced by 59±17% (p<0.001). These results demonstrate that activation of sGC results in a potent antiplatelet activity, and BAY 41-8543 may offer an new approach for treating cardiovascular diseases including thrombotic disorders.

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CONSTRUCTION OF A NEW FIBRINOLYTIC USING THE SINGLE-CHAIN-ANTIBODY TECHNOLOGY

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Fatal bleeding complications are the major adverse effects of thrombolytic therapy. Fibrin targeting may allow a lower systemic concentration of fibrinolytics and thus bleeding complications may be reduced. We describe the construction and functional evaluation of a recombinant plasminogen activator consisting of a single-chain antibody fragment, that is specific for the amino terminus of the fibrin b-chain (Bb15-22, also termed b-peptide), and a low-molecular-weight form (residues leu144 – leu 411) of single-chain urokinase-type plasminogen activator (scuPA). The variable regions of the heavy and light chains of the fibrin-specific antibody were amplified by polymerase chain reaction (PCR) using hybridoma DNA as template. The cDNA of scuPA was produced by reverse transcription of endothelial (ECV) cell mRNA, amplified by PCR and genetically fused to the C-terminus of the variable region of the light chain. The fusion protein was produced in E. coli, purified by affinity chromatography and characterized by its size on SDS-Page (56 kDa), by western blotting and by its binding to b-peptide. The proteolytic function of the new construct was evaluated by cleavage of the chromogenic substrate S2444. An urokinase activity of 14 000 IU/mg could be determined. Both, fibrin-targeting and plasminogen activation could be demonstrated in a S2444 assay on immobilized b-peptide in comparison to an unspecific control peptide (P<0.001). Finally, the ability of the designed fusion protein to lyse clots was tested. In direct comparison to equimolar amounts of native urokinase a 6-8 fold (P<0.001) higher efficiency in the lysis of 125I-labeled fibrin clots in human plasma could be demonstrated.
Conclusion: The newly designed fusion protein demonstrates a fibrin targeted plasminogen activation and is highly efficient in clot lysis. Thus, an efficient clot lysis with a low risk of bleeding complications may be achieved. The efficient and fast production at low cost should facilitate the further evaluation and potential clinical use of this new fibrinolytic.

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PLATELET SURFACE EPITOPES IN PATIENTS FOLLOWING HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Patients following hematopoietic stem cell transplantation (HSCT) may have an increased bleeding tendency in spite of a normal platelet count. On the other hand, an association between chronic graft versus host disease (cGvHD) and a thrombophilic state has been observed even in thrombocytopenic patients. We suspect a central role of platelets in these transplantation-associated hemostatic complications.
We evaluated 27 long-term survivors of HSCT (13 without cGVHD, 14 with cGVHD) by flow cytometric analysis of the platelet surface receptors GPIa/IIa, GPIIb/IIIa and GPIb/V/IX, markers of activated GPIIb/IIIa receptor (PAC-1, LIBS-1) and surface expression of the granula markers P-selectin and GP53. Mepacrine staining was performed to quantify the dense bodies. The two HSCT groups did not differ concerning their medication (aciclovir, ketoconazole, cyclosporine A, mycophenolic acid, steroids).
Patients with cGVHD have a significantly lowered surface expression of GP Ib and GP IIa, whereas GP IIb surface antigen is within the normal range. HSCT patients without cGVHD have significantly lower GP IIa levels, whereas PAC-1 surface binding is elevated in this group when compared to the controls. All HSCT patients have elevated levels of LIBS-1 surface expression and a low mepacrine staining indicating fewer dense bodies than normals. In HSCT patients without cGVHD a preexisting degranulation of lysosomes occurred. Patients in the cGvHD group had a 7fold increased, HSCT patients without long-term complications a 2fold increased ratio of microparticles over the normal baseline value.
The observed platelet receptor defects and the in-vivo activation of platelets might be the consequence of disturbed platelet production and immunological processes. The role of inflammation is underscored by the high levels of circulating microparticles in cGVHD patients and might explain the thrombophilic state in cGDVH patients.

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HEMOSTATIC COMPLICATIONS IN ALLOGENEIC BONE MARROW TRANSPLANTATION: A RETROSPECTIVE ANALYSIS OF 447 PATIENTS

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Hemostatic disturbances represent a frequent problem in patients undergoing allogeneic bone marrow transplantation and are closely related to the most important severe complications graft-versus host disease (GvHD), sepsis, hepatic veno-occlusive disease (VOD) and microangiopathic hemolytic anemia (MAHA). We analyzed the history of allogeneic bone marrow recipients in order to determine type, frequency, severity and manifestation time of hemorrhagic or thrombotic events.
447 Patients (274 allogeneic/related, 90 allogeneic/unrelated, 78 autologous, 5 syngeneic transplants) were evaluated retrospectively for the presence of hemostatic complications (bleeding, thrombosis, VOD, MAHA) beginning from start of the conditioning. In addition, degree of acute and chronic GvHD, immunosupressive medication and coagulation laboratory test (PT, aPTT, fibrinogen, antithrombin, platelet count) were registered.
83.2% of the patients presented with at least one either hemorragic or thrombotic complication during the investigational period. Whereas most bleeding episodes were within the first 4 weeks of transplantation and less severe (71.9%), major hemorrhage occurred in 27.1%. 1.1% of the events were fatal gastrointestinal or intracerebral hemorrhage and happened after the fourth week of transplantation in 92.3%. Bleeding was strongly associated with acute or chronic GvHD, hemorrhagic cystitis additionally with cyclophosphamide treatment.
Thromboembolic events like extremity thrombosis or pulmonary embolism occurred in 9.5% of the patients, were especially frequent in allogeneic transplanted patients with an incidence of 14.6% and strongly associated with acute GvHD and uptake of steroids or cyclosporine A. Hepatic veno-cclusive disease occurred in 4.7% of allogeneic transplant recipients and was associated with a high rate of fatal outcome. Busulfan conditioning increased the VOD risk 2.6fold. MAHA occurred in 14.6% of allogeneic transplant recipients, leads to an increased overall mortality and is associated with acute GvHD.
The data of this study demonstrate the enormous impact of hemostatic complications on the course of allogeneic bone marrow transplantation and the narrow interactions between hemostasis and immune system, that may cause lethal complications in the course of transplantation and thereby strongly influence morbidity and mortality of patients following hematopietic stem cell transplantation.

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ACTIVATED INTEGRIN ALPHA VBETA3 IS REQUIRED FOR TUMOR CELL ARREST UNDER DANYMIC FLOW CONDITIONS

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During hematogenous metastasis, shear resistant arrest of circulating tumor cells can be mediated by tumor cell integrin alphaVbeta3 and involves plasma proteins immobilized on activated platelets. We used video epifluorescence microscopy in order to analyse human M21 melanoma cell arrest on fibrinogen (Fg), von Willebrand Factor (VWF) and fibronectin (FN) matrices under flow in a buffer perfusion system. M21 cells that came in contact with immobilized Fg, VWF or FN during perfusion at venous flow, arrested abruptly and began to spread immediately. Cell attachment was resistant to increasing wall shear rates, including arterial levels. M21 cell arrest on Fg, VWF and FN was mediated by alphaVbeta3: the alphaV integrin-lacking M21-L cells failed to arrest to either ligand, but arrest was fully restored in M21-L4 cells that were transfected to express alphaVbeta3; M21 cell arrest was inhibited by anti-alphaVbeta3 mAb (inhibition: >95% on Fg, >90% on VWF and >75% on FN) and completely blocked by RGD peptide. Under static conditions, alphaVbeta3 was the only receptor that mediated M21 cell adhesion to Fg and VWF, but cell contact with FN during stasis recruited the integrins alpha5beta1 and alphaVbeta1 in addition to alphaVbeta3. In contrast, under dynamic flow conditions, beta1 integrins did not participate in melanoma cell arrest on FN. This is uniquely mediated by alphaVbeta3. To support tumor cell arrest during flow, integrin alphaVbeta3 had to be activated: arrest was measurable in the presence of Mn2+ but not Ca2+. Treatment with Mn2+ failed to induce M21 cell adhesion when actin polymerisation was inhibited with Cytochalasin D. Even though beta1 integrins were also activated by Mn2+ (enhanced M21-L cell adhesion on FN under static conditions), activated beta1 integrins did not contribute to tumor cell arrest in the presence of shear forces (Mn2+ treated M21-L cells did not arrest on FN under flow). During flow, M21 cell arrest on a Fg matrix was enhanced by soluble Fg, which served as cross linking ligand between attached and circulating tumor cells. Activated integrin alphaVbeta3, in contrast to beta1 integrins, is required for shear resistant M21 cell arrest. This specific ability of alphaVbeta3 functionally involves the cytosceleton and is enhanced by soluble plasma proteins. The presence of alphaVbeta3 in an activated state may provide a unique advantage for circulating tumor cells by promoting their arrest under blood flow conditions.

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THE 23 BP INSERTION IN THE ENDOTHELIAL CELL PROTEIN C RECEPTOR (EPCR) GENE IN PATIENTS WITH ISCHAEMIC STROKE

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Background: Apart from thrombomodulin, a second endothelial cell specific transmembrane protein, termed the endothelial cell protein C receptor (EPCR), is involved in the protein C anticoagulant system. Biguzzi et al. (2001) recently identified a 23 bp insertion in exon 3 of the EPCR gene in 4/198 patients with premature myocardial infarction (MI) and 3/194 patients with deep vein thrombosis (DVT) but also in 4/575 healthy individuals. Functional studies suggest that patients carrying the 23 bp insertion should have a diminished capacity for activating protein C, thus this insertion might predispose for thrombotic events.
Patients: A total of 99 patients presenting with ischaemic stroke were included in this study.
Methods: Genomic DNA was extracted from peripheral blood, and exon 3 of the EPCR gene including intron-exon boundaries was amplified. PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.
Results: Of the 99 patients under investigation, 98 showed only the PCR fragment characteristic for the wildtype sequence. One patient besides the wildtype fragment displayed an additional PCR product of an approximately 20 bp larger size, indicating the insertion mutation.
Conclusions: The 23 bp insertion in the EPCR gene seems to be a rare event in patients with ischaemic stroke. Nevertheless, in vitro studies indicate that this EPCR gene mutation is a candidate risk factor for venous and/or arterial thrombosis. Additional studies especially in families of carriers of this mutation are required to establish the clinical importance.
Reference: Biguzzi E et al. Thromb Haemost 2001; 86:945-948

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COAGUTCHEK® S INSTRUMENT PERFORMANCE: METER-TO-METER VARIABILITY

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Background: In Germany the concept of patient self management (PSM) of oral anticoagulation therapy (OAT) has been successfully implemented. The vast majority of patients performing PSM are using the CoaguChek®/CoaguChek® S system for determining their INR values at home. The CoaguChek® S system was introduced in early 2000 and replaces the CoaguChek® system. It offers reduced size and weight, improved software features and better access to the sampling port. Here we present data on meter-to-meter variability internally determined at Roche Diagnostics during product release testing.
Method: Samples are taken from every 160 instruments during meter manufacturing. In release testing 10 meters are compared with a qualified reference meter. 10 replicates are determined on each meter using CoaguChek® Quality Control solution. The relative deviation of the mean result in seconds against the mean on the reference meter is calculated for each meter.
Results: The distribution of relative deviations from the reference meter is given for 657 instruments tested in the time period between May 1 and August 14, 2001. They represent a total manufacturing output of approximately 7000 CoaguChek® S instruments. More than 99% of the meters are within 3% from the reference meter. These data in seconds correspond to a meter-to-meter coefficient of variation (CV) of approximately 1.5% in INR.
Conclusion: The CoaguChek® S instruments show a very low meter-to-meter variability. A similar magnitude of the meter-to-meter CV was found in the CoaguChek® S evaluation study using venous blood as sample material (CV=1.1%, 24 meters. Data presented at GTH congress 2000, Freiburg).

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COMPARISON OF INR RESULTS OF THE COAGUCHEK® S PT AND COAGUCHEK® PRO PTN TEST WITH INTERNATIONAL REFERENCE THROMBOPLASTINS

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Background: The CoaguChek® S and the CoaguChek® Pro system (Roche Diagnostics, Mannheim) are designed for near patient testing (NPT) in coagulation. To give INR results, both systems are calibrated according to WHO recommendations: A master lot of test strips or cartridges, respectively, is standardized against an international reference preparation (IRP), and used as the manufacturer's in-house standard. The subsequent production lots are calibrated against the master lots. In this study the agreement of INR results of a routinely manufactured CoaguChek® PT test "mini" strip lot (#218) and of a sublot of the CoaguChek® Pro PTN reference lot (#200076-05) with the IRPs CRM149S and rTF/95 was investigated.
Method: A total of 184 samples were collected at two study sites (Zwolle, NL, and Indianapolis, USA). PT tests were performed using venous whole blood for the NPT systems and using the corresponding citrate plasma for the IRPs. INRs were recorded as displayed by the NPT systems, or calculated for the IRPs, respectively, using the assigned ISI and the locally determined MNPT. Agreement of results was assessed according to the methods of Passing-Bablok (PB) and Bland-Altman (BA). 95% confidence intervals (CI) are given. Additionally the mean absolute relative deviation (MRD) and the coefficient of correlation r were calculated.
Results:

CoaguChek® versus

	r	Slope (PB; 95% CI)	Bias ((BA; 95% CI)	MRD
CRM149S	0.956	0.99 (0.95–1.03);	–0.08** (-0.13- -0.03)	10.5%
RTF/95	0.932	1.03 (0.98–1.08)	–0.07** (-0.13- -0.01)	11.8%

CoaguChek® Pro PTN versus

	r	Slope (PB; 95% CI)	Bias ((BA; 95% CI)	MRD
CRM149S	0.951	1.02 (0.98–1.06	0.004 (-0.05-0.06)	10.6%
RTF/95	0.935	1.09* (1.04–1.14	0.02 (-0.05-0.08	11.2%

*/**: statistically significantly different from *=1, or **=0

No trends could be detected over the measured INR range in the Bland-Altman-Plots. Scatter of data points increases with increasing INR values.
Conclusions: Clinically, the level of the INR values measured by the NPT systems fully agrees with the level of the INR values determined with the IRPs CRM149S and rTF/95. All bias are found <0.1 INR. These results prove the reliability of the manufacturer's calibration process.

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***QUALITY ASSURANCE IN HEMOPHILIA A THERAPY: A PHARMACOVIGILANCE EVALUATION WITH REFACTO® IN GERMANY – 3 YEARS OF CLINICAL EXPERIENCE**

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A Pharmacovigilance evaluation (PE) is a tool to access the therapeutic efficacy, tolerance and safety of a pharmaceutical product in usual care settings after marketing authorization. It also supports physicians in their obligation to monitor therapy and their commitment to drug safety monitoring.
In Germany a PE with ReFacto® was initiated immediately after launch of this first albumin-free formulated recombinant FVIII-product for prophylaxis and treatment of hemophilia A in 1999. The PE baseline documentation comprises the following parameters: medical history, prior treatment, concomitant treatment, concomitant medication and patient symptoms before inclusion. In the follow up evaluations concomitant treatment, dosage, investigator and patient assessment of safety and efficacy, adverse events, laboratory results and subjective health reporting are obtained. A component of this PE are anonymized data from the patient diaries. These diaries are an obligatory component of the treatment according to the "German transfusion law". Additional information about treatment regimen, FVIII consumption, bleeding episodes and concomitant medication can be derived.
Data are available today for 30 months of ReFacto® PE. 136 Patients with severe hemophilia A from 27 centers are included. Patients of the PE were classified as "prophylactic-" and "on-demand-patients" as follows: Routine prophylaxis was defined as at least two consecutive substitutions without occurrence of bleeding within a four-week-interval combined with at least eight substitution within this four-week-interval. On demand treatment was defined as at maximum one substitution without occurrence of a bleeding within the four-week-interval or less than eight substitutions within the four-week-interval. When treatment scheme changed, we defined the very therapeutic scheme, that was applied in 75% of the intervals and assigned the patient to that group. For patients, whose individual treatment still did not fit into one of these groups, we defined a third group. According to this, 81 patients can be classified clearly. 67 patients receive a prophylactic treatment scheme whereas 14 patients are treated on-demand. 56 patients are still under evaluation. A much higher FVIII consumption has been documented under prophylaxis treatment scheme which has to be related to a lower bleeding tendency under a prophylaxis treatment and to long-term prevention of hemophilic arthropathy. Taken together these data indicate, that ReFacto® is a safe and efficient option in Hemophilia A therapy.

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COMPARISON OF PROPHYLAXIS- AND ON-DEMAND TREATMENT: EVALUATION DERIVED FROM THE GERMAN PHARMACOVIGILANCE EVALUATION WITH REFACTO®

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A German pharmacovigilance evaluation (PE) with ReFacto was initiated immediately after launch. This PE serves as a monitoring tool and as an instrument to evaluate the safety and efficacy of the treatment with ReFacto under routine therapeutic conditions in Germany. Data are available now for 30 months of ReFacto PE. 136 Patients with severe hemophilia A from 27 centers are included. We classified patients of the PE in-

to “prophylactic-” and “on-demand-patients:” routine prophylaxis was defined as at least two consecutive substitutions without occurrence of bleeding within a four-week-interval combined with at least eight substitution within this four-week-interval. On demand treatment was defined as at maximum one substitution without occurrence of a bleeding within the four-week-interval or less than eight substitutions within the four-week-interval. When treatment scheme changed, we defined that therapeutic scheme, which was applied in 75% of the intervals and assigned the patient to that group. For patients, whose individual treatment still did not fit into one of these groups, we defined a third group. According to this, 81 patients could be clearly classified into 67 patients with a prophylactic treatment scheme and 14 patients with an on-demand treatment scheme. 56 patients are still under evaluation. With respect to the treatment schemes, the 67 patients receiving a prophylactic treatment had 0.4 bleeding episodes/month. 25 patients had 0.3 joint bleedings/month; for 42 patients no joint bleedings were documented. 55 patients required 0.5 additional substitutions for bleedings. All patients on prophylaxis received 13.9 substitutions/month with a dose of 29.8 IU/ED/kg. The dose /month and kg bodyweight applied to a prophylaxis patient was 370.2 IU. The 14 patients receiving an on-demand treatment had 2.8 bleeding episodes/month. 12 patients had 1.5 joint bleedings; for 2 patients no joint bleedings appeared. These patients received 3.2 substitutions/month with a dose of 25.7 IU /ED/kg. The total dose/month and kg bodyweight applied to an on-demand-patient was 69.7 IU. (all data calculated as median). With regard to our data we confirm a much higher consumption under prophylaxis treatment scheme which has to be related towards a lower bleeding tendency under a prophylaxis treatment scheme and to the long-term prevention of hemophilic arthropathy. Long term data will show the significance of prophylactic treatment.

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THE JAGGED/NOTCH EXPRESSION IN DIFFERENTIATING TO ENDOTHELIUM HUMAN UMBILICAL BLOOD PROGENITOR CD 34$^+$ CELLS

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Formation of new tubular structures by proliferating endothelial cells (EC) is regulated by growth factors, PPAR delta/gamma agonists as well as by cell/matrix, cell/cell adhesive interactions. Jagged/Notch system is a transmembrane receptor complex. The Jagged/Notch interaction inhibits cell differentiation, thus expression of this proteins on EC may be the marker of cell proliferation but not angiogenesis. The homeobox (Hox) morphoregulatory genes encode transcription factors that play an essential role in organogenesis during development and induce tissue remodeling in adults. Hox D3 promotes the invasive or migratory EC behavior, when Hox B3 is required for capillary formation by proliferating and differentiating EC. Since CD 34+ EC progenitors (ECP) are recently experimentally used for stimulation of angiogenesis of ischemic tissue, and because some of angiogenic factors influence differentiation of human EC by the Jagged/Notch based mechanism and Hox DNA-binding protein the influence of PPAR gamma agonists on the expression of Jagged/Notch and Hox genes in EC and CD34+ ECP was investigated.
Methods: EC were isolated from human umbilical vein, when the CD34+ cells were isolated from cord blood by ficoll-paque method. Cells were grown in EBM medium. VEGF, or bFGF, or leptin (1-50 ng/ml) or PPAR gamma agonists 15d-PGJ2 or ciglitazone (1-30 mM) were added to tissue medium for 24 hours. Gen expression was determined by RT-PCR, and angiogenesis was evaluated by the tube formation assay in matrigel. Participation of Hox B3 transcription factor in tube formation was verified by transfection with Hox B3 antisense plasmid.
Results: bFGF, leptin but not VEGF down-regulate expression of Jagged/Notch genes and activate the tubule formation in EC. PPAR-gamma activation and the HoxB3 antisense completely prevented the EC differentiation (tubule formation) by EC activated by all angiogenic factors in 3D model of angiogenesis.
Conclusion: Our preliminary results indicate that endothelial progenitor cells CD34+ demonstrate the expression of Jagged-1, Notch-1, Notch-2 genes like EC and stimulated by VEGF and bFGF demonstrate the ability of tube formation in the Matrigel model angiogenesis. Thus PPAR gamma activation may be used for controlling of ECP differentiation monitored by Jagged/Notch expression during the procedure of CD34+ culturing before retransplantation.

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ANTICOAGULANT AND ANTIPROTHROMBINASE ACTIVITY OF INHIBITORS OF FACTOR XA

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Synthetic inhibitors of the clotting enzymes represent a new class of drugs for the treatment of cardiovascular thrombotic disorders. For more than two decades the development has been concentrated on thrombin inhibitors, however, up to now only a few compounds have entered clinical trials. Therefore, the interest of developing inhibitors of factor Xa (FXa) has markedly increased. The inhibition of FXa may present a more favorable strategy for anticoagulation because of its suggested reduced bleeding risk.
With several inhibitors of FXa there is a discrepancy between the inhibition of the free enzyme and their anticoagulant activity. It has been assumed that differences in the structure-activity relationship for inhibition of the free enzyme and for inhibition of the prothrombinase complex are the reason for a comparatively lower anticoagulant activity of some types of inhibitors. We determined the anticoagulant activity (concentration necessary for doubling clotting times, EC200) and the inhibition of the prothrombinase complex (IC_{50}) for 40 FXa inhibitors of 6 different scaffolds with Ki values for inhibition of FXa from 0.01 – 1 µM.
There was a strong correlation between anticoagulant activity (aPTT or PT) and inhibition of the prothrombinase complex. In contrast, a considerably less strong correlation was found between the Ki values for inhibition of FXa and the IC_{200} values representing anticoagulant activity. The same applies for the correlation with the IC_{50} values for inhibition of the prothrombinase complex. However, when eliminating highly hydrophobic compounds from the calculation, there was an increase in the strength of this correlation.
High plasma protein binding is well known as a reason for reduced anticoagulant activity of highly hydrophobic inhibitors of FXa. We can now demonstrate that the binding of inhibitors into the prothrombinase complex is reduced with increasing hydrophobicity. Obviously, hydrophobic inhibitors are less capable of binding to the active site of FXa-prothrombinase complex.

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ROTATION THROMBELASTOGRAPHY DEMONSTRATES POTENTIAL UTILITY OF FACTOR XIIIA INHIBITION IN THROMBOLYSIS

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Factor XIIIa (FXIIIa) is the terminal enzyme of the blood clotting cascade. FXIIIa exhibits transglutaminase activity and is responsible for covalently cross-linking fibrin monomers and alpha2-antiplasmin during the formation of the blood clot by indroducing carbonamide bonds between glutamine and lysine residues of the adjacent fibrin fibers. Inhibition of FXIIIa has been suggested as a biological target for enhancing fibrinolysis in arterial and venous thrombotic disorders.
We were able to demonstrate the beneficial effect of FXIIIa inhibition on coagulation and fibrinolysis after clotting activation in combination with fibrinolytic agents (uPA, tPA, streptokinase). FXIIIa inhibitors used in this study included both novel small molecules and recombinant agents. The dynamic and kinetic parameters of thrombi formation and lysis were measured in a rotation thrombelastography system (ROTEG®) provided by Pentapharm GmbH (Munich, Germany).
Thrombelastographic experiments showed the influence on clot formation and significant improvements of fibrinolytic pattern in terms of clot formation time, maximum clot firmness and the fibrinolysis index (per cent fibrinolysis at specific times) when adding FXIIIa inhibitors to standard fibrinolytic agents.
The data suggests that FXIIIa inhibition may become a valuable adjunct to current anticoagulation and thrombolytic therapy with the potential benefit of improving clinical outcomes in thrombotic disorders. Although thrombelastography using whole blood represents a biological system that mimics physiological conditions, additional studies in validated animal models of thrombosis are necessary to evaluate the effects of FXIIIa inhibition.

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ASSAY SELECTION AND INTERPRETATION FOR THE LABORATORY TESTING OF HEPARIN-INDUCED THROMBOCYTOPENIA: DIAGNOSTIC IMPLICATIONS

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The diagnosis of heparin-induced thrombocytopenia (HIT) is based on clinical observations that may be confirmed by detection of HIT antibodies in the laboratory. Traditional assays to detect HIT antibodies are in vitro functional assays that infer the presence of HIT antibodies based on their heparin-dependent platelet-activating properties. ELISAs have been developed to detect binding of HIT antibodies to an immobilized target. Easier technical performance of the ELISA has prompted laboratories to use these ELISAs to screen for HIT, with follow-up confirmation by an activation assay, or to test by ELISA alone. We retrospectively analyzed specimens from undiagnosed patients referred to our laboratory for HIT testing between 1/1/99 and 6/30/01, in order to compare results between the ELISA and the serotonin release assay (SRA). ELISAs were carried out per manufacturer's instructions (Stago, Asnieres, France; GTI Brookfield, WI). Out of 2248 patient samples tested, 244 (10.8%) were positive by either ELISA or SRA. Of these 99 (4.4%) were SRA+ and 188 (8.4%) were ELISA+. However, these two patient populations were only partially overlapping; only 43 (<2%) were positive in both tests. Of the known SRA+ samples, approximately one half (43.4%) were ELISA+. If either the ELISA test alone, or the approach of ELISA screening/SRA confirmation approach were used in this population, 56 SRA+ patients would have been diagnosed as HIT negative. There are reports that ELISA methods are as sensitive as SRA in detecting the presence of HIT. However, these studies were conducted on in pre-selected groups of confirmed clinical HIT patients, and do not represent the true performance characteristics of these assays. Our study documents the frequency with which heparin-dependent antibodies that cause serotonin release from washed platelets in vitro, go undetected by the available anti-heparin:PF4 antibody ELISAs. This data suggests that the ELISA-screening/SRA-confirmation approach and ELISA stand-alone testing, are not optimal approaches for the laboratory diagnosis of HIT. Clearly, both of the HIT assay methods used here contribute independent information. This study underscores the fact that as yet no single assay is ideal for diagnosing HIT, and results obtained from the currently available tests need to be interpreted with caution.

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FACTOR-XA-DEPENDENT ACTIVATION OF MATRIX METALLOPROTEINASE-2 (MMP-2) AND ITS ROLE FOR MITOGENESIS AND MATRIX INVASION OF HUMAN VASCULAR SMOOTH MUSCLE CELLS

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Background: Matrix metalloproteinases (MMPs) are secreted as inactive proenzymes (pro-MMPs). They are activated extracellularly by proteolytic cleavage of the propeptide. This study demonstrates induction of active MMP-2 (65 kDa) by activated Faktor-X (FXa) in the cell culture medium of human vascular smooth muscle cells (SMC) and a contribution of this mechanism to DNA synthesis and matrix invasion by these cells.
Methods: MMP-2 was detected by gelatin zymography, DNA synthesis by [3H]thymidine incorporation and cellular invasion by a matrix invasion assay.
Results: Upon cellular stimulation with FXa (3-300 nM), a concentration-dependent induction of 65kDa-MMP-2 in the medium of SMC was detectable. In conditioned cell-free medium, containing secreted pro-MMP-2 from SMC, a cell-independent cleavage of pro-MMP-2 to 65kDa-MMP-2 was observed, which was much weaker as compared to cell stimulation. This cleavage was inhibited by the specific FXa inhibitor DX-9065a (DX, 0.3-100 µM). Stimulation with FXa (100 nM) resulted in a 5-fold stimulated DNA synthesis. This was significantly inhibitable by the MMP inhibitor GM6001 (100 nM) by about 20%. Cell count of SMC migrated through a matrix gel was 1.5-fold enhanced by FXa (100 nM) and was reduced to control levels by DX and GM6001.
Conclusion: These data suggest, that FXa cleaves secreted pro-MMP-2 and releases active MMP-2 from the cells. These mechanisms might contribute to the mitogenic potency of FXa and to the invasion of human vascular SMC into the extracellular matrix.

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***HEMOSTASIS IN PEDIATRIC CARDIOLOGY**

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Congenital heart disease affects approximately 1% of all life births and can be responsible for a wide spectrum of hemostatic alterations in children. The nature of cardiac malformation may be closely related to the severity of coagulation disturbances: Complex cardiac defects, by chronic hypoxemia, can lead to high red blood cell count, to hepatic stasis with diminished synthesis of coagulation factors and inhibitors and to low organ perfusion. These mechanisms may cause thromboembolism and threaten in particular children born with only one functional ventricle for both, the systemic and the pulmonary circulation. For a majority of them the so-called modified Fontan operation is the only therapeutic option: The ventricle is used as systemic pump while venous blood flows passively through the lungs. This situation represents an intriguing example for hemodynamic and hemostatic interaction: Increased hepatic venous pressure appears to be responsible for low levels of protein C and may contribute together with prosthetic tube grafts to coagulation activation. Some of the children are not only prone to thrombus formation but suffer embolic complications by shunting blood from the right to the left side of the circulation. Reported incidences of venous thromboembolism and stroke range from 3 to 16% and 3 to 19%, respectively! Current strategies for primary postoperative prophylaxis include therapeutic amounts of heparin followed either by aspirin or oral anticoagulation therapy. Interestingly, Fontan children seem to require less warfarin than other children to obtain the same target INR.
Non-cyanotic heart disease has also been shown to generate changes in coagulation: In a probably underestimated number of children sharing a common hemodynamic pattern the acquired von Willebrand syndrome may trigger important hemorrhage, but can also remain clinically silent. A high velocity jet associated with large blood flow in left-to-right shunting cardiac defects may contribute to shear-induced destruction of the largest von Willebrand multimers even in small infants. This bleeding disorder can be "cured" by surgery or interventional cardiac catheterization. However, close collaboration between pediatric cardiologists and hemostaseologists is necessary to establish the correct diagnosis before hemorrhage complicates the procedure. This is also true for re-achievement of vessel patency in thrombosis at the puncture site following cardiac catheterization. Failure to do so in arterial clots means progressive growth retardation of one limb. Particularly younger children were found to be at increased risk, mainly depending on the size of sheath used. Studies on thrombus formation and the extent of coagulation activation throughout different kinds of intervention have not yielded a generally accepted regimen for primary prophylaxis so far. Most centers use unfractionate heparin as bolus or flush solutions, some support prolonged administration. Ongoing studies investigate the benefit of low molecular weight heparin for this indication.

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***TISSUE FACTOR IN CIRCULATING BLOOD AND CORONARY THROMBOSIS**

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Tissue factor (TF) on cell-derived microparticles is present in the blood of patients with acute coronary syndromes. Increased TF level in the circulation has been associated with a heightened blood thrombogenicity not only in acute coronary syndromes but also in patient with hematological disorders, sepsis and disseminated intravasal coagulation. TF-positive microparticles in blood originate from ruptured atherosclerotic plaques, activated endothelial cells and leukocytes. Recently, we have reported that leukocytes transfer TF-positive particles to platelet thrombi enabling them to propagate thrombus growth. These findings have questioned the original understanding that vessel injury and TF exposure within the vasculature to blood is sufficient for inducing arterial thrombosis. Propagation of thrombus growth involves coagulation reactions and platelet deposition on the luminal thrombus surface. Vessel wall-derived TF has to diffuse from the vascular site through the deposited thrombus to the luminal surface to be present at the site of thrombus growth. With respect to diffusion and mechanical obstruction by overlaying thrombi, TF vesicles present on the platelet surface at the site of blood flow would reduce this diffusion distance, thus, increasing rate of thrombus growth. The transfer of leukocyte derived TF-particles is dependent on the interaction of CD15 and TF with platelet thrombi. This points to TF as a protein, which exhibits adhesive properties. Ultrastructural data on the location of TF on cells revealed TF at the cell surface in a spotty pattern at the apex of budding processes and in close proximity

to actin-rich regions. TF cooperates with integrin-mediated adhesion on matrices that contained ligands for integrins and the TF-VIIa complex. We found that anti-hTF antibodies, which inhibited factor VIIa binding to TF, reduced the TF-vesicle attachment to platelets. The adhesiveness of TF-positive microparticles in combination with their high thrombogenicity explains why therapies, such as anti-TF antibodies and inhibited FVIIa, are antithrombotic while causing no bleedings. Blocking the extrinsic coagulation system at the level of TF/factor VIIa and factor Xa is effective in reducing thrombosis in disorders associated with increased level of circulating TF such as acute coronary syndromes.

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IDENTIFICATION OF THE QUANTAL STRUCTURE FOR PLATELET ADHESION AND MICROPARTICLE FORMATION IN ARTERIAL FLOW

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Platelet function requires a transition from circulating blood to arrest onto vascular surfaces, which in fast arterial flow depends on glycoprotein (GP) Iba binding to the A1 domain of immobilized von Willebrand factor (VWF). Here we demonstrate in real time the formation of membrane tethers and microparticles. The responsible platelet-surface contacts are initiated by a limited number of discrete adhesion points (DAPs), each involving a membrane area of 0.1-0.25 mm^2. Single DAPs resist detachment against force exceeding 160 pN, but in the process they may become separated from the platelet body that moves in the direction of flow, forming the tethers and microparticles. The number of bonds in a DAP, which contains a calculated 100-375 GP Iba receptors, depends on VWF A1 domain density and determines the upper shear rate compatible with adhesion. Approaching this limit, DAPs tend to exhibit first-order dissociation kinetics, suggesting that multiple homogeneous bonds act in synchrony under maximal tensile stress. Thus, plasticity of the cellular membrane and multivalent receptor function are defining properties of the quantal unit that initiates platelet thrombus formation during normal hemostasis or pathological arterial occlusion.

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INHIBITION OF PROLIFERATION AND INDUCTION OF DIFFERENTIATION OF ENDOTHELIAL CELLS BY ANTITHROMBIN

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Antiangiogenic and anti-tumor activities of latent and cleaved antithrombin have been previously described, and in vitro, the serpin inhibited proliferation of endothelial cells. We have recently observed that direct cellular effects of antithrombin are mediated by syndecan 4, a heparan sulfat proteoglycan known to bind to the heparin-binding site of antithrombin. Syndecan 4 is known to affect proliferation and differentiation of a variety of cell types. We have, therfore, studied direct effects of intact antithrombin on endothelial cells. Human umbilical vein and calf pulmonary artery endothelial cells were studied in the presence or absence of antithrombin concentrate or monclonal antibody purified antithrombin with and without concomitant presence of synthetic pentasaccharide. Proliferation was assessed in BrDU incorporation and MTT assays. For testing endothelial cell differentiation, capillary tube formation was investigated in Matrigel assays. Proliferation of the two types of endothelial cells was significantly inhibited by 1 to 10 U/ml of both antithrombin concentrate and antibody-purified antithrombin. Capillary tube formation induced by Matrigel was augmented by the presence of 1 to 10 U/ml of antithrombin concentrate which was partly reversed with pentasaccharide. Results show that in vitro effects of antithrombin on angiogenesis-related endothelial cell functions may be directly exerted by the intact serpine and can be antagonised by pentasaccharide.

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THROMBIN-INDUCED MITOGENESIS IN BOVINE SMOOTH MUSCLE CELLS: STUDIES ON THE SIGNALING PATHWAY

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Background: Thrombin is a potent mitogen for vascular smooth muscle cells (SMC), but the signalling pathway by which thrombin mediates its mitogenic response are not yet fully understood. In the present study, the involvement of epidermal growth factor (EGF) receptor, on one hand, and the role of Gi-proteins, on the other hand, in thrombin-induced mitogenesis in bovine coronary artery (BCA) SMC was examined.
Methods: Activation of p44/42 and p38 mitogen-activated protein kinases (MAPK) were analyzed by Western blotting using phospho-specific antibodies. Mitogenesis was measured by [3H]thymidine incorporation. Intracellular cAMP concentrations were detected by radioimmunoassay after stimulation of adenylyl cyclase by forskolin.
Results: It was found that thrombin (10 nM) did not transactivate the EGF receptor, because the selective inhibitor of EGF receptor kinase (AG1478; 2-10 µM) had no effect on the response to thrombin, neither on MAPK activation nor on DNA synthesis. MAPK activation induced by the thrombin receptor activating peptide (TRAP, 100 µM), which mimics many of the cellular actions of thrombin, was also not affected by AG1478. However, in contrast to thrombin TRAP is not mitogenic for SMC. To estimate the role of Gi-proteins, cells were preincubated with pertussis toxin (PTX). Thrombin induced mitogenesis was not reduced after PTX treatment. Moreover the adenylyl cyclase was not inhibited, because levels of intracellular cAMP were not decreased after thrombin or TRAP stimulation.
Conclusions: These results demonstrate that in BCA-SMC the thrombin and TRAP induced activation of p44/42 MAPK is not EGF receptor dependent and the thrombin induced DNA synthesis is not mediated by Gi-proteins. This suggests that other intracellular signal transduction pathways are involved by thrombin to act as a mitogen.

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***THE ROLE OF MULTIPROTEIN COMPLEXES IN COAGULATION PROTEASE SIGNALING**

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The crucial role of cell signaling in hemostasis is clearly established by the action of the downstream coagulation protease thrombin that cleaves platelet-expressed, G-protein coupled protease activated receptors (PARs). Certain PARs are cleaved by the upstream coagulation proteases factor Xa (Xa) and the tissue factor (TF)-factor VIIa (VIIa) complex, but these enzymes are required at high, non-physiological concentrations and show limited recognition specificity for the scissile bond of target PARs. However, defining a physiological mechanism of PAR activation by upstream proteases is highly relevant, because of the potent anti-inflammatory *in vivo* effects of inhibitors of the TF initiation complex. Recent results indicate that activation of substrate factor X (X) by the TF-VIIa complex produces enhanced cell signaling in comparison to the TF-VIIa complex alone, free Xa, or Xa that is generated *in situ* by the intrinsic activation complex (factors VIIIa-IXa-X). Macromolecular assembly of X into a ternary complex of TF-VIIa-X is required for proteolytic conversion to Xa, and product Xa remains transiently associated in a TF-VIIa-Xa complex. By trapping this complex with a unique inhibitor that preserves Xa activity, it was shown that Xa in this ternary complex efficiently activates PAR-1 and PAR-2. These results support the novel concept that efficient proinflammatory Xa signaling depends on the TF/VIIa/Xa complex and thus is mechanistically coupled to the TF-VIIa initiated coagulation pathway, rather than a late event during excessive activation of coagulation and systemic generation of proteolytic activity. Furthermore, the data support the general concept that efficient signaling by coagulation proteases depends critically on the assembly of cell surface multiprotein complexes that position the protease appropriately for PAR activation.

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OCCURRENCE OF ANTIPHOSPHOLIPID ANTIBODIES IN PATIENTS TREATED WITH THERAPEUTIC DOSAGES OF UNFRACTIONATED AND LOW-MOLECULAR-WEIGHT HEPARINS AND WHO DEVELOPED ANTI-HEPARIN-PF4 ANTIBODIES

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To investigate the prevalence of antiphospholipid (APL) antibodies in patients who developed high titers of anti-heparin platelet factor 4 (AHPF4) antibodies during the therapeutic course with unfractionated heparin (UFH) and a low-molecular-weight heparin (LMWH), clivarin (Reviparin; Knoll AG, Ludwigshafen, Germany), a sub-study was conducted using plasma samples obtained during the CORTES trial. Citrated plasma samples were collected prior to treatment and after 5 to 7 days and 3 to 4 weeks of therapy. The three treatment groups included UFH for 5 to 7 days (Arm A), clivarin s.c b.i.d. for 5 to 7 days (Arm B), and clivarin

s.c. o.d. for 26 to 30 days (Arm C). An ELISA-based method was used to subtype the APL antibodies (American Diagnostica, Inc., Greenwich, CT). 233 samples from 157 patients across three arms were positive for AHPF4 antibodies (GTI, Brookfield, WI). In Arm A, 102 samples from 70 patients were positive. In Arm B, 56 samples from 34 patients were positive and in Arm C, 64 samples from 44 patients were positive for AHPF4 antibodies. All of these samples were tested for the presence of IgG and IgM isotypes of APL antibodies. In Arm A, 4/70 patients were found to be positive in APL antibodies (1 IgG+/IgM+, 2 IgG+ and 1 IgM+). In Arm B, 4/34 patients were APL antibody positive (1 IgG+/IgM+, 3 IgG+). In Arm C, 2/44 patients were positive for APL IgG antibodies. Overall in this sub-study, of 157 AHPF4 antibody positive patients, 10 patients were positive for APL antibodies (2 IgG+/IgM+, 2 IgM+ and 6 IgG+). Out of these 10 APL positive patients, 1 was positive before the treatment only, 3 patients were positive pre- and post-heparin (6 to 21 days) and 6 were positive after heparin-treatment. Thus, in Arm A, 4 out of 70 (5%), in Arm B, 4 out of 34 (12%), and in Arm C, 2 out of 44 (4%) patients exhibited the APL antibodies titer. These data indicate a low prevalence of APL antibodies in patients who developed AHPF4 antibodies. The presence of these antibodies appeared to be lowest in the clivarin o.d. group. This observation further reinforces the relatively higher safety profile of LMWHs.

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A REVISED VIEW OF ANTITHROMBIN III's MECHANISM OF ACTION

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Introduction: A significant decrease of antithrombin III (ATIII) plasma levels, in particular if acquired for instance in the course of a DIC, is correlated with a fatal outcome of the patient. Substitution with an ATIII concentrate supports the efforts to regain the physiological balance. Research during the last decade provided an accumulating body of evidence that ATIII`s abilities are not limited to the control of blood coagulation, but that it fulfills important tasks as a modulator of inflammatory and cellular reactions.
Modulation of coagulation: ATIII is the most important protease inhibitor in plasma forming covalent complexes with a number of activated factors. At the vessel wall, ATIII is bound to glycosaminoglycans (GAGs), which locate the inhibitor at the vascular surface thereby protecting the endothelium from excessive proteolytic and coagulant events. Antiinflammatory properties: ATIII`s reactions with vascular and blood cells are likely to have a modulating impact on the release of pro- and anti-inflammatory protagonists. In addition to ATIII`s stimulatory impact on endothelial cells to release prostacyclin, leukocytes are down-modulated in their response to procoagulant, inflammatory and chemotactic stimuli. Upon exposure to LPS, the expression/release of IL-6, IL-8 and tissue factor from e.g. monocytes was significantly reduced in vitro. Incubation with ATIII limited the neutrophil chemotaxis toward fMLP or IL-8. These in vitro studies provide an explanation for the in vivo observations that the enhanced leukocyte-endothelium interactions during inflammation are down-modulated to a physiological level by ATIII.
Conclusion: ATIII supports the organism in regaining its physiological balance at procoagulant and inflammatory challenges by its dual mechanism of action.

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THE CONTRIBUTION OF FACTOR VII-ACTIVATING PROTEASE (FSAP) TO COAGULATION AND FIBRINOLYSIS

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Introduction: The factor VII- and prourokinase activating protease (FSAP) is a recently discovered serine-protease present in human plasma at 12 µg/ml on average. It was reported to be able to drive coagulation via the extrinsic pathway thereby displaying a FVIII bypassing activity. The ability of FSAP to activate single-chain plasminogen activators very potently provides evidence for a profibrinolytic function as well. This study contributes information on the functional role of FSAP in both hemostatic systems, coagulation and fibrinolysis.
Methods: FSAP proenzyme was prepared by immune-adsorption as reported recently. Its procoagulant and fibrinolytic properties were studied by plasma thrombelastography and coagulation assays according to Schnitger and Gross. Coagulation of citrated plasma was initiated by either recalcification or tissue factor (TF) addition. Activation of plasmatic FSAP proenzyme was induced by dextran sulfate (DXS). Prourokinase was added to facilitate enhanced fibrinolysis. Inhibitory and non-inhibitory monoclonal antibodies (mAb) to FSAP as well as FSAP deficient plasma (DP), prepared by immune adsorption, served as tools to address FSAP-specific functions in plasma.
Results: Incubation of plasma with DXS activated FSAP as demonstrated by SDS-PAGE/Western blotting In the presence of a functionally blocking mAb or in FSAP-DP, recalcification times of plasmas following DXS pre-incubation were significantly prolonged. When TF was used to initiate coagulation, no significant acceleration of clotting was observed. TF induced clot formation led to an accelerated lysis in prourokinase containing plasma, in particular in the presence of DXS. Addition of the FSAP inhibitory mAb delayed clot lysis significantly in comparison to a control mAb.
Conclusion: These results support our hypothesis that FSAP plays a dual role in hemostasis. In plasma, activated FSAP contributes to coagulation, but participates in clot lysis in the presence of single-chain plasminogen activators.

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MONOCLONAL ANTIBODIES SPECIFIC FOR LATENT ANTITHROMBIN III

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Introduction: Antithrombin III (AT) is the most important inhibitor of hemostasis. AT's latent or locked conformation (l-AT), essentially having lost the properties to inhibit its target proteases and to interact with glycosaminoglycans, recently has been reported to display potent inhibitor function on tumor associated angiogenesis in mice by an as yet not clarified cellular interaction. It is unknown whether (active-site loop cleaved and) l-AT represents a physiological principle to modulate tumor angiogenesis. Studies of its presence in body fluids and tissues were not yet possible due to the lack of suitable assays differentiating l-AT from active AT. We report monoclonal antibodies to l-AT facilitating further investigations.
Methods: L-AT was prepared from the AT concentrate Kybernin P by removal of active AT by adsorption to immobilized heparin. Mice were immunized with l-AT. AT positive clones were identified by ELISA. The specificity of antibodies to l-AT were confirmed by comparative investigation of active AT (alpha- and beta-AT) and l-AT employing ELISA and biomolecular interaction analysis (BIACORE).
Results: From the 2400 clones obtained from immunization attempts, 255 were found to be positive for AT. Out of those, only 16 clones produced antibodies differentiating AT and l-AT. Three clones were identified to be suitable for further investigations, which were of IgG1 type. The l-AT specificity and binding characteristics render them suitable for specific l-AT immunoassays and as potential tools for histological and cell binding studies.
Conclusion: To our knowledge it is the first time to report suitable antibodies specific for l-AT facilitating studies of its potential occurence in body fluids or in/on cells and tissues in health and disease.

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SUBCUTANEOUS LOW MOLECULAR WEIGHT HEPARIN (LMWH) VERSUS INTRAVENOUS UNFRACTIONATED HEPARIN (UFH) BOLUS IN PEDIATRIC CARDIAC CATHETERIZATION

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Venous or arterial thrombosis after cardiac catheterization is a well known complication. Anticoagulation with UFH reduces the risk of thromboembolic complications during cardiac catheterization in both children and adults. In adults, advantages of LMWH application during coronary intervention have been found. We investigated in our study 65 pediatric patients with 2 different antithrombotic covers: 40 children received a 100 IU/kg bodyweight UFH bolus, in 25 patients LMWH (enoxaparin) was administered 4 hours before cardiac catheterization subcutaneously. Anti Xa activity, F1+2, D-Dimer, and ACT were determined before, 5 min, 30 minutes after puncture, and at the end of catheterization. Anti Xa enoxaparin activity showed more consistent dose to plasma activity than UFH. ACT >200 seconds reflected UFH levels greater than 0.8 IU/ml. Clotting activation (F1+2, D-Dimer) did not differ in the 2 groups. Clotting activation was related with younger age in the heparin group. F1+2 and D-Dimer formation were not dependent on duration and type of the procedure (interventional/diagnostic).
Our preliminary study showing more consistent dose to plasma activity and no increased markers of clotting activation with 10mg/kg bodyweight subcutaneous LMWH than with a bolus of 100 IU/kg BW UFH suggests that the well known advantages of LMWH may also apply to pediatric cardiac catheterization. While groups did not differ in the incidence of bleeding and thrombosis, larger clinical trials will be necessary to prove the safety of LMWH in pediatric cardiac catheterization.

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ATYPICAL MANIFESTATION OF HEPARINE INDUCED THROMBOCYTOPENIA TYPE II

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A 40-year old woman comes to the emergency room stating to suffer from pain in the lower left leg for the past 5 days. Multiple arterial thromboembolic occlusions in both lower legs are diagnosed by Doppler sonography and confirmed by angiography. The most severe occlusions are located in the left leg. The laboratory screening does not reveal any signs of thrombocytopenia. All common markers for thrombophilia are within the normal range.The therapeutic urokinase fibrinolysis does not show any effect. The following rtPA fibrinolysis proves to be ineffective, too. Both fibrinolytic treatments lead to the formation of new thrombotic material. In addition to the fibrinolytic treatment the patient receives intravenous and intraarterial heparin. A computer tomography of the thoracic arteries and a transesophageal echocardiography reveal no potential sources of embolic material. In the course of further laboratory diagnostics we find antibodies against PF4-Heparin.The patient states to have been treated with a heparin ointment while being hospitalized for a period of six weeks. Any other heparin treatment is negated by the patient. After exchanging heparin with orgaran the number of thrombotic occlusions show a rapid decline. Thus, a heparin-induced thrombocypenia type II can be diagnosed without thrombocytopenia after transcutaneous heparin treatment. The patient has been dismissed from hospital with oral anticoagulation and possesses a HIT pass.

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GP IB ALPHA: IMPACT OF THE VNTR POLYMORPHISM ON CORONARY ARTERY DISEASE

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The variable number of tandem repeats (VNTR) polymorphism is located in the macroglycopeptide region of glycoprotein (GP) Ib alpha, which is known as the binding site of von Willebrand-factor (vWF). The VNTR alleles A, B, C and D encode four different isoforms of GP Ib alpha. The smallest isoform (D) contains one sequence of 13 amino acids (aa) within a region comprising aa Ser399 to Thr411, whereas the largest isoform (A) contains four such sequences. The addition of repeats increases the length of GP Ib alpha and projects the vWF binding domain farther away from the platelet surface. Thus, it was hypothesized that such molecular changes may have an impact on ligand-receptor interactions and might represent an inherited risk factor for coronary artery disease.
We genotyped DNA samples from 2127 male patients who underwent coronary angiography for diagnostic purposes and 535 healthy control individuals as determined by physical examination and standard questionnaire. We found no association between the A, B, C and D alleles with coronary artery disease (n=1729) or myocardial infarction (MI, n=1065).
As GP Ib alpha is known to form homodimers in the platelet membrane, we also addressed the question, whether there is a difference between homozygous (CC) and heterozygous (BC or CD) individuals. Whereas there was no evidence for an association between heterozygosity and MI (p=0,127) or CAD (p=0,066), significance was found in the subgroup of patients with CAD and elevated fibrinogen levels (p=0,014).
In conclusion, our data indicated that length of GP Ib alpha do not associate with an increased risk for CAD or MI. However, heterozygosity in terms of different long counterparts of the homodimer may possibly influence the receptor's susceptibility of vWF binding. Data gained from adhesion experiments with CHO transfectants addressing this problem will be presented.

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BERNARD-SOULIER-SYNDROME DUE TO HOMOZYGOUS ASN45SER MUTATION IN PLATELET GLYCOPROTEIN IX IN A PATIENT MISDIAGNOSED WITH IDIOPATHIC THROMBOCYTOPENIA

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Bernard-Soulier-Syndrome (BSS) is a rare bleeding disorder due to quantitative or qualitative abnormalities in the platelet receptor GPIb-IX-V complex. We examined a 63 years old patient with a history of chronic thrombocytopenia and mild bleeding tendency who had been diagnosed with idiopathic thrombocytopenia (ITP) and treated with steroids without response. A peripheral blood smear showed giant platelets. Ristocetin-induced platelet aggregation was absent. Quantitative flow cytometry disclosed a greatly reduced surface expression of glycoprotein (GP) IX (18%), GP Ib alpha (14%) and GP V (29%) in comparison to normal control platelets. Western blot analysis showed reduced amounts of GP Ib alpha, GP Ib beta and GP V, whereas GP IX was absent. DNA sequence analysis of GP IX revealed a single nucleotide exchange in position 1826 (A/G) changing Asn45Ser. Nucleotide sequencing of the entire GP Ib alpha and Ib beta revealed no other mutations. Restriction digestion of PCR fragments proved that the patient as well as his affected siblings are homozygous for this mutation.
Today, seven different mutations of GP IX have been reported in patients with BSS, but only the Asn45Ser has been found in several families in different European countries, supporting the theory that this is an ancient mutation. We present here the first case in Germany.
However, this case also demonstrates, that in patients with chronic thrombocytopenia the differential diagnosis between ITP and BSS should be studied thoroughly in order to avoid inappropriate treatment.

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SCREENING OF LYMPHATIC TISSUE MAY BE A POTENTIAL TOOL FOR RISK ASSESSMENT OF VCJD TRANSMISSION WITH BLOOD PRODUCTS

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Purpose: Transmissible spongiform encephalopathies (TSEs) are neuropathological diseases which are caused by prions. Prions are infectious particles (PrPSc) which can induce bovine spongioform encephalopathy and most likely also a related infectious disease (new variant of Creutzfeldt-Jakob disease=vCJD). The exposure of humans to the BSE agent in contaminated meat products presumably led to the emergence of vCJD. According to the oral infection route, in vCJD, prions can be detected immunohistochemically not only in neuronal tissue but also in lymphoreticular tissue. vCJD is of significance in transfusion medicine because of the hypothetical transmission of prions by blood products. No data exist on the frequency of vCJD carriers in the population.
Methods: An immunohistochemistry test system has been established allowing epidemiological screening for vCJD in human lymphoreticular tissue.
Results: PrPSc can be detected in the cerebrum and cerebellum of sCJD patients and in the lymph nodes, tonsils and spleen of vCJD patients. As a major advantage this method works in fixed specimens which are routinely saved in departments of pathology and therefore allows screening of large numbers of archived human lymphoreticular tissues in different regional areas and of different time points and of autopsies of hemophiliacs. Scrapie positive lymphoreticular sheep tissue which is available in sufficient amounts reacts as human tissue of vCJD affected patients and can be used as positive control in screening programs.
Conclusion: A method is provided which is a feasible tool for an epidemiological screening program to assess the prevalence of the assumed infectious agent of vCJD, PrPSc, in various populations.

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GENOMIC DIAGNOSIS IN HAEMOPHILIA A AND B-REPORT OF FIVE FAMILIES FROM COSTA RICA

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Haemophilia A and B are the most common bleeding disorders with a frequency of about 1 per 4.000 to 7.000 and 1 per 25.000 to 30.000 male birth respectively. Haemophilia A and B are X-linked diseases. Only males are effected, female carriers are phenotypic healthy. Theoretically carriers have clotting factor level around 50% of normal, which is generally sufficient for normal haemostasis. So far the application of clotting and immunolocical methods allow to detect only about 80% of the carrieres due to the random inactivation of one X-chromosome in females. Anxiety about the risk of haemophilia affecting their offspring is the reason, why possible carriers ask for counselling. The precondition for an effective counseling are reliable detection of female carriers and the possibility of prenatal diagnosis. Mutation in both genes exhibit extreme diversity and in most cases involve changes of single nucleotides. Sequence analysis for FVIII and FIX for mutation detection is expensive and is offered only by a few specialized centres. That's why many laboratories in developing countries assesss the genetic status of potential carrieres through the use of indirect genetic markers linked to the FVIII- or FIX gene respectively. Such markers are e.g. RFLPs, STS or VNTRs which can be detected by Southern blotting or PCR and gives good results in most families. The detection of an inversion in FVIII gene in about 45% of patients with severe haemophila A opened the possibility of direct mutation analysis in those familes by southern blotting or PCR. In about 5% of the patients with severe haemophilia deletions of the FVIII/FIX genes can be detected.
First results of molecular diagnosis in Costa Rica are presented. In three families with haemophilia A and two families with haemophilia B preconditions, practicability, facilities of molecular analyse but also restrictions of the indirect molecular diagnosis are demonstrated. In one family with haemophilia A and both families with haemophilia the causative mutation could be detected by Southern blotting (inversion in FVIII gene, deletion in FIX gene) or sequence analysis.

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EVALUATION OF AN ATHROMBOGENIC FILTER SYSTEM FOR MEASURING PLATELET FUNCTION (RETENTION TEST HOMBURG – RTH)

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Background: Platelet adhesion and aggregation was measured using a standardized filter system (Eppendorf). In contrast to other test systems (PFA, O'Brien, Hellem) athrombogenic material (polyurethane filter Eppendorf) is used. Analytical - and the "between laboratories" variability as well as the biological variance was evaluated in citrated PRP and whole blood.
Methods: Platelet retention index (RI) was measured using the standardized Eppendorf filter cups under defined centrifugal conditions (138 g/ 15 minutes for PRP, and 110 g/5 minutes for platelet retention respectively). The RT-H assay was performed 30 minutes up to 180 minutes after the withdrawal of blood. Furthermore electron microscopy was used to analyze the platelets adhering or aggregating in the Eppendorf filter. In cooperation with other laboratories (P. Hellstern - Ludwigshafen/Rh., S. Monien, A. Grigorov - Berlin, and M. Willmer - Basel) the "between laboratories" feasability was evaluated.
Results: We analyzed the interindividual variance ("normal values") of the RT-H in apparently healthy blood donors (n=50), which was found to be 13.6% to 30.3% using citrated PRP, respective 72.9%+-2.1% in citrated whole blood. The coefficient of variation for the platelet count in multiple measurements in series is found to be 2.6% to 7.6% (- from "day to day:" 5.3% to 8.3%). The coefficient of variation for the RI ranges below 20%. Transmission electron microscopy demonstrated that activated platelets adhere to the polyurethane surface and spread or form aggregates. Platelet retention values obtained from our department were comparable with those of three other laboratories. However different citrate solutions, counter systems and strategies to prepare PRP resulted in significant RI differences.
Conclusions: The assay is simple and technically reliable. Furthermore the biological variability of the filter system was found to be rather low. Additionally "between laboratory" evaluations have to be carried out.

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ON THE INFLUENCE OF PLATELET SIZE ON PLATELET RETENTION

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Background: The aim of this study was to evaluate the retention of total platelet populations (RI) and the retention of large platelets ("LCRI" - large cell retention index) in patients with decreased (v.Willebrand syndrome - "vWJ.-S") as well as increased platelet retention (postsurgical patients) by the retention test - Homburg (RT-H).
Patients and methods: 7 patients with mild vWJ-S. type I (group I) and 10 healthy subjects receiving daily doses of 500 mg aspirin (group II) were examined during (day 3) and 6 weeks after cessation of aspirin administration. Furthermore we investigated 14 patients with coronary heart disease undergoing thoracic surgery with a postoperative decrease of platelet count below 80000/µl (group IIIa) and a further group of patients without a postoperative platelet drop (group IIIb). Platelet rich plasma was (PRP) was obtained 30-180 minutes after the withdrawal of citrated whole blood by centrifugation at 138 g for 15 minutes. 500 µl PRP was exposed to the "Eppendorf retention tubes") and centrifuged again at 110 g for 5 minutes after an incubation of 10 minutes. Platelet count (PC) and platelet large cell ratio (P-LCR) were calculated before and after the filter passage using a Sysmex counter. The retention index (RI), and LCRI was calculated as "PC before" minus "PC after" divided through "PC before" (%). Statistical analysis was performed using the ANOVA-assay.
Results: Group I - Significantly lower LCRI-values were obtained (13.54%; $p<0.0005$) in comparison with healthy individuals (25.9%). However RI´s were not different from normal RI-values (22.98% vs.20.9%). Group II - There was a significant increase of LCRI ($p < 0.0005$) (29.6 % to 36.9% - means) and RI-values (21.3% to 30.8%) six weeks after cessation of aspirin treatment compared with the measurements performed on day three. Group IIIa - RI: 26.7%; LCRI: 41.2%; Group IIIb - RI: 40.9%; LCRI: 56.7%. The retention of large platelets (LCRI) is significantly higher than the retention of the total platelet population (RI) in both groups. However in thrombocytopenic patients (group IIIa) the RI was found to be not abnormal, in contrast to the LCRI findings and also to the results obtained from group IIIb.
Conclusions: The LCRI is more sensitive than RI-values to diagnose "hyporetention" which may also be true for "hyperretention." Six weeks after taking aspirin RI and LCRI significantly increased. Platelet counts do not influence the LCRI/RI ratio.

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ON THE PROPHYLACTIC/THERAPEUTIC EFFICACY AND DOSE DEPENDENT INCREASE OF PLATELET COUNT BY THE USE OF DANAPAROID IN PATIENTS WITH HEPARIN INDUCED THROMBOCYTOPENIA

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Background:Danaparoid was approved for the treatment and prevention of thrombosis by the US Food and Drug Administration in 1/2000. It is used as an effective anticoagulant agent for both prophylaxis and therapy in patients with HIT. To date the most effective way to treat patients with HIT - namely the most promiseful dose is unclear.
Patients and methods: We aimed to evaluate the clinical outcome and platelet course of 21 patients with thromboembolic complications and 24 patients without clinical signs/symptoms related to HIT but thrombocytopenia using danaparoid during the hospitalization lasting four to five weeks.
Results: Twice daily given subcutaneous doses of danaparoid (10 Anti-Xa U/kg BW) corresponding with anti Xa levels ranging from 0.2 to 0.3 U/ml led to normalized platelet counts within five to seven days. Before the alternative anticoagulation the platelet count was found to be 83000/µl±74 000/µl (mean ± SD).
For therapy 181.3+-58.1 U/h (mean±SD) danaparoid were given. These doses correlated with anti Xa levels ranging about 0.44+-0.15 U/ml (mean±SD). In contrast to prophylactic doses the effect on platelet count was more pronounced a higher dosed intravenous administration of danaparoid. Furthermore the platelet count increased from 651000/µl±465000/µl within three days. No severe side effects were seen in both groups. Neither bleeding complications, nor any progression of the thrombotic process could be objected. No amputation became necessary, as well as no letal outcome was registered.
Conclusion: The increase of platelets dose dependently and significantly occured due to danaparoid administration. Higher, intravenously given doses led to an earlier normalization of platelet count compard with prophylactically given doses. Patients with and without thromboembolic

complications and suspicious for HIT may profit from “therapeutic” doses of danaparoid. Higher doses, as also demonstrated did not increase the risk of bleedings. We suggest that anti-Xa levels ranging about 0.4 U/ml may be as effective as safe.

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ON THE INFLULENCE OF THE C 677 T METYLENTETRAHYDROFOLATE POLYMORPHISM ON PLATELET FUNCTION IN THROMBOPHILIC PATIENTS AND ASYMPTOMATIC INDIVIDUALS

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Background: Retrospective studies as well as prospective trials demonstrated a significant influence of genetic abnormalities of the enzyme methylentetrahydrofolate reductase (“MTHFR”) on the progression of atherosclerosis and homocysteine. We therefore aimed to evaluate the role of this gene defect on platelet function and homocysteine in serum.
Patients and Methods: We analyzed platelet function (spontaneous platelet aggregation acc. to Born (1962) - normal range of “angle alpha:” >9 degrees; platelet adhesion acc. to Hellem, 1970 (normal range: 3% to 38%) and homocysteine serum levels (normal range: <15 µmol/l) in 100 individuals with a proved homozygous (T/T-), or heterozygous (C/T-) genotype for MTHFR. In 65 cases (group I) venous/arterial thrombotic complications did occur whereas 35 individuals (group II) were asymptomatic. Both groups were homogenous for gender and age (group I: 44.8±5.9 years - females : males=35:30; group II: 39.1±15.8 years - females: males=19:16). Statistical analysis has been performed using the Chi Square assay.
Results: In 40% of all cases (27 patients, 13 healthy individuals) hyperhomocysteinemia coincided with the gene defect for MTHFR. Hyperaggregability (acc. to Born’s method) could be objected in 54 cases implicating that 46 individuals with a heterozygous or homozygous genotype for MTHFR demonstrated normal platelet aggregation. At least the severity of the genetic defect of MTHFR does not influence the homcysteine level in serum.
Conclusion: The mutation for MTHFR (C 677 T polymorphism) may not necessarily coincide with hyperaggregation of platelets. Platelet adhesion remains uninfluenced by abnormal genotypes. Furthermore this gene defect is not significantly correlated with the level of homocysteine measured in serum.

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PLATELET ACTIVITY, REACTIVITY AND PLATELET-LEUKOCYTE-CONJUGATE FORMATION AFTER STRENUOUS ENDURANCE TREADMILL EXERCISE

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Background: Strenuous exercise is well known to cause changes in the haemostatic system. However, conflicting results concerning exercise induced platelet activation have been reported recently.
Methods: 17 healthy male non-smokers (age: 26±6 years; rel. VO2 peak: 56.4±5.9 ml/min/kg)) underwent a treadmill exercise at 90% of their individual anaerobic threshold (IAT) with a duration of minimal 60 and maximal 120 min. Blood samples were taken after 30min rest, immediately, and 2h after exercise for measuring changes in platelet count, -activity (CD62P), -reactivity (CD62P, TRAP-6 stimulated) and platelet-leukocyte-conjugate formation using flowcytometry. Additionally, markers of thrombin generation were investigated via thrombin-antithrombin III complex (TAT) and prothrombinfragment 1+2 (F1+2) analysis.
Results: Immediately after exercise an increase in platelet count of 45% ($p<0.001$) was observed. CD62P expression of unstimulated platelets was not significantly changed (1,65 vs. 1,73% pos. cells), while the number of TRAP-6 stimulated, CD62P positive platelets rose by 17% ($p<0.05$) after exercise. Furthermore the percentage of platelet-leukocyte-conjugates increased by 38% ($p<0.01$) after exercise. Both changes were reversible after 2 h. In addition, markers of thrombin generation were elevated (TAT: 49%; F1+2: 25%; $p<0.01$ each), but no correlations between these changes and changes in platelet reactivity or conjugate-formation could be found.
Conclusion: Platelet reactivity and the formation of platelet-leukocyte-conjugates is increased after strenuous endurance exercise. In contrast, activity of unstimulated platelets is not altered. This increase of platelet reactivity and conjugate formation is reversible after 2h and seems to be independent of thrombin generation.

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SUSTAINED DELIVERY OF THERAPEUTIC CONCENTRATIONS OF HUMAN CLOTTING FACTOR IX – A COMPARISON OF ADENOVIRAL AND AAV GENE THERAPY VECTORS ADMINISTERED IN UTERO

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Prenatal somatic gene therapy has been considered for genetic disorders presenting with morbidity already at birth. Haemophilia is associated with an increased risk of catastrophic perinatal bleeding complications such as intracranial haemorrhage, which could be prevented by gene transfer in utero. Prenatal gene therapy may be more promising than postnatal treatment, as the fetus may be more amenable to uptake and integration of therapeutic DNA and the immaturity of its immune system may permit life-long immune tolerance of the transgenic protein, thus avoiding the dominant problem in haemophilia treatment, the formation of inhibitory antibodies. In this study adenoviral or AAV vectors carrying human clotting factor IX (hfIX) cDNA or a reporter gene were administered to late-gestation mouse fetuses. Mice treated in utero by intramuscular injection of an adenoviral vector carrying hfIX cDNA exhibited high-level gene expression at birth and therapeutic - albeit continuously decreasing - plasma concentrations of hfIX over the entire 6-months time course of the study. Systemic vector spread was detected by PCR. Intramuscular, intraperitoneal or intravascular application of AAV vectors carrying hfIX cDNA led to much lower plasma concentrations of hfIX shortly after birth, which declined during the first month of life but stabilised in some of the mice at detectable levels. No signs of immune responses were found, neither against the different viral vectors nor against hfIX.
This study demonstrates for the first time that sustained systemic delivery of a therapeutic protein can be achieved by prenatal gene transfer. It thus shows the feasibility of gene therapy in utero and provides a basis for considering this concept as a preventive therapeutic strategy for haemophilia and perhaps also for other plasma protein deficiencies.

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THROMBOTIC THROMBOCYTOPENIC PURPURA: MUTATION SCREENING OF THE ADAMTS13 GENE TO DIFFERENTIATE BETWEEN THE FAMILIAL AND THE ACQUIRED FORM

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Background: Thrombotic thrombocytopenic purpura (TTP) is caused by severe deficiency (<5%) of the von Willebrand factor cleaving protease (VWF-CP) that has recently been identified as a member of the ADAMTS family of proteins. ADAMTS 13 gene defects cause the familial form of this disease that is inherited in an autosomal recessive fashion. Many cases, however, are due to an acquired antibody against the VWF-CP. Although such an antibody can be identified by conventional methods, in several cases, a familial form cannot completely be excluded.
Patients: We investigated four patients with TTP from four unrelated families. VWF-CP activity was <5% in each of them. Two young adolescent patients presented with classical TTP with frequent episodes that could be managed by means of plasma exchange and plasma infusions, respectively. Antibodies against VWF-CP were absent. In two patients, children of 4 years of age, antibodies against the VWF-CP were inconsistently found. Interestingly, both patients were primarily diagnosed with chronic ITP, although they additionally presented with mild hemolytic symptoms and 2-3% fragmentocytes. None of these two patients, however, had ever suffered from thrombotic episodes as the two other patients did, but one of them also had a high titre inhibitor of 50 BU against Factor XI.
Methods: The VWF-CP in plasma was determined by measuring the decrease in plasma VWF collagen binding before and after incubation with a specific buffer. Patients with a severe VWF-CP deficiency (<5%) were then screened for mutations of the ADAMTS13 gene by means of PCR and direct sequencing using an automatic sequencer.
Results and discussion: Two of the index patients were compound-heterozygous for three novel mutations of the ADAMTS13 gene. Both carried an A-insertion (4143insA) in exon 27 on one chromosome and the nonsense mutation R1034X encoded in exon 24, or the missense mutation S263C encoded in exon 7 on the other chromosome, respectively. These are the first mutations identified in Germany. They have to be considered as causal due to their nature and since they do not re-

present known polymorphisms or variants. Thus we could clearly demonstrate the genetic background in these patients one of whom also had an antibody against VWF-CP. In the third patient with a presumed familial form, no mutations were detected, nor in the patient with the additional FXI inhibitor, speaking for the acquired form of the latter.

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SPECTRUM OF MUTATIONS AND GENOTYPE-PHENOTYPE ANALYSIS OF VON WILLEBRAND DISEASE IN GERMANY AS A BASIS FOR RATIONAL MOLECULAR GENETIC TESTING

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Background: Von Willebrand disease (VWD) is a highly heterogeneous bleeding disorder, reflected by a diversity of different types and subtypes and is caused by mutations of the von Willebrand factor (VWF) gene. In many cases, attempts to classify patients by phenotypic analysis alone fail and molecular testing is the only choice for a final diagnosis. However, due to the size of the gene (178 kb) and its fragmentation into 52 exons this task would be rather laborious, if the region of interest could not be narrowed. Due to our continuous work on phenotype-genotype correlation in von Willebrand disease during the last years we can now offer a tailored mutation analysis on the basis of a standardized assessment of the phenotype.
Patients: In our secondary laboratory, 489 samples were found to fulfill the criteria of VWD during the course of one year. Among them, 303 (62%) had the hereditary form which was the subject of our study.
Methods: Conventional phenotypic parameters included VWF:AG, functional assays like collagen binding and FVIII binding of VWF, and VWF multimer analysis. The latter method was refined by photo-imaging evaluation of the luminescent Western blot which allowed to differentiate between even subtle deviations from the normal multimer pattern. Phenotypic analysis was followed by direct sequencing of particular domains of the VWF gene.
Results and discussion: Depending on the phenotypic analysis, mutation screening could be concentrated on distinct regions of the gene in cases of VWD type 2A and its subtypes, as well as in types 2B, 2M and 2N. A correlation of phenotype and genotype close to 90% impressively confirmed our preliminary diagnosis or helped to find the correct one. The situation in severe VWD type 3 is different, since mutations causing this type are distributed over the whole gene. The molecular evaluation of VWD type 1 is currently under investigation by a joint European project that will certainly result in a higher diagnostic power even in this problematic type of VWD.
Conclusion: A high quality phenotypic evaluation of VWD should always precede molecular testing, since targeting the gene regions of interest can cut down the duration and the costs of such an analysis to less than 10%.

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RELEASE OF TISSUE FACTOR FROM PLATELETS AND ITS TRANSFER TO MONOCYTES: ROLE OF PLATELET-DERIVED MICROVESICLES AND CD62P

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Background: Tissue factor (TF) is the most important initiator of intravascular coagulation. Platelets contribute to TF exposure on monocytes, but the mechanism is not completely understood. Recently, it was shown that platelets contain and release TF upon stimulation. Activated platelets also release microvesicles by shedding of membranes from the surface. Platelet-derived microvesicles (PMV) expose platelet-specific antigens on their surface and can adhere to leukocytes in a CD62P-dependent manner. Here we provide evidence that PMV transfer TF from platelets to monocytes.
Methods: After incubating human citrated platelet-rich plasma (PRP) with collagen, platelets were removed by centrifugation (5,000× g, 5 min) and the obtained plasma was mixed with a sediment of red and white blood cells. After a further incubation, monocytes were analysed by flow cytometry for the platelet-specific antigens CD42a and CD62P as well as for TF. Levels of CD62P and TF in plasma were measured by ELISA.
Results: When PRP was incubated with collagen there was an increase in the plasma levels of TF and CD62P from 135±43 to 268±51 pg/ml and from 20±8 to 67±6 ng/ml, respectively ($p<0.005$). Incubation of plasma obtained from collagen-stimulated PRP with a red and white cell sediment resulted in an increase in the number of monocytes that express not only CD62P and CD42a, but also TF on their surface. Blocking CD62P by a specific antibody or removal of PMV by high speed centrifugation reduced the number of TF positive monocytes from 38±10 to 21±10 and 21±9%, CD42a positive monocytes from 76±15 to 12±1 and 60±14% and CD62P positive monocytes from 96±2 to 9±4 and 80±6%, respectively ($p <$ at least 0.02). MPV removal reduced all three antigens to about those levels that were measured in control experiments with unstimulated PRP. In contrast the anti-CD62P reduced only TF to control values, but reduced CD42a and CD62P to values that were much lower than in controls.
Conclusion: The data indicate that PMV that are released from collagen-stimulated platelets may carry TF, CD62P and CD42a and may transfer these antigens to the surface of monocytes in a CD62P-dependent manner. Platelets and/or PMV that are already present on monocytes in the sediment seems not to express TF, but can be displaced from the monocyte surface by the anti-CD62P antibody.

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RELATION BETWEEN THE THROMBIN ACTIVATABLE FIBRINOLYSIS INHIBITOR, THE CIRCULATING AMOUNTS OF THROMBIN IN PATIENTS WITH MILD AND SEVERE HEMOPHILIA A AND B AND VAN WILLEBRAND DISEASE

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Background: The Thrombin Activatable Fibrinolysis Inhibtor (TAFI) is activated by the Thrombin-Thrombomodulin-Complex. Because of low concentrations of F VIII:C and F IX in these patients a reduced thrombin generation leads to lowered activation of TAFI followed by a decreased down-regulation of fibrinolysis. This hypothesis is an additional mechanism to explain bleeding in such patients.
Material and Methods: We investigated 94 patients with severe to mild hemophilia A or B or van Willebrand disease from the Centre of Coagulation disorders from the University of Leipzig. To measure the amount of circulating thrombin we used the Endogenous Thrombin Potential (ETP) as described by Hemker. The F VIII, F IX and Ristocetin Cofactor Activities (RCoF) were determined with reagents from Dade Behring (Germany) at the BCS as one stage clotting assay and as turbidimetric assay respectively. For the TAFI-determination we used the COALIZE TAFI from Chromogenix (Italy).
Results: We found a correlation between TAFI and ETP in patients with hemophilia B (r^2=0,54) patients and patients with von Willebrand disease (patients with RCoF <30%, r^2=0,66). A correlation to F VIII-activities in hemophilia A-patients is missing, there is only a tendency. But in all cases we find, the higher the ETP, the higher the TAFI. We also find a relation between ETP and F VIII:C-activities with the following values (F VIII 0-10%, mean ETP 116,8 AU, F VIII 10-20% mean ETP 152,4 AU, F VIII 20-30% 178,3 AU, F VIII>30% ETP normally). The same tendency we observe in patients with F IX-deficiency. If we include all three groups with mild forms, we find no differences in TAFI between hemophilie A/B and Willebrand disease, but we find a concentration dependent down regulation of TAFI in the severe forms.
Conclusiones: The circulating amount of thrombin measured as the ETP influences the activity of the TAFI. The TAFI is also influenced from the concentration of coagulation factors, especially F VIII:C, F IX and RCoF. Long term observations are necessary to find out a correlation with the bleeding risk in different situations.

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THROMBOSIS – A CLUE OF POOR PROGNOSIS IN PRIMARY NON-METASTATIC BREAST CANCER?

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In breast cancer the true incidence of venous thromboembolism (VTE) is not known but after diagnosis of non-metastatic breast cancer VTE will occur in about 6% of patients during adjuvant treatment. An association between the occurrence of thrombosis in a patient with breast cancer and poor prognosis of cancer has been discussed for a long time but not formally tested.
From 1/1991 until 6/1996, 366 consecutive patients with breast cancer admitted for primary surgery, were screened for VTE using non-invasive methods and followed over a maximum of 7.5 years. Eight-teen patients (4.9%) had metastases at the initial evaluation. Of the remaining 348 patients without metastases, 19 (5.5%) developed VTE during adjuvant treatment. Total observation-time was 23,211 patient months (mean of

66.7 months/patient). 126/348 patients (36.2%) developed metastases while 60/348 (17.2%) died. 12/116 patients (10.3%) who developed metastases and 12/60 patients (20.0%) who died of progressive cancer, previously had VTE. Accordingly, patients with VTE had a 2-fold increased risk for subsequent cancer-relapse (Odds Ratio: 2.00; 95% CI: 1.37-2.92; P=0.008) and a more than 4-fold increased risk of dying of cancer compared to patients without VTE (Odds Ratio: 4.32; 95% CI: 2.81-6.67; P<0.0001). Median disease-free (p=0.004) and overall survival (p=0.01) in patients with VTE was significantly shorter as compared to patients without VTE.
VTE occurred more often in the presence of a tumor greater than 5 cm, in nodal positive women having more than 9 positive axillary-lymph-nodes, and in women with a history of thrombosis.
These results support the idea that the development of VTE is linked to the progression of cancer. Anticoagulant-treatment and low-molecular-weight-heparin in particular might not only reduce thrombosis incidence during chemotherapy but also improve survival in certain patients.

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ANTIPHOSPHOLIPID ANTIBODY SYNDROME IN GYNECOLOGIC CANCER PATIENTS

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Background: The condition under which certain disease i.e. thrombosis, recurrent miscarriage, and so forth coincide with the presence of high antiphospholipid antibody (APA) is termed antiphospholipid antibody Syndrome. A number of smaller studies have revealed a higher frequency of elevated APA in some patients with miscellanous malignancies. We routinely estimated anticardiolipin antibodies (ACA) and the Lupus anticoagulants (LA) in gynecologic cancer patients prior to the commencement of therapy and evaluated the clinical outcome of patients.
Methods: Blood samples were drawn from 394 consecutive cancer patients between 8:00 and 11:00 am the day before primary cancer surgery. Another 292 unselected women with histological proof of non – malignant neoplasia also underwent these tests prior to their surgery. Lupus anticoagulants was determined by a commercial test (RVVT) and a ratio above 1.2 was considered as positive. Anticardiolipin-antibodies were estimated by an ELISA (Stago, Paris), and a titer above 5 IgG GPL Unit was defined as ACA positive. No further stratification was performed.
Results: Fifty-six patients with ovarian malignancy, 255 with primary none – metastatic breast cancer, and 83 with uterine cancer were included. Before primary surgery there were significantly more cancer patients with positive LA (22.6%) than among controls (15.4%; p=0.02) while ACA incidence was similar in groups (11.6% vs. 11.4%). The prevalence of LA positive patients was significantly higher among those with a later diagnosis of thrombosis (20/60, 33.3% vs 69/334, 20.6%; p=0.04) and in patients that died of cancer (23/57; 40.4% vs. 66/271 19.6%; p=0.001) as compared to women without thrombosis and still alive. The rate of positive ACA titers allowed not to distinguish between patients with and without these events. In the univariate analysis the LA was a significant risk factor for both the development of thrombosis (RR: 2.2 95% CI: 1.21–4.16; p=0.009) and to die of cancer (RR: 2.2 95% CI: 1.30–3.82; p=0.003).
Conclusion: One out of 5 patients with gynecologic cancer was LA positive and these patients had a 1.6 fold risk for subsequent thrombosis and a more than 2 fold risk to die of cancer. Every 3rd patient who died of cancer had the LA. Our results give evidence that the antiphospholipid antibody Syndrome is a frequent condition in gynecologic malignancy and that the presence of LA is a risk factor for thrombosis and indicator of poor survival.

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INHERITED THROMBOPHILIA DEFECT IN GYNECOLOGIC CANCER

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Background: A high incidence of thromboembolic events (TEE) in the presence of impaired coagulation activation and fibrinolysis is a common observation in patients with gynecologic cancer. By contrast, neither estimations of a wide range of sophisticated coagulation tests before treatment nor monitoring with such markers during therapy allowed identification of patients with a subsequent TEE. A Medline search revealed no studies on the prevalence of hereditary thrombophilia defects in a homogenous group of cancer patients.
Methods: Blood samples were drawn from 218 unselected patients with a primary diagnosis of gynecologic cancer who are under steady surveillance every 3 to 6 months in our oncological unit. Another 154 unselected women in whom a suspected diagnosis of cancer was excluded histologically served as controls. Investigations of inherited thrombophilia defects included Factor V leiden mutation, Prothrombin mutation, homozygous Methylen Tetrahydrofolat reductase mutation (MTHFR) [Inst Genetic Research Wiesbaden], and 4G/4G variant of PAI length polymorphism [Inst Hemostaseology DKD Mainz].
Results: Patients and controls were comparable with respect to the age. None of the patients had metastasis at the time of admission. The prevalence for none of the mutations was significantly different between patients and controls: F V Leiden: 5.9% vs 8.44; p=0.4; FII: 3.74 vs. 3.4, p=1.0; MTHFR: 23.0% vs. 24.7%, p=0.86; PAI: 41,9 vs. 30.7, p=0.17. 16.7% of patients who developed TEE during follow up were heterozygous for the F V mutation compared to 3.9 % of patients without TEE (p=0.009). Moreover, carriers of the F V mutation had a more than 5 fold increased risk upon wild type carriers to die of cancer (RR: 5.29, 95% CI: 1.89–14.84; p=0.006). All patients who died had progressive stage of the malignancy. None of the remaining mutations was significantly different between cancer patients who had such an event and those who did not. All FV Leiden carriers had homozygous mutation.
Conclusion: There is an unexpectedly high prevalence of the F V Leiden mutation in patients who died of cancer. These findings would support the hypothesis, that coagulation activation may promote the spread/invasion of the cancer and thus signal poor outcome of patients. An approximately 17% rate of this mutation was present among cancer patients with TEE which is comparable to the prevalence found in patients with thrombosis in the general population.

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PLASMA VISCOSITY AND PROGNOSIS OF BREAST CANCER

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Tumor growth leads to tissue hypoxia and tissue hypoxia, in turn, is a strong stimulus for expression of genes encoding factors that promote tumor growth. A marker of the presence of tissue hypoxia may be the presence of high blood viscosity, which is found in a number of neoplastic diseases. From September 1992 to September 1998, 285 consecutive patients with localized breast cancer admitted for primary surgery were entered into this study and followed over a maximum of 7.3 years. 125 healthy women and 164 women awaiting surgery for benign tumors served as controls. Plasma viscosity (pv) was determined using a capillary tube viscosimeter (KSPV 1 Fresenius, Bad-Homburg). The day prior to primary breast cancer surgery, the mean pv was 1.33 SD:0.13 mPas which was significantly higher when compared to both patients with benign tumors (1.27 SD:0.1mPas;p<0.0001) and to healthy women (1.29 SD:0.09 mPas;p<0.0001). The total follow-up observation-time was 15,699 patient months (mean: 55.1 months/patient). Within this period, 50 of the 285 patients (17.5%) died. Patients dying of cancer had had significantly higher initial pv (1.40 SD:0.18 mPas;p<0.0001) when compared to patients not dying of cancer (1.30 SD:0.10mPas). In multivariate proportional hazard regression analysis, next to tumor size (p=0.03) and nodal status (p=0.004), pv was an independent prognostic marker for overall survival of breast cancer patients (RR=130.2;95%CI:11.6 to 1,460.6; p<0.0001). An optimized preoperative cut-off value above 1.40 mPas was significantly associated with poor outcome in the Kaplan Mayer survival-estimates, even in node-negative breast cancer.
Like-wise an increase fibrinogen/fibrin-turnover and breakdown-products characteristically associated with tumor-cell-dissemination contribute to the increased plasma viscosity while the hematocrit, leukocyte-, and platelet count contributed little to the increased blood viscosity in patients with breast cancer.

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POLYMORPHISMS IN FV GENE ASSOCIATED WITH FV DEFICIENCY – FIRST RESULTS

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Coagulation factor V plays an important role in maintaining the hemostatic balance in both the formation of thrombin in the procoagulant pathway as well as in the protein C anticoagulant pathway. The autosomal rezessive inherited FV deficiency is a rare bleeding disorder with variable phenotypic expression. Homozygote FV deficiency has a frequency od app. 1 in 1 million, for heterozygotes a frequency of 1 in 1.000 is expected. FV deficiency is characterized by reduced FV anti-

gen and/or activity. Heterocygous deficiency states are generally unrecognized because of a lack of significant clotting time prolongation or bleeding risk. The molecular basis od FV deficiency is still largely unexplored. Because of the seize of the gene (80 kb) a sequence analysis is expensive. Among the large number of polymophisms described in the gene, a few are reported to be assoziated with a lowered FV activity. A correlation between the R2 allele of FV and a slightly reduced FV activity has been reported earlier. The Arg712stop mutation, the Glu1608Lys and Tyr1702Cys variants previously have been reported more than 1 time in nonrelated FV deficient Italien patients (Castoldi, 2001). These genetic variants have been characterized in 14 German patients with FV deficiency and 95 healthy blood donors. Whereas the Arg712stop mutation could be detected neither in patients nor in the controls, the other 3 variants show allele frequencies of 0.076 (R2), 0.001 (1608Lys) and 0.021 (1702Cys) in the population. The R2 and 1702 Cys alleles were more frequent in FV deficient patients than in the controls (allele frequenciey 0.076 vs. 0.107 and 0.021 vs. 0.071 resp.) but this difference is not yet statistically significant. 1608 Lys is significantly more frequent in the patients compared to the controls (0.001 vs. 0.107; X2=89.3, p=0.008). 3 out of 14 probands with reduced FV activity and 2 of 95 controls were heterozygous for this mutation. Both, the Glu1608Lys as well as the Tyr1702Cys variants are assumed to be causative for a FV deficiency as far as they are located in the A3 domain region which is involved in the anchoring of FV to the phospholipid membrane. Thus the stability of the prothrombinase compex could be influenced by that mutations. Further phenotype/genotype analyses and expression studies have to be done to verify that assumption.

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FACTOR V LEIDEN AND OTHER THROMBOTIC RISK FACTORS IN CHD AND MYOCARDIAL INFARCTION

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Several polymorphisms in genes regulating coagulation and haemostasis have been described as risk factors for venous thrombosis. However, their role in artherial thrombosis is still unresolved. We have investigated FV Leiden (FVL Arg506Gln), FV HR2 haplotype, FII G20210A, FVII Arg353Gln, FXIII Val34Leu, MTHFR C677T and the insertion/deletion polymorphism in intron 16 of the ACE gene in 132 angiographically characterized patients with coronary heart disease, admitted to the Clinic for Internal Medicine at University Hospital Greifswald. 89 of them had a history of non-fatal myocardial infarction (MI). Regarding the results of angiography 38 had a single vessel disease, 39 a double vessel disease and 53 a triple vessel disease. As a control group we investigated 155 participants of a population-based cross-sectional epidemiological study of the same region having no sign of CHD. We found that the prevalence of FVL mutation was higher in patients than in controls (12.1% and 5.8% resp.), although this effect was statistically not significant. All the other factors investigated had the same distribution in patients and in controls. Patients with a history of MI had a significantly higher prevalence of FVL than controls (13.5%, p=0.04). Stratified for degree of coronary atheroscleosis it appears that FVL was significantly more common in patients with single vessel disease than in controls (18.4%, p=0.012). There was no difference for the other groups. The effect seems to be confined largely to women. The prevalence of FVL for female CHD patients beeing 16.7%, for women with MI 18.8% and for women with single vessel disease rising up to 40.0%. When adjusted for major cardiovascular risk factors age, hypertension, diabetes, BMI, level of cholesterol, triglycerides and fibrinogen in serum in women only, the Odds ratio for CHD was 4.7 (95% CI1.02-21.62) and for MI 7.71 (95% CI 1.16-51.0). The risk was increased 6 times (OR=6.11, 95% CI: 1.78-20.88) for all patients with single vessel disease carrying the FVL mutation. Our results did not demonstrate any importance of the genetic polymorphisms in FII, FV R2, FVII, FXIII, MTHFR or ACE I/D polymorphism for artereial thrombosis. However, the frequency of the prothrombotic mutation FVL is statistically significant increased in groups of CHD patients being at conventionally low risk (lower degree of coronary stenosis, women). In these groups FVL is associated with an increased risk for CHD and myocardial infarction.

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FACTOR XIII ANTIGEN LEVELS AND ACTIVITY IN PATIENTS WITH CORONARY ARTERY DISEASE INVESTIGATED BY ANGIOGRAPHY

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Since the description of a protective effect of FXIIIVal34Leu against thrombotic diseases, FXIII has gained attention in this field, however the role of FXIII antigen levels and activity in coronary artery disease (CAD) still remains not clear. In a case-control study higher FXIII A- and B-subunit antigen levels and lower FXIII activity were found in CAD patients compared to controls. However, those patients were insulin-resistant, a condition which has been shown to be associated with higher FXIII antigen levels, and control subjects had no angiogram to exclude asymptomatic CAD.
We investigated 516 patients admitted for angiography for suspected CAD. 365 patients had 1, 2 or 3 vessel disease, 151 were free of CAD and were used as controls. Blood samples were taken from the iliac vein and from the ostium of the left coronary artery. FXIII A-subunit antigen levels were determined by a sandwich ELISA. FXIII activity was measured by an incorporation assay.
CAD patients had a statistically not significant trend towards lower venous and intracoronary ostial FXIII antigen levels compared to controls (venous 118.2 vs. 122.9%; ostial 108.9 vs. 114.3%, p>0.5). In venous but not ostial blood, there was also a trend towards lower FXIII antigen levels with increasing number of affected vessels. Considering the total number of atherosclerotic plaques (irrespective their extent), patients with only one or two plaques had lower venous and ostial FXIII antigen levels than controls (venous 116.5 vs. 123.1%; ostial 106.8 vs. 114.7%, p>0.5), and patients with 6-14 plaques had lower ostial but not venous antigen values, but again there were no statistically significant differences. FXIII activity showed no differences between controls and CAD patients. There was no association between FXIII activity and the total number of atherosclerotic plaques.
For the first time, we present data about FXIII antigen levels and activity in controls who are angiographically verified to be free of CAD. Additionally, FXIII measures have not been performed in arterial intracoronary blood before. In our study population, there were no significant differences in FXIII antigen levels or activity between CAD patients and controls. Therefore we do not attribute an important role to FXIII antigen levels neither in development of CAD nor as a marker for the extent of underlying CAD.

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THROMBIN ACTIVATABLE FIBRINOLYSIS INHIBITOR (TAFI) LEVELS IN PATIENTS WITH CORONARY ARTERY DISEASE INVESTIGATED BY ANGIOGRAPHY

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Activated thrombin activatable fibrinolysis inhibitor (TAFI) leads to a potent attenuation of tPA-induced fibrinolysis. High TAFI antigen levels may therefore contribute to an increased risk for thrombotic disorders, but there are only little and contradictory data available about the role of TAFI antigen levels in patients with coronary artery disease (CAD):
A study in men with verified CAD found significantly higher TAFI levels in patients than in controls. Another study presented lower TAFI levels in survivors of myocardial infarction (MI). However, control subjects had no angiogram to exclude asymptomatic CAD.
We investigated 352 patients admitted for angiography for suspected CAD. 218 subjects had 1, 2 or 3 vessel disease, 134 were free of CAD and were used as controls. Blood samples were taken from the iliac vein and from the ostium of the left coronary artery. TAFI antigen levels were measured with a sandwich ELISA.
As earlier described a broad range of TAFI levels were found. Age, gender, body mass index and smoking had no influence on TAFI antigen levels. Control subjects with a family history of MI showed a trend towards higher TAFI levels than controls without a family history (121.0 vs. 107.9%, p=0.066). CAD patients had higher venous (116.8 vs. 109.3%, p=0.065) and intracoronary ostial (112.9 vs. 101.4%, p<0.05) TAFI levels than control subjects. Patients with one-vessel disease had higher ostial TAFI levels than controls (115.2 vs. 102.6%, p<0.05). Regarding the total number of atherosclerotic plaques (irrespective of their extent), the biggest differences in TAFI levels were seen between patients with only one or two plaques and controls (116.2 vs. 101.4%, p<0.05).
For the first time, we present data about TAFI antigen levels in patients with CAD and control subjects who are angiographically verified to be free of CAD. Additionally, TAFI antigen levels have not been measured in

arterial intracoronary blood before. Our results suggest, that CAD is associated with higher TAFI antigen plasma levels. Furthermore, since TAFI levels show a trend towards increased values in control subjects free of CAD but with a family history of MI, higher TAFI levels may represent a risk factor, which is under genetic control, and may therefore contribute to the development of CAD.

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***REFACTO® AF: CLINICAL PLAN FOR AN ALBUMIN-FREE B-DOMAIN DELETED FVIII-PRODUCT**

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The manufacturing process for ReFacto® was modified to eliminate all human or animal derived raw materials (including monoclonal antibodies), to provide a rFVIII product of the so called 3rd generation, providing an improved viral safety profile for hemophilia A patients. ReFacto® AF produced by this process is comparable to ReFacto® produced by the current licensed process by all biochemical, biological, structural and functional assessments.
The clinical plan now encompasses two protocols. Study 1 will be a pharmacokinetic crossover study to evaluate relative bioavailability. This pharmacokinetic study is designed to establish the comparability of the drug substance manufactured by the modified process and that manufactured by the current process. The proposed protocol will be limited to a double-blind randomized crossover evaluation of the pharmacokinetics of ReFacto® AF and ReFacto® in severe hemophilia A patients. Up to 30 patients will be enrolled to ensure that 24 will complete the study. Patients enrolled in the PK crossover study will be offered the opportunity to enroll into an open-label multinational study with ReFacto® AF. Patients in this study will receive the study medication on a routine prophylaxis regimen and/or on-demand for acute bleeding episodes based on standard-of-care dosing for a minimum of 50 EDs. Up to a total of 80 additional patients over the initial 24 PK patients will be enrolled to ensure 80 evaluable patients (defined as patients who have attained at least 50 EDs). Patients will maintain treatment records and return to the clinic after approximately 30 days and every 3 months thereafter for safety evaluations. Efficacy of ReFacto® AF during surgical procedures will be assessed as applicable. Clinical trials have been initiated recently.

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PHARMACOKINETICS OF PHOSPHOPENTOMANNAN SULFATE (PI-88) IN A NON-HUMAN PRIMATE MODEL: CLINICAL IMPLICATIONS FOR THERAPEUTIC DRUG MONITORING

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PI-88 is being developed as an anti-tumor agent due to its anti-angiogenic and anti-heparanase properties. Previous studies have reported on the potent anticoagulant effects of PI-88 The aim of this study was to identify a monitoring method for PI-88 in order to profile its pharmacokinetics in a non-human primate model. Groups of primates (n=4-6) were administered various dosages (2.5, 5.0, and 7.5 mg/kg) of PI-88 intravenously (i.v.) or subcutaneously (s.c). Citrated plasma samples were collected at various time points up to 6 hrs. The anti-IIa (AIIa) aPTT, Heptest, and ecarin clotting time (ECT) assays were used to measure the anticoagulant effects of PI-88. Neither the Heptest nor the AIIa methods were reliable to accurately calculate the circulating concentrations of PI-88. However, the pharmacokinetics of PI-88 could be evaluated by using the aPTT and ECT assays. In the i.v. studies, the area under the curve (AUC, mg/ml*min) for PI-88 was found to be: 1.2±0.3 and 1.0+/-0.1 at 2.5 mg/kg, 2.8±1.4 and 2.2±1.1 at 5.0 mg/kg, and 6.9±1.0 and 5.4±1.5 at 7.5 mg/kg, using the aPTT and ECT, respectively. In the s.c. studies, the AUC for PI-88 was determined to be 2.0±0.4 and 1.0±1.2 at 2.5 mg/kg, 3.3±1.1 and 2.5±3.0 at 5.0 mg/kg and 5.6±0.4 and 5.6±0.6 at 7.5 mg/kg, using the aPTT and ECT respectively. This data clearly shows a dose response in the AUC. In the i.v. studies, the mean clearance (CL) was calculated as 1.3±0.3 ml/(min*kg) using the aPTT assay and 1.4±0.5 ml/(min*kg) using the ECT assay. In the s.c. studies, the mean CL was determined to be 1.2±0.4 ml/(min*kg) using the aPTT and 1.2±0.3 ml/(min*kg) using the ECT. The bioavailability of PI-88 was calculated to be 1.2 and 1.0 using the aPTT and ECT data, respectively. These data indicate that PI-88 exhibits a predictable pharmacokinetic profile with a high bioavailability, which can be characterized by the aPTT and ECT assays. The increased levels of PI-88 measured by the aPTT are due to additional protease inhibitory effects, which are not measurable by the ECT. Because of the similarities in the coagulation system, the methods used in the primates can be applied to study the pharmacokinetics/pharmacodynamics of PI-88 in humans. Clinical trials to evaluate the pharmacokinetics of PI-88 in tumor patients are in progress.

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REVERSIBILITY OF GP IIB/IIIA BLOCKER INDUCED CONFORMATIONAL CHANGES OF THE PLATELET INTEGRIN RECEPTOR GP IIB/IIIA

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GP IIb/IIIa blockers can induce a conformational change from a low to a high affinity GP IIb/IIIa receptor and thereby reveal an intrinsic activating property. The reversibility of this conformational change may determine procoagulant effects of GP IIb/IIIa blockers. The conformational change of GP IIb/IIIa is monitored by a newly produced mouse IgG antibody directed against a ligand-induced binding site (anti-LIBS/Clone-145). In flow cytometry, anti-LIBS/CI145 demonstrates background binding to non- activated platelets (mean fluorescence (MF)): 4.5±0.15) and increased binding to ADP-stimulated (MF: 20±0.3, p<0.001), to GRGDSP-peptide (2mM)-incubated (MF: 34±0.2, p<0.001) and eptifibatide (10µg/ml)-incubated (MF:43±2.3, p<0.001) platelets. Dissociation of the blocker was achieved by washing platelets in Tyrode's buffer after 30 min incubation with GRGDSP or eptifibatide. Indeed, anti-LIBS/CI145 binding returned to background binding. In contrast, anti-LIBS/CI145 binding remained when a physiological concentration of fibrinogen (3 g/l) was present in the washing-buffer. To further evaluate the time course of the reversibility of receptor conformation, we mixed GRGDSP incubated platelets with untreated platelets. Directly after mixing, flow cytometry showed two separate cell populations. The fluorescence levels of these populations were approaching each other continuously and finally, within 15 minutes, formed one common population, which was located in the middle of both former cell populations. To exclude platelet specific artifacts, the findings described above were proven with CHO cells that were stably transfected with GP IIb/IIIa. In conclusion, we could demonstrate a fast reversibility of the GP IIb/IIIa blocker-induced conformational change of GP IIb/IIIa. Nevertheless, the presence of fibrinogen can inhibit this reversibility by its binding to the receptor.

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INCIDENCE OF ISOLATED CALF MUSCLE VEIN THROMBOSIS FOLLOWING LONG-HAUL FLIGHTS

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Background: The risk of venous thromboembolism after long-haul flights is still controversial. There is no data on the incidence of isolated calf muscle thrombosis (ICMVT) in flight passengers.
Objective: To evaluate the risk of deep venous and ICMVT in passengers of long-haul flights compared with a control group.
Methods: From August to October 2001 we evaluated the incidence of thrombosis in 160 passengers returning from long-haul flights (flight duration 8 hours and more) using venous compression ultrasound. The baseline examination was performed within one week before the onward flight and the second examination within 48 hours after the return flight. We used a standardized examination protocol covering all venous segments of the leg including the calf muscle veins. To evaluate the relative risk of deep venous and ICMVT, we compared the results with an age- and sex-matched control group of 160 non-traveling volunteers who were examined in the same way. In addition, we investigated common acquired and inherited risk factors for venous thrombosis in all participants. We excluded persons who were treated with antithrombotic or anticoagulant drugs or who used compression stockings during the flight.
Results: We diagnosed ICMVT in 4/160 (2.5%) flight passengers compared with 1/160 (0.6%) controls. There are no clear trends for distribution of acquired and inherited risk factors in this patients. In all persons with ICMVT, the thrombosis was located in the soleal muscle veins. All persons with ICMVT were over 55 years old. Deep vein thrombosis was not observed in either group.
Conclusion: Our results suggest an increased risk for ICMVT after long-haul flights. A larger study to confirm this preliminary result is currently under way.

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SAFETY OF COMPLETE COMPRESSION ULTRASOUND (CCUS) FOR THE DIAGNOSIS OF PROXIMAL AND DISTAL THROMBOSIS – PRELIMINARY RESULTS OF A PROSPECTIVE OUTCOME STUDY

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Background and aim of the study: Compression ultrasonography (CUS) has been shown to be the most accurate non-invasive test for the diagnosis of proximal deep vein thrombosis (DVT), but is currently not unequivocally recommended for the diagnosis of distal DVT. We developed a standardized protocol of complete compression ultrasound (CCUS) covering all venous segments of the leg. A prospective outcome study was performed to assess the clinical safety of CCUS. Main outcome measure was the incidence of venous thromboembolic complications during 3-months follow-up in patients in whom anticoagulation was withheld on the basis of normal CCUS results.
Patients and Methods: Between July 27, 2000 and October 12, 2001 1706 consecutive patients >18 years, presenting with clinical suspected acute thrombosis of the lower extremity to the vascular diagnostics unit were included. 681 patients showed abnormal results at diagnosis, life expectancy <3 months or need for anticoagulation for other reasons than thrombosis and were not chosen eligible for follow-up.
Results: Until November 15, 2001 3-months follow-up could be completed in 730/1025 patients, contacted by phone. Initially clinical probability for thrombosis was high in 270 patients, medium in 363 patients, and low in 97 patients. After 3 months 11 patients had died, one with high probability of pulmonary embolism 7,5 weeks after negative ultrasound. In 2 patients DVT was confirmed 6 and 10 weeks after inclusion into the study (venous thromboembolism 3/730=0,41%/95% CI=0,11-1,15%).
Conclusion: This preliminary result indicate that withholding anticoagulation after a single negative CCUS using a standardized examination protocol in patients presenting with suspected DVT is safe. The safety figures are within the same rage or even more favorable as with combined algorithms.

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CAMPUS – AN INTERACTIVE CASE-BASED PROGRAM AS NEW STRATEGY IN TREACHING COAGULATION DISORDERS AS DEMONSTRATED IN VON WILLEBRAND'S DISEASE

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New teaching strategies become more and more important in medical education. CAMPUS, a computer- and case-based, multimedia learning system (available as local and web application: www.medicase.de), was developed by the MediCase working group of the laboratory for Computer-Based Training in Medicine of the University of Heidelberg. With CAMPUS, authentic medical cases can be used for medical teaching in an interactive and almost realistic way. The student has to take the patient´s history, to order physical, laboratory or technical examinations, make diagnostic decisions and propose a therapy. The users can get help via expert comments or context-sensitive systematic knowledge which is available in addition to the case data on demand. Questions can be defined by the author to enhance active knowledge processing and interactivity. The aim is that users improve and test their problem-solving competence.
We developed for CAMPUS an interactive case report to study pathophysiology, a variety of laboratory tests and differential diagnoses in coagulation disorders. In this case report we present a seven year old girl with recurrent nose bleedings and hematomas. Using the interactive taking-history part of CAMPUS the student gets the information that she had a history of prolonged bleeding after adenectomy and after two dental surgeries. Now he has to decide which clinical and laboratory investigations should be performed. Then he has to determine the differential diagnoses and after further several diagnostic steps, he has to find the final diagnosis (in this case: von Willebrand disease). Finally he is asked to induce the adaequate therapy. CAMPUS provides assistance at all these different steps. After solving this case report the student should be able to handle a coagulation disorder in a logical manner thereby saving unneccesary costs, tests and blood.
CAMPUS is an innovative teaching program which has been developed to improve education in the medical school. CAMPUS cases can be used in addition to conventional medical text books, but are not intended to replace bed side practical clinical training by offering virtual patients.
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HIT II AFTER FONTAN PROCEDURE – TREATMENT WITH LEPIRUDIN

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Introduction: After Fontan-procedure or total caval-pulmonal anastomosis (TCPC) patients in many centers receive heparin treatment followed by long-term oral anticoagulation. Heparin-induced thrombocytopenia is a potentially dangerous complication of heparin-therapy in children as well as in adults and may cause life-threatening thromboembolic events.
Case report: A 2½ year old boy with complex congenital heart disease (double inlet left ventricle, VSD, ASD and hypoplastic mitral valve) received TCPC-surgery 1.5 years after a bidirectional Glenn-anastomosis. The boy developed an increasing heparin-demand (max. 29 IE/kg/h) to achieve a therapeutic aPTT-prolongation. Between day 6 and 9 after surgery platelet count decreased from 217 G/l to 73 G/l (about 66%). So far, there was no sign for a thromboembolic event, however, the prothrombin fragments 1+2 were slightly increased (1.2 nmol/l). The suspected HIT-II diagnosis was confirmed by ELISA and Heparin-induced platelet aggregation (HIPA). After cessation of heparin the patient was treated with lepirudine for 8 days. A therapeutic aPTT prolongation was reached with a lepirudin dose between 0.09-0.12 mg/kg/h. Over the following days platelets increased to normal values. Drainage of pleural effusion and insertion of a chest tube were performed without bleeding complications. 6 days after initiation of phenprocoumon treatment, lepirudin was stopped.
Conclusion: Only a few reports address treatment of lepirudin in children with HIT II. In case of a decrease of platelet count and heparin-resistance HIT II should be considered. The use of lepirudin in our patient resulted in an effective anticoagulation with stable aPTT-values and a recovery of the platelet count, no thrombosis was observed. The change to oral anticoagulation with phenprocoumon under protection with lepirudin was uncomplicated.

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FUNCTIONAL ANTITHROMBIN-III LEVELS IN PATIENTS ADMINISTERED LOW-MOLECULAR-WEIGHT HEPARIN (CLIVARIN) VS. UNFRACTIONATED HEPARIN FOR THE THROMBOPROPHYLAXIS IN HIP AND KNEE REPLACEMENT SURGERY

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Hip and knee replacement surgery are associated with a high risk of thromboembolic complications (40-70% incidence). Post-surgical prophylaxis is highly desirable in these patients. The LMWHs are now considered as a gold standard and have been recommended by the American College of Chest Physicians as the drugs of choice for this indication. Clivarin (Reviparin; Knoll AG, Luwigshafen, Germany) represents a nitrous acid depolymerized LMWH (M.W. ~4 kD, potency 110 anti-Xa IU/mg). This drug has been extensively investigated for both the prophylaxis and treatment of thrombotic disorders. Unlike UFH, this drug exhibits superior safety and efficacy profiles. This sub-study was designed to determine the differential effect of 4,200 anti-Xa IU of clivarin administered subcutaneously once-a-day and 7,500 IU of UFH twice-a-day for 11 to 14 days (ECHOS Study) on plasma ATIII levels. Both drugs were initiated pre-operatively. Citrated blood samples were drawn prior to, 2 to 4 days, 11 to 14 days, and 6-8 weeks post-treatment. The ATIII levels were measured using a chromogenic substrate method. In the interim analysis performed on the first 100 patients, the functional ATIII levels were found to be decreased (<75% of baseline) in 25% of patients treated with UFH. In contrast, only 8% of patients treated with clivarin showed an ATIII level <75% baseline. Despite this difference, the circulating levels of anti-Xa activity, Heptest anticoagulant effect, and tissue factor pathway inhibitor release, were consistently higher in the clivarin-treated patients. These differences were not seen in samples collected at 6 to 8 weeks, post-treatment/surgery. Interestingly, the relative prevalence of anti-heparin-platelet factor 4 (AHPF4) antibodies did not differ significantly at any time point except on days 11 to 14, where a much higher AHPF4 antibody titer was observed in the UFH-treated patients. The clinical results indicated that clivarin produced significant reduction in thromboembolic complications in comparison with UFH. The surrogate marker analysis in the relative sparing of functional ATIII in the clivarin-treated patients is highly suggestive of the increased efficacy of this LMWH

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RANDOMISED CROSS-OVER STUDY OF NEAR PATIENT TESTING VERSUS HOSPITAL LABORATORY TESTING WITH COMPUTER ASSISTED DOSING IN A COMMUNITY ANTICOAGULANT CLINIC

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Near patient testing monitors allow the introduction of anticoagulant services outside hospital. The study looked at the reliability of International Normalised Ratios (INR) measured by a near patient device (CoaguChek) used in a community clinic over a period of 12 months compared with INR measured in the hospital laboratory. To ensure valid comparison between the groups it was based on a six month cross over design. There were two aims to assess:

1. Any measurable difference in patients' time in INR range or frequency of clinic attendance.
2. Patients' satisfaction with a community based service using a near-patient testing monitor.

Methods: Forty-Six patients were enrolled and divided into two groups. Group one had the INR measured by the CoaguChek (CC) device for the first 6 months. In the second six months the INR was determined by the hospital laboratory. In group two the order was reversed. Each patient dosed using the result from the CC device had a second sample sent to the hospital laboratory and the result of the INR on this sample was determined for comparison. Similarly, each patient dosed using the INR obtained in the hospital laboratory had a simultaneous CC reading. All patients were dosed using computer assisted dosing (Dawn 4S). The results of this study were analysed using a Bland-Altman plot.
Patients completed a questionnaire before and at the end of the study to assess patients' satisfaction with the community clinic compared to the hospital based service.
Results: The Bland-Altman plot indicated that the INR differences increased as the average INR increased, but the mean relative deviation was below 10% up to an INR of 4.0. There was no evidence of a significant difference between the geometric mean INR with the monitor and the laboratory systems. There was no evidence of a difference in the mean percentage of time within target INR range with either the monitor or laboratory INR testing. Results of the questionnaire circulated to patients at the end of the study found that 98% of patients expressed a preference for a community clinic.
Conclusion; This study shows that equal success can be achieved in a community based clinic with control by home PT monitor, rather than relying on traditional laboratory testing in a hospital clinic. Patients expressed greater patient satisfaction with home PT monitor testing in the community clinic.

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***COFACTOR- AND SUBSTRATE-INDUCED ACTIVATION OF FACTOR VIIA AND FACTOR IXA**

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Factor VIIa initiates the extrinsic coagulation cascade; this event requires a delicately balanced regulation that is implemented on different levels, including a sophisticated multi-step activation mechanism of factor VII. Its central role in hemostasis and thrombosis makes factor VIIa a key target of pharmaceutical research. We succeeded, for the first time, to recombinantly produce N-terminally truncated factor VII (rf7) in an *E. coli* expression system by employing an oxidative in vitro folding protocol, which critically depends on the presence of ethylene glycol. Activated recombinant factor VIIa (rf7a) was crystallised in the presence of the reversible S1-site inhibitor benzamidine. Comparison of this 1.69 Å crystal structure with that of an inhibitor- and sulphate-free, but isomorphous crystal form identified structural details of factor VIIa stimulation. The stabilisation of Asp189 and Ser190 by benzamidine, and the capping of the intermediate helix by a sulphate ion appear to be sufficient to mimic the disorder – order transition conferred by the cofactor TF and the substrate factor X. Factor VIIa shares with the homologous factor IXa, but not factor Xa, a bell-shaped activity modulation dependent on ethylene glycol. The ethylene glycol binding site of rf7a was identified in the vicinity of the 60-loop. Ethylene glycol binding induces a major conformational rearrangement of the 60-loop. This region serves as a recognition site of the physiologic substrate, factor X, which is common to both factor VIIa and factor IXa. These results provide a mechanistic framework of substrate-assisted catalysis of both factor VIIa and factor IXa.

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MEASUREMENT OF ANTITHROMBIN ACTIVITY: COMPARISON BETWEEN BERICHROM ANTITHROMBIN AND COAMATIC LR ANTITHROMBIN

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There exist two principles to determine antithrombin activity; the thrombin-dependent methods and the factor Xa-dependent methods. It is reported in the literature that under heparin therapy thrombin-based methods overestimate the antithrombin activity because the dependence of heparine cofactor II (HC II). The aim of our study is to compare the influence of HC II on two relatively widely used AT assays, Berichrom® Antithrombin (thrombin-dependent) and COAMATIC® LR Antithrombin (factor Xa-dependent).
For the measurement of the AT activity we used the Berichrom AT (Dade Behring) and the Coamatic LR AT (Haemochrom Diagnostica) test kits. The heparin levels were controlled with Berichrom Heparin calibrated with the LMWH-standard from Haemochrom Diagnostics. The HC II was measured with the Unitest HC II (Unicorn Diagnostics Ltd.).The PTT was measured with Pathromtin SL (Dade Behring). All investigations were carried out with a BCT (Dade Behring). We included patients with hereditary and acquired AT deficiency with (UFH and LMWH)/without heparin treatment and patients with AT III deficiency.
The mean AT III concentration with the Berichrom assay and the COAMATIC assay was 72.0% (95% CI 70.2–73.8) and 73.0% (95% CI 71.3–74.7), respectively, and there was no statistically significant difference (p=0.53). There is no significant difference between the two assays up to anti-Xa levels of 1 U/ml. The influence of HC II is similar for both IIa and Xa dependent tests. The thrombin- dependent assay overestimates AT III levels only at very high concentrations of HC II. The influence of this cofactor seems generally to be low.
Both the thrombin - dependent and the factor Xa - dependent assay give nearly identical results of AT activities in patients under heparin treatment (both unfractionated and LMWH). That means the influence of the HC II on the thrombin - dependent test is only marginal. This is an effect of the use of bovine thrombin that minimises the influence of HC II. The data are influenced by the changes on the test components, particularly the origin of the thrombin used, and the changes in the incubation time. Therefore, these results cannot be extrapolated for other thrombin - dependent antithrombin assays. The influence of HC II can be minimised by selecting suitable test conditions.

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HIT II DIAGNOSTIC AT THE BCT (DADE BEHRING)

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The HIT II is a severe complication under heparin therapy. The laboratory tests to recognize a HIT are time-consuming and no test has a sensitivity and specifity of 100%. The best of all available and practicable tests is the original HIPA test from GREINACHER at a microtiter plate. The tests are adapted to the aggregometers and also to automated systems like BCT from Dade Behring. The aim of our study was to compare the GREINACHER method adapted to an aggregometer with an automatized version coagulation analyzer.
Patients with HIT II (defined as positive in the GREINACHER assay and positive in the ELISA test for antibodys against the PF4-heparin complex (Diagast assay with an IgG antibody) and clinically defined HIT II were reinvestigated at the BCT with three to four blood donors and a control aggregation with ADP. The test is a modification from the original method adapted to the BCT. The observation time is 20 minutes and the investigated heparin concentrations are 0.2, 0.5 and 100 U/ml of heparin. The reinvestigation at the BCT was done simultaneously with a reinvestigation at the aggregometer with the same donor thrombocytes to ensure the comparability of the results.
There is a good aggreement of both methods. The patients with positive HIT II were also found at the BCT method. All patients with signs of HIT II (laboratory and clinically definition) were also recognized at the BCT method. The investigation time is about 60 minutes testing three blood donors.
The advantage of the BCT method is the time saving. The method is expansible to more tested blood donors without additinal work for the technicians. It is possible do define a formula to calculate the result in percent of aggregation and to test more heparin concentrations (for example also 0.1 U/ml) because we know from our experience that there exists a time dependent reaction with different therapeutic heparin concentrations. The curves are useful to document the results and is possible to measure two ore more patients in one run. The disadvantage is the same like in all HIPA-tests. The results are a function of the functionality of the prepared thrombocytes of the blood donors.

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F VIII:C MEASUREMENT – COMPARISON BETWEEN CHROMOGENIC AND COAGULOMETRIC METHODS IN HEMOPHILIA A PATIENTS UNDER THERAPY WITH THE B-DOMAIN-DEPLETED RECOMBINANT F VIII-PRÄPARATION REFACTO

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Introduction: Since any years the recombinant F VIII from Wyeth/Genetic Institute is also available in Germany. The difference between ReFacto with the active substance Moroctocog alfa is the lack of B domain. This leads to problems in the coagulation laboratories in monitoring the f VIII-activities in patients with ReFacto. An alternative way to obtain comparable results is to use the ReFacto laboratory standard. With this special standard (without charge) it is possible to measure comparable values with the one stage clotting assay and the as reference method evaluated chromogenic substrate assay.
Patients and Methods: In two patients with severe hemophilia A and ReFacto-therapy we made a recovery over 24 hours (baseline, 30 min., 60 min., 120 min., 2, 3, 6 and 12 hours after injection of 20000 U ReFacto). We measured the F VIII:C-levels with two one stage clotting assays (as aPTT reagent Pathromtin SL from Dade Behring and DappTin from Progen Immuno, both Germany) and three chromogenic assays (F VIII chromogenic from Dade Behring, Immunochrom F VIII, Progen Immuno and COAMATIC F VIII, Haemochrom Diagnostica, also Germany).
Results: There is a good correlation between all three chromogenic assays (r^2>0.96). There is also a good agreement between chromogenic and coagulometric measurement of F VIII:C (r^2>0.95 for DappTin and r^2>0.81 for Pathromtin SL) with the F VIII laboratory standard. If we use a plasma standard the one stage clotting assay underestimates the F VIII-value , whereby there are great differences between the aPTT-reagents. The DappTin method is in closer agreement with the chromogenic substrate method.
Conclusions: Patients under treatment with the B domain depleted recombinant F VIII ReFacto couldn't be controlled with the conventionally used one stage clotting assay and the corresponding plasma standards because of the underestimation of F VIII activities. An alternative way is the use of ReFacto laboratory standard. This phenomenon is due to the decreased thrombin activation of F VIII because of the lack of the B domain in this recombinant F VIII preparation.

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SUBSTANTIAL ANTI-XA ACTIVITY REMAINS FOLLOWING NEUTRALISATION OF LMW HEPARIN – AN MAJOR CAUSE OF IMPROPER DIAGNOSIS

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Objective: Low molecular weight (LMW) heparins have proven to be effective and safe in both prophylactic and therapeutic treatment of deep vein thrombosis. LMW heparins are characterized by an antiXa to antiIIa-activity ratio >1,5. Anti-Xa activity remains uninfluenced following neutralization by protamine. It is unclear, whether heparin antidotes supplied with testkits are sufficient for inactivation of conventional heparin as well as LMW heparin. Previously we described significantly higher protein S (PS) activities than free PS concentrations in patients under treatment with LMW heparin. The aim of this study was to investigate the influence of LMW heparin on protein S activity, diluted Russel viper venom time (dRVVT) and tissue factor pathway inhibitor (TFPI) activity in factor Xa dependent assays.
Methods: Reference plasma was incubated with Fraxiparinâ in various concentrations (final concentration 0,37 U/ml; 0,78 U/ml and 1,34 U/ml) for 10 min. PS activity and dRVVT were determined using two different commercial kits. The influence of LMW heparin on TFPI activity was investigated by incubation of different TFPI standards.
Results: All applied LMW heparin concentrations lead to prolonged clotting times in the PS activity assay based on factor Xa, which falsely indicates normal or increased PS activity. Within the therapeutic range LMW heparin showed no influence on evaluated dRVVT-assays. In samples with anti-Xa activities >1,0 U/ml an influence of LMW heparin has to be considered in dependence of the specific assay. The incubation of TFPI standards with LMW heparin >1,0 U/ml lead to lower absorbance in standards >0,06 U/ml TFPI and fakes a false high TFPI activity.
Conclusion: Heparin antagonists supplied with different assays neutralize conventional and LMW heparin in different ways. Substantial anti-Xa activity remains following neutralization of LMW heparin containing samples. The assessment of different haemostaseological parameters in factor Xa-dependent assays can lead to improper diagnoses in cases with unclear or missing information on therapeutically applied LMW heparins.

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MOLECULAR CLONING OF TEPI MUTANTS: FIRST EXPRESSION OF [P151L]TFPI IN INSECT CELLS

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Tissue factor pathway inhibitor (TFPI) is an important regulator of the extrinsic blood coagulation pathway. We recently discovered the first natural TFPI polymorphism which significantly correlates with a higher risk for venous thrombosis. In order to study the biochemical and pathophysiological role of this 536C→T mutation, we have now developed a system for the expression of TFPI and its variants in insect cells.
Therefore, RNA was isolated from human SW1353 chondrosarcoma cells and the TFPI coding cDNA was obtained by reverse transcription polymerase chain reaction (RT-PCR). The amplified 915 bp fragment was cloned into the vector pCR2.1 and the sequence was verified by double-stranded sequencing.
The 536C→T mutation was introduced into the cloned TFPI cDNA by site-directed mutagenesis. For protein expression in insect cells the wildtype and mutant TFPI cDNA constructs lacking the signal sequences were subcloned into the expression vector pMIB/V5-HisC. High Five insect cells were then transiently transfected with the cloned vectors and the expression of the TFPI-V5 fusion protein was monitored in cell culture supernatant for 96 hours. Using a total TFPI ELISA a maximum expression rate of 6.4 ng/mL (SD±0,79 ng/ml) was measured in cell cultures transfected with the wildtype and the mutant TFPI plasmids. No quantitative differences were found between the expression of the mutant and the wildtype protein, and TFPI expression was not detected in the controls (untransfected cells and cells transfected with the chloramphenicol acteyltransferase control vector, detection limit: 0.36 ng/ml) either.
In summary we have successfully expressed TFPI in insect cells and have developed a new model system as an essential prerequisite for the detailed analysis of the biochemical properties of TFPI. Furthermore, our expression system is suitable for all other variants of this important blood coagulation inhibitor.

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ANALYTICAL CHARACTERIZATION OF DIFFERENT PLASMA QUALITIES USED FOR FRACTIONATION

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Human plasma is the valuable starting material for the production of functional proteins used in the therapy of coagulation, hematological and immunological disorders. The quality of this heterogeneous biomaterial depends on two main factors: the health of the blood or plasma donor and the method of plasma preparation. Currently assessment of plasma quality is focused on the common serological and PCR tests to minimize the risk of transmission of blood-borne pathogens. Little attention is paid to the variables, which ensure a consistent manufacturing process for plasma fractionation. Defined raw materials are a major concern for a manufacturer of therapeutics. We therefore studied selected biochemical and hematological variables as markers of different plasma separation techniques and plasma pre-treatment procedures.
We investigated six different plasma types: source plasma (Baxter Fenwal: Autopheresis-C, Haemonetics: PCS 2), recovered plasma (classic) and three different types of inline filtered plasma prepared from leukocyte-reduced whole blood using three different filter systems. Selected representative analytical variables were assayed by standard test systems: protein content, coagulation proteins (FVIII, vWF), activation markers of coagulation (TAT, prothrombin fragments 1+2, activation of complement system (complement factor C3a), thrombogenicity marker (FXIIa), residual cell content (platelets, erythrocytes, leukocytes using FACS analysis), and the cell-specific variables of lactate dehydrogenase, platelet factor 4 and beta-thromboglobulin.
We found substantial differences in complement activation as indicated by C3a measurements, TAT values and, to a minor extent, FXIIa levels. The most striking differences were found in residual cell content and the levels of the platelet-associated proteins, platelet factor 4 and beta-thromboglobulin. We could show that the passage of blood through leukocyte reduction filters disrupts cells and remaining platelets, and thus promotes the release of platelet factor 4 and beta-thromboglobulin. In addition, we could show that the inline filtration process of whole blood can lead to activation of the complement system. Thus, biochemical and cellular surrogate markers are valuable discriminators of different plasma types. This finding might help to standardize plasma as a source material for biopharmaceutical drugs.

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CHARACTERIZATION OF FACTOR VIII/VON WILLEBRAND FACTOR – COMPLEX CONCENTRATES BY EXAMINATION OF PLATELET-BINDING PROPERTIES UNDER SHEAR STRESS CONDITIONS

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Von Willebrand disease (vWD) can be treated by replacement therapy with high and intermediate purity factor VIII (FVIII) concentrates purified from human plasma, which also contains von Willebrand factor (vWF). Although all concentrates lack the highest molecular weight multimers found in plasma, there is evidence that they control bleeding in patients with different vWD-types. Currently characterization of von Willebrand factor is based on methods as ristocetin cofactor- and collagen binding assays done under static conditions. To assess whether FVIII-vWF-concentrates can promote platelet adhesion under shear stress we characterized them by simulating in vivo flow conditions. We characterized vWF in two flow models by preparing different perfusates by supplementing mixtures of erythrocytes and platelets with varying amounts of vWF from plasma-derived FVIII/vWF concentrates. These mixtures were subjected to the platelet function analyzer, PFA-100 under standard conditions using ADP cartridges. We also compared the potency of the different vWF-containing samples to promote platelet adhesion to a collagen surface in the parallel plate perfusion chamber, described by Sakariassen at the high shear rate of 2500 sec^{-1}. Platelet-poor plasma and human serum albumin were used as controls. The potency to facilitate platelet adhesion to a collagen surface was compared in three high purity and one intermediate FVIII/vWF concentrate. All concentrates lacked the highest molecular weight multimers and were found to promote platelet adhesion in a dose-dependent manner. This result was confirmed by applying the same artificial whole blood to the platelet function analyzer, PFA-100, equipped with ADP-cartridges. For the FVIII/vWF concentrate, showing the highest efficacy in the parallel plate perfusion, definite closure times, although above the normal range (71-118 sec), could be measured when vWF concentrations higher than 1.0 RCoF IU per ml of total perfusate were applied. With the other concentrates no closure time could be determined. Mixtures of erythrocytes and platelets supplemented with platelet-poor plasma served as controls for the normal range. Our data show that FVIII/vWF concentrates can promote platelet adhesion under flow at high shear stress even if they lack the highest molecular weight multimers.

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REFERENCE VALUES OF COAGULATION GLOBAL TESTS IN CHILDREN ARE SIGNIFICANTLY BUT IN PRAXI NOT RELEVANTLY DIFFERENT TO THOSE FROM ADULTS

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Laboratory tests play an important role in differential diagnosis of our patients. They have, however, little practical impact, until clinical studies have ascribed various states of health or disease to certain values of the tests. So-called reference values/intervals describe the usually obtained results from apparently healthy or physiologically normal individuals who are commonly adults in the age range between 20 and 50 years. Values for other age groups, older people and especially children, are only rarely available.
We compared the results of the three global tests APTT (Actin FS), PT (Innovin), TT (Thrombin Reagenz Roche), the activity rates of antithrombin (Chromogenix), and the derived fibrinogen concentration from 196 adults in the younger age range of 19 to 30 years with the values from 87 children (newborn until the age of 18 years). The children were exclusively those who did not have a bleeding tendency or any thromboembolic complications due to other underlying diseases such as systemic lupus or malignancy in their past or present medical history. Consecutive blood samples were evaluated from routine laboratory specimens from children mostly scheduled for elective surgery. The group of adults comprised healthy controls without thromboembolic or hemorrhagic events in their medical history.

test	units	children		adults		p ≤
		mean	mean±SE	mean	mean±SE	
APTT	sec	30,25	27,103±33,40	28,86	24,14±33,58	0,0000
PT	percent	95,87	75,18±116,57	109,36	80,14±138,59	0,0002
TT	sec	18,39	15,37±21,41	18,19	15,60±20,79	0,7118
fibrinogen	g/l	2,82	1,82±3,81	2,99	1,64±4,34	0,6368
AT	percent	1,12	0,98±1,27	1,04	0,88±1,19	0,0081

Results are given in the table. APTT, TT and AT were significantly, the absolute values, however, were only slightly different, i. e. clinically not important. These findings are in contrast to other laboratory groups which could measure substantially higher values especially for the APTT in children. Possibly prolongations due to the common finding of lupus anticoagulants in those patient groups could explain this.

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POSTTHROMBOTIC SYNDROME IS A RARE FINDING IN PATIENTS AFTER DEEP VEIN THROMBOSIS (DVT) – 3 TO 45 YEARS F FOLLOW-UP

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The postthrombotic syndrome is characterized by heaviness, pain, and swelling of the affected leg. Cumulative incidences of 3% after one year have been reported (Prandoni 1996). No apparent relationship between recurrent DVT and the development of the syndrome has been found. Since thrombolysis therapy will faster lead to patent vessels than anticoagulation alone, the postthrombotic syndrome was seen in those patients with lesser frequency (Hirsh, Lensing 1996). Since 1986 1300 patients with DVT were treated with lysis therapy in the former Luebeck thrombophilia center. High Patency rates (up to 80%) were usually achieved with lysis. For the past 3 years we have begun a follow-up program for the treated patients as well as the patients who did not undergo lysis therapy for various reasons.
We have so far seen 401 patients (205 females, age 18 to 87 years at the time of follow-up), 128 patients with DVT and lysis therapy, 151 patients with contraindications for lysis, 122 additional healthy volunteers served as controls (mean age in all 3 groups: 57 years). In all patients, the past medical history was thoroughly taken, physical examination focused on DVT and a Doppler ultrasound of the affected leg was performed. The main risk factors for thromboembolic complications were determined, and no statistical difference was found. The use of supportive stockings or of coumadin was the same in both DVT groups. A positive family history for DVT was found to the same extent in all three groups.
Both DVT groups did not differ concerning the feeling of heavy legs, the number of calf cramps, the verification of varicosis, hyperpigmentation, or ulcerations (only 2 patients without lysis therapy). A significant difference concerning the tendency for swollen feet or legs could be found only in patients without lysis therapy.
The effect of lysis therapy can only be demonstrated during the acute phase of DVT, in which a quick recanalization of an occluded vessel occurs more rapidly. This effect fades over time. However, patients with DVT, regardless of the therapy they received, show a higher incidence of subjective and objective complaints in comparison to a control group.

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CRYOPRESERVATION OF PLATELET CONCENTRATES COMPARED TO ROUTINE BLOOD BANK METHODS BY FLOW CYTOMETRY (QUANTIBRITETM)

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Purpose: This study was designed to compare cryopreserved platelet concentrates (PCs) with PCs stored on a horizontal flat bed agitator. During storage of PCs a constant decrease in platelet function due to the storage lesion is seen. It is associated with in vitro activation of platelets and shows in a higher surface expression of activation dependant neoepitopes (CD62p).
Methods: 36 PCs were obtained by plateletpheresis procedures. Half of the split PCs were stored under routine blood bank conditions on a horizontal flat bed agitator (LPR1, Melco Engineering Corp., Glendale, USA) with an agitation rate of 60 shakes/min at room temperature (22±2°C) for five days (samples taken every day (d1-5)). The other 18 PCs were cryopreserved. Before cryopreservation the first sample (before) was taken. Autologous plasma was frozen and thawed separately. PCs were transferred into special freezing containers and 1.2 ml of storage solution (ThromboSol (TC) , LifeCell Corp., Branchburg, USA) were added. PCs were placed into an aluminium cassette and directly put into the gas phase of a nitrogen tank (-196°C; one week), thawed and resuspended in the autologous plasma. PCs were allowed to rest for 1h before a sample (1h) was taken. PCs were stored for further 24 hrs followed by final analysis (24hrs). Flow cytometric analysis was performed with a FACSCalibur® cytometer (Becton Dickinson, USA). A QuantiBriteTM PE quantitation kit was used and platelets were incubated with monoclonal anti-

bodies (PE/antibody ratio 1:1) in saturating concentrations. Results were expressed as antibodies bound per cell (ABC).
Results: Cryopreserved PCs (1h) display significantly more ABC than horizontally stored PCs at d1. Compared to d5 of horizontal storage there was no significant difference between ABC.

	1h	d1	d5
CD62p	1475±296	879±323	1579±349
p-value		<0.05	>0.05

Table 1: ABC 1 h after cryopreservation compared to d1 or d5 of horizontal storage
Conclusions: Cryopreserved PCs show a significant increase in ABC over the course of measurement (before,1h 24hrs; data not shown). Compared to horizontally stored PCs at d1 and d5 of storage cryopreserved PCs display an amount of ABC as horizontally stored PCs at d5. We conclude that cryopreservation of PCs results in a higher platelet activation than horizontal storage of PCs. Expression of CD62p is moderate as it is comparable to ABC of PCs at d5.

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A ROLE FOR P38 MAPK IN THE PPARGAMMA-MEDIATED INHIBITION OF PLASMIN INDUCED ACTIVATION OF HUMAN PERIPHERAL MONOCYTES
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It has been proposed that activated peroxisome proliferator-activated receptors (PPARs) belonging to the family of nuclear receptors may convey antiinflammatory activity in monocytes and macrophages. We have previously shown that the serine protease plasmin, but not catalytically inactivated plasmin, triggers chemotaxis and NF-kappaB and AP-1-mediated induction of tissue factor and proinflammatory cytokines in human peripheral monocytes.
We have now investigated the effects of PPAR ligands on the plasmin-mediated activation of human peripheral monocytes. Monocytes express primarily PPARgamma, but only traces of PPARalpha. PPARgamma ligands such as 15-deoxy-delta12,14-PGJ2 and citglitazone, but not the PPARalpha ligand clofibric acid, inhibited the plasmin-induced chemotaxis of monocytes as well as cytokine biosynthesis through inhibition of NF-kappaB and AP-1 as shown by electrophoretic mobility shift assays. Activation of PPARs in human monoctes was analysed in nuclear extracts by surface plasmon resonance. It could be shown that the PPARgamma ligands used indeed triggered binding of PPARgamma to PPAR response elements immobilized on the sensor chip. WY14,643, generally considered as PPARalpha activator, was identified as a promiscuous ligand activating PPARgamma as well. In agreement with the low abundance of PPARalpha in monocytes, there was no significant PPARalpha activation after stimulation with clofibric acid or WY14,643. In order to further elucidate the mechanism of the inhibitory efficacy of PPARgamma ligands on the plasmin-induced monocyte activation, we investigated the effect of PPARgamma activation on the plasmin-induced phosphorylation of p38 MAPK. The p38 MAPK is able to activate transcription factors such as AP-1. Experiments with the specific p38 inhibitor SB203580 revealed that p38 is essential for both, plasmin-induced monocyte migration as well as for the proinflammatory gene expression. Indeed, with phosphospecific antibodies it could be shown that plasmin triggers p38 phosphorylation required for activation; this phosphorylation was effectively inhibited by PPARgamma ligands. By contrast, FMLP led only to a weak p38 activation that was dispensable for the FMLP-induced chemotaxis. Thus, PPARgamma activation leading to inhibition of the p38 activation might represent the mechanistic basis for the PPARgamma-mediated inhibition of plasmin-triggered proinflammatory monocyte functions.

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EXPRESSION OF FUNCTIONAL PROTEASE-ACTIVATED RECEPTORS IN HUMAN PERIPHERAL MONOCYTES AND ANTIGEN PRESENTING CELLS
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Protease-activated receptors (PARs) belong to the G-protein coupled receptor family. PARs are proteolytically and irreversibly activated either by thrombin, as shown for PAR-1, PAR-3 and PAR-4 or by trypsin, coagulation factor VIIa and Xa, as demonstrated for PAR-2. We have now investigated the expression of PARs in human peripheral monocytes and professional antigen-presenting cells.
Monocytes isolated by Percoll gradient centrifugation were free of platelet contamination as judged by the analysis of CD41 both by RT-PCR and flow cytometry. Monocyte-derived macrophages were differentiated in vitro by exposure to macrophage colony-stimulating factor (M-CSF) over a period of eight days. Monocyte-derived dendritic cells were differentiated in vitro by the addition of granulocyte-macrophage colony-stimulating factor (GM-CSF) combined with interleukin (IL)-4 over a period of six days. PAR expression was analysed in these cells both at the mRNA level by semiquantitative RT-PCR with gene-specific primers, and at the protein level by flow cytometric analysis.
Monocytes express mainly PAR-1and PAR-3, and very little PAR-2. During differentiation into macrophages expression of PAR-1, PAR-2 and PAR-3 was maintained or slightly upregulated. In contrast, in dendritic cells a strong downregulation, both at the mRNA and protein level was observed. Although dendritic cells still contained small amounts of PAR-1, PAR-2 and PAR-3 mRNA, PAR expression in terms of protein could not be detected at the cell membrane. Experiments with IL-4-treated macrophages revealed that the anti-inflammatory cytokine IL-4 is responsible for the transcriptional down-regulation observed in dendritic cells. In all cell types investigated PAR-4 remained undetectable. Functional activity of PARs was shown in monocytes and macrophages by stimulation with receptor-specific activating peptides (TRAPs) and FURA-2-based analysis of cytosolic calcium levels. The data indicate that monocytes and macrophages express functional PAR-1 and PAR-3 receptors on their cell surface, despite the fact that in other cell types PAR-3 was claimed to be non-functional. Consistent with the flow cytometric results on PAR expression, no calcium mobilization was detectable for PAR-1, PAR-2 and PAR-3 stimulation in dendritic cells and for PAR-2 in monocytes and macrophages.
Together these data suggest that PARs might be involved in the regulation of monocyte and macrophage functions in vascular inflammation.

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***EXPERIENCES WITH CLOPIDOGREL IN PATIENTS WITH CORONARY HEART DISEASE: ARE ALL PLATELETS INHIBITED?**
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Thienopyridines (ticlopidine and clopidogrel) are widely used effective antiplatelet drugs for the prevention of thrombotic events in ischemic cardiac, cerebrovascular, and peripherial arterial diseases. Thienopyridines prevent ADP-induced platelet activation and aggregation by inhibition of the platelet $P2Y_{12}$ ADP receptor. Although the combination of thienopyridines with aspirin has been found very effective in the prevention of thrombotic events after invasive coronary interventions, the risk of coronary sub-acute stent thrombosis still remains between 1% and 2%. This indicates that the inhibition of platelet activation may not be sufficient in certain patients despite therapy with thienopyridines.
We have developed a novel flow cytometry based method using phosphorylation-specific antibodies for the quantitation of the phosphorylation state of the vasodilator-stimulated phosphoprotein (VASP), a signalling molecule involved in platelet inhibition. This assay allows determination of the platelet inhibition state and exact quantification of inhibitory effects of thienopyridines.
Based on this assay, we carried out a prospective evaluation of this functional platelet assay with regard to the efficiency of the thienopyridine-aspirin regimen and the occurrence of coronary sub-acute stent thrombosis. In a series of 734 consecutive stented patients, a strong correlation was found between the results of the flow cytometric platelet VASP assay reflecting weak or strong platelet inhibition and the occurrence or non-occurrence of coronary stent thrombosis, respectively. All patients who presented a sub-acute stent thrombosis revealed insufficient platelet inhibition as detected by the platelet VASP assay. Three groups of patients could be identified based on this assay: 1) patients with a strong response to thienopyridines and a strong inhibition of ADP-induced platelet activation within a few hours after start of thienopyridine treatment, 2) patients with a delayed response showing significant platelet inhibition later than 3 days after start of thienopyridine treatment, and 3) patients who showed weak or no inhibition of ADP-induced platelet activation despite thienopyridine treatment. In a follow-up study, we determined the percentage of thienopyridine non-responders and analyzed the molecular basis of their lack of thienopyridine effects with regard to plasma levels of clopidogrel metabolites, ADP receptor signalling and function of intracellular signalling pathways.
Identification of thienopyridine non-responding patients as well as monitoring of thienopyridine effects will certainly help to prevent the occurence of thromboembolic events in patients at risk.

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INHIBITION OF PLATELET-LEUKOCYTE CONJUGATE FORMATION BY A P2Y12 ADP-RECEPTOR ANTAGONIST (AR-C69931)

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On platelet activation, platelets adhere to leukocytes mainly via platelet expressed P-selectin and its receptor on leukocytes (PSGL-1), as well as via fibrinogen bridging between GP IIb/IIIa on platelets and CD11b/CD18 on leukocytes. Upon adhesion a new cell type is formed, platelet-leukocyte conjugates, and these conjugates are characterized by specific properties, such as a transcellular metabolism or the expression of tissue-factor activity on monocytes. An increased number of platelet-leukocyte conjugates in blood samples of patients with different diseases, such as coronary artery disease, unstable angina, sepsis or after angioplasty or cardiopulmonary bypass operations, have been reported by several authors. The contribution of these conjugates to the development of these diseases or for complications during operations has been discussed. Therefore, an inhibition of platelet-leukocyte conjugate formation seems to be an important target in the treatment of these diseases or for the reduction of complications occurring during angioplasty or cardiopulmonary bypass operations.
We have shown previously that GP IIb/IIIa antagonists (GR 144053F or abciximab), which clearly inhibited platelet aggregation, actually potentiate the formation of platelet-monocyte conjugates. Here we show, that an ADP-receptor antagonist (AR-C69931), inhibited both platelet aggregation and platelet-leukocyte conjugate formation. Platelet aggregation was measured in PRP and whole blood and the conjugate formation was analyzed by flow cytometry using anti-CD14 and anti-CD42a monoclonal antibodies. Platelet activation markers as analyzed by anti-CD62P or PAC-1 were also measured in PRP as well as in samples prepared from whole blood. The data were obtained from blood anticoagulated with citrate or with hirudin.
The results show that AR-C69931 is a powerful inhibitor of both platelet aggregation as well as platelet-leukocyte conjugate formation. Also platelet activation is inhibited by the ADP-receptor antagonist which might explain the capacity of the compound to inhibit platelet-leukocyte conjugate formation. The data demonstrate the superior potential of the ADP-receptor antagonist as compared with some GP IIb/IIIa antagonists which offers a potential for improving current antiplatelet therapy.

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REGULATION OF EXPRESSION OF HUMAN FACTOR IX USING AN ADENOVIRUS MEDIATED TETRACYCLINE GENE EXPRESSION SYSTEM

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The regulation of gene expression in human gene therapy protocols is important for both therapeutic and safety reasons. Thus, it should be possible to maintain transgene expression within the therapeutic range via an exogenous control of expression. For this aim, the high gene transfer capacity of adenoviral vectors (AdV) and the high inducibility of the tetracycline-regulatable expression system were combined in this study to develop a tetracycline-regulatable AdVs for the expression of human factor IX (hFIX) just downstream of a CMV-promoter. In vitro studies showed a dose-dependent and high induction potential of this hFIX-expression system resulting in a 65-fold increase of hFIX after induction by doxycycline. The expression of hFIX could either be maintained by continuous induction of doxycycline or up- and down-regulated by mutual presence and absence of doxycycline. Likewise the CMV-promoter, the use of the liver-specific hAAT-promoter have been shown to induce a high expression of hFIX in HepG2 (liver carcinoma) cells. In contrast to this, the liver-specific hAAT-promoter showed very low or no hFIX expression when tested in three non-hepatic human cell lines. When mice were injected with an AdV/hFIX-construct and induced by doxycycline, an inducible and therapeutic levels of circulating functional hFIX have been demonstrated. Altogether, these results report on the development of an adenoviral-mediated, tissue-specific and regulatable expression system for hFIX in vitro and in vivo. This system should be useful not only for gene therapy protocols for hemophilia B but also for diverse applications in gene therapy studies.

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ARE ANTI-XA MEASUREMENT HELPFUL IN THE USE OF DANAPAROID AND ARE PROPHYLACTIC DOSES OF DANAPAROID EFFECTIVE

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Background: Danaparoid has proved to be effective as prophylaxis in elective hip surgery, for the treatment of deep venous thrombosis, and for the management of patients with HIT. To date it is unclear if prophylactic doses of danaparoid are effective. Furthermore there might also be no need to control anti Xa activity using rather low doses of danaparoid. Routinely 10 U/kg are given subcutaneously two to three times per day. We aimed to evaluate the efficacy of prophylactic as well as therapeutic doses of danaparoid in patients with heparin induced thrombocytopenia.
Patients and Methods: 21 patiens with thromboembolic events and 24 patients with an isolated form of thrombocytopenia highly suspicious for HIT were given danaparoid intravenously, and subcutaneously. For prophylaxis 10 U/kg BW were given twice daily. For therapy 100 to 200 U/h were applied. Statistical analysis was performed using the Chi Square assay.
Results: The administration of danaparoid corresponded with anti Xa levels ranging from 0.14 to 0.36 U/ml (as prophylaxis), and 0.19 to 0.7 U/ml (as therapy) respectively. There was a weak correlation between the dose of danaparoid applied and the inhibition of factor Xa measured amidolytically (Coatest, Chromogenix-Mölndal/Sweden). This phenomenon became more apparent using higher, therapeutic doses (r=0.31). Prophylactic doses more strongly correlated with the anti Xa activity (r=0.7). However no severe side effects,especially no bleedings or further thrombotic complications occured.
Conclusion: There is a weak dose-relationship of danaparoid with the anti Xa levels measured by a chromogenic substrate assay. Prophylactic as well as therapeutic doses of danaparoid demonstrated to be effective. Further studies are necessary clarify if higher doses than routinely used for prophylaxis so far - as suggested by Schenk et al (GTH/Erfurt-2002) - can improve the patient´s outcome. Anti Xa measurements may therefore not be avoidable also with regard to the safety of this compound.

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***ALTERNATIVE METHODS OF ANTICOAGULATION IN HEMODIALYSIS TREATMENT**

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Heparins are the standard anticoagulants in long-term hemodialysis treatment. However, due to their side effects as well as an increasing incidence of heparin-induced thrombocytopenia, there has been an intensified search for alternative anticoagulation. The direct-acting antithrombin hirudin and its analogues, have several potential advantages over heparin; not require a cofactor such as AT III, are active against clot-bound thrombins, and do not interact with other plasma proteins. Recombinant Hirudin(Refludan®) exclusively undergoes glomerular filtration with an elimination half time (t1/2ß) of 1-3 hours. With an increasingly impaired renal function the t1/2ß is prolonged by 25-60 hours in patients with renal insufficiency, and up to 80-120 hours in those with advanced chronic renal failure.
Refludan® was used in the treatment of 18/113 dialysis patients (15,9%) with HIT II; three with classic HIT (2,6%) and 15 patients with abortive HIT (13,3%). Length of treatment totaled 29 patient years.
In the hemodialysis treatment of patients with a creatinine clearance of >5ml/min, a bolus injection was set to 0,1 mg/kg KG before the beginning of dialysis, with a clearance of <5 ml/min the dose was set at 0,085 mg/kg KG from the second treatment, with a clearance of <2 ml/min the doseage was reduced to 0,075 mg/kg KG, in the following treatments, a every two weeks therapeutic monitoring was initiated using the Ecarin clotting time (ECT) . The therapeutic blood concentration in HD was 0,4–0,8 µg/ml, in HDF 0,3–1,2 µg/ml.
All treatments were without complications, the disturbed coagulation variables and numbers of platelets were normalized. Impaired coagulation, in the form of increased bleeding or clotting in the extracorporeal blood circulation, did not occur. The cost of an intermittent, as well as a maintenance treatment, were not above that of a therapy with fractionated Heparin. Patients who receive anticoagulant therapy with Refludan® are able to undergo kidney transplantation; the dose therapy with Refludan must be adjusted according to the degree of renal impairment and the necessity for dialysis.
Conclusion; Hirudin is a suitable alternative for anticoagulation therapy for patients on dialysis who experience side effects from Heparin. The advantages of a therapy with Hirudin, in comparison to Heparin, should be the subject of further studies.

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EFFECT OF GLYCOPROTEIN IIB/IIIA ANTAGONIST ABCIXIMAB ON PLATELET INDUCED MONOCYTE TISSUE FACTOR EXPRESSION

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Background: Tissue Factor (TF), the major physiological initiator of the coagulation cascade is normally not expressed by cells within the circulation. However, activated platelets induce monocyte TF expression. We studied the effect of abciximab, a glycoprotein IIb/IIIa antagonist used in percutaneous coronary interventions (PCI), on platelet-monocyte cross talk and monocyte TF expression in vitro.
Methods: Expression of TF as well as total (CD42) and activated (CD62P, CD40 Ligand) platelet load was determined on CD14 positive monocytes by a four colour whole blood cytometric technique after stimulation with thrombin receptor agonist (TRA; 12.5 µM) in the absence or presence of abciximab (50 µg/ml).
Results: Percentage of activated platelets (CD62P) on monocytes correlated positively with total monocyte platelet load (CD42; r=0.97, p<0.001) and CD40 Ligand in timecourse (r=0.99, p<0.001). TF mean fluorescence intensity (MFI) was positively correlated with platelet activation measured by CD62 MFI in timecourse (r=0.86, p<0.001) After preincubation with abciximab percentage of TF positive monocytes was significantly reduced at 1, 10, 30 and 60 minutes (see table1).
Conclusion: We demonstrate reduced TF expression due to altered total and activated platelet load on monocytes by abciximab. Next to inhibition of platelet aggregation this my further contribute to the efficacy of abciximab in preventing thrombotic complications in PCI.
Table 1: percentage of TF positive monocytes (mean± sd) in time course after TRA stimulation w/o abciximab (n=5)

	0 min	1 min	5 min	10 min	30 min	60 min
TRA	12.0±4.1	27.5±±5.6	22.0±12.1	22.2±7.2	35.5±8.3	31.0±10.3
TRA + abciximab	12.4±4.1	10.1±4.6	12.6±5.8	10.7±3.5	12.3±8.0	15.2±10.1
p-value	1.0	0.001	0.15	0.012	0.002	0.049

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ANTI-ANGIOGENIC THERAPY WITH THALIDOMIDE IN ACUTE MYELOID LEUKEMIA

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Emerging data suggest an involvement of angiogenesis in the pathophysiology of acute myeloid leukemia (AML). Thus, anti-angiogenic therapy could constitute a novel strategy for the treatment of AML. To test this hypothesis, we performed a phase I/II dose escalating trial to study the efficacy and safety of thalidomide, a putative inhibitor of angiogenesis, in 20 patients with AML not qualifying for intensive cytotoxic chemotherapy. Thirteen patients were assessable for both response and toxicity tolerating a maximum dose of 200-400 mg daily for at least one month. Seven patients had to be withdrawn prematurely from drug administration due to progressive disease and death (3 patients), personal decision (2 patients) or intolerance of thalidomide (2 patients). Overall adverse events of the drug comprised fatigue (12 patients), constipation (9 patients), rash (5 patients) and neuropathy (4 patients). In 4 patients, a partial response defined as reduction in the blast cell infiltration of the bone marrow of at least 50% accompanied by increases in the platelet counts and hemoglobin values was observed. One additional patient showed a haematological improvement without fulfilling the criteria of a partial response. In parallel, microvessel densities significantly decreased in these 5 patients during treatment with thalidomide (p<0.05). This decrease was accompanied by declining plasma levels of vascular endothelial growth factor and basic fibroblast growth factor, the most potent angiogenic growth factors. In conclusion, single-agent thalidomide has anti-angiogenic and anti-leukemic activity in AML, although a causal relationship between both effects has still to be proven.

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IODINE CONTAINING COMPOUNDS INTERFERE WITH FIBRIN FORMATION – EXPLANATION OF CONTRAST MEDIA INDUCED EFFECTS ON COAGULATION?

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Introduction: Data shows that ionic and nonionic contrast media alter coagulation. Global clotting tests are mainly based on fibrin formation. Elucidation of the mechanism was performed by ro-TEG analysis of thrombin-induced fibrin formation in the presence of contrast media agents, organic iodine compounds and an inorganic iodine compound. Inhibition of thrombin by these compounds containing iodine was studied by an amidolytic assay.
Materials and Methods: Ionic+ and nonionic- contrast media agents (iotroxin+, iotrolan-, iodixanol-, iotalamin+, iomeprol-, iohexol-, ioxaglin+), organic iodine compounds (2, 4, 6-triiodobenzoic acid, povidone-iodine, sodium iodine acetate), sodium iodine and sodium acetate as a control agent were analysed for fibrin formation using ro-TEG (Pentapharm, München) equipment as follows: 150 µl fibrinogen solution (20 mg/ml, Haemocomplettan®) was mixed with 150 µl of contrast media agents, one molar solution of organic and inorganic compounds and corresponding dilutions (>1:4 until restored fibrin formation) with 50 µl thrombin (6 NIH/ml). The ro-TEG coagulation analysis is based on coagulation time, clot formation time and maximum clot firmness. Amidolytic determination of thrombin inhibition was performed as follows: 50 µl thrombin (0.5 IU/ml) was mixed with 50 µl of the compounds containing iodine. After an incubation period of 180 sec the chromogenic substrate (Pefachrome® TH, Pentapharm) was added and pNA formation measured using a Behring Coagulation System (BCS). Results: Chromogenic substrate analysis of thrombin in the presence of contrast media, organic and inorganic cmpounds containing iodine revealed no inhibition of the enzymatic activity. However, fibrin formation by ro-TEG analysis was inhibited by ionic and nonionic contrast media agents. Iiotalamin+, iomeprol-, ioxaglin+ and iohexol- showed the best effects. The tested iodine containing organic and inorganic substances showed similar effects; sodium acetate as control compound showed no effect.
Discussion: Contrast media containing iodine and organic and inorganic compounds containing iodine inhibit fibrin formation. Due to basic biochemistry, iodine is known to act as a lyotropic agent synonymous with the principle in splitting hydrogen bonding. These investigations demonstrate that iodine is the essential component of ionic and nonionic contrast media agents interfering with fibrin formation and may explain the effect on coagulation.

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***UROKINASE-MEDIATED CONTROL OF SMOOTH MUSCLE CELL MIGRATION AND VASCULAR REMODELING**

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The processes of cell adhesion, migration and invasion are regulated both by the number of membrane adhesion receptors and by enzymatic cascades of the serine- and metallo-protease families. One of the extracellular proteolytic cascades which participates in cell migration and tissue remodeling, is the plasminogen activator(s)(PA)/plasmin system. Urokinase consists of tree domains – protease-, kringle- and growth factor-like-domain (PD, KD and GFD, respectively). A protease domain of urokinase generates plasmin, whereas the growth factor-like domain mediates uPA binding to a specific receptor (uPAR) that provides tight localization of the enzyme on the cell surface and activation of intracellular signaling with promotion of cell migration.
Recently we demonstrated that uPA induces cell migration by binding of its kringle domain to the yet unidentified target (kringle-binding target, Kr-BT) and uPAR. We examined the downstream intracellular targets that regulate cell migration, induced by urokinase. uPA binding to Kr-BT induces phosphorylation of myosin light chain (MLC) and caldesmon. We did not observe a rise in the intracellular Ca2+ level when SMC were stimulated by uPA. MAP-kinase cascades can mediate MLC and caldesmon phosphorylation in Ca^{2+}-independent manner. Using different recombinant urokinase derivatives we demonstrated that proteolytic activity of uPA was required for activation of p42/p44erk1,2, whereas the kringle domain was essential for p38 MAP-kinase activation. We suggest that 1) urokinase binds to a tandem of receptors, which include a GPI-anchored uPAR and Kr-BT, to induce cell migration; 3) MAP-kinase cascades can transduce the uPA-induced signal from yet unidentified Kr-BT to the components of actomyosin complex to activate SMC migration regardless of intracellular Ca^{2+}.
We observed that the periadventitial application to the injured carotid artery of recombinant uPA stimulated neointima and neoadventitia formation in rats as well as cell proliferation and migration *in vivo*. In contrast,

tissue-type plasminogen activator (tPA) reduced the number of neointimal smooth muscle cells and neointimal area and increased both lumen area and the area, encompassed by the external elastic laminae, after balloon catheter injury of the rat carotid artery. We conclude that the ability to stimulate neointima and neoadventitia formation is specific for uPA. Our experiments suggest that this property, which could not be induced by tPA, provide a specific functional target for attenuating lesion growth.

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MRNA-BINDING PROTEINS IN HUMAN PLATELETS: HUR VERSUS AUF1

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Backgrounds: Stability of specific transcripts are regulated by Auf1p (hnRPD) and HuRp (HuAp or ELAVL1p) by binding to cis-acting AU-Rich Elements (AREs). Auf1p, 4 isoforms from alternative pre-mRNA splicing 37, 40, 42, and 45 kD (Dempsey L.A. et al. Genomics 49:378), is known to destabilize ARE carrying mRNA (Larola G. et al. Science 284: 499). HuRp has the ability to stabilize mRNA with AU-rich sequences (Fan X.C. et al. EMBO J. 17:3448). Platelets are cytoplasmatically transcriptionally silent but have functional transcripts (Weyrich et al. PNAS 95:5556, Houng et al. Eur-J-Biochem 243:209). We therefore examined which proteins are responsible for mRNA stability regulation due to ARE in platelets?
Methods: We prepared leukocyte-free platelet concentrates by differential centrifugation (822 g for 10 min) from filtered platelet concentrates, tested by PCR with LCA, AUF1, and HUR specific primers to isolate platelet specific mRNA and proteins. Auf1p and HuRp were determined by immunoblotting using murine anti-Auf1 IgG (Dreyfuss G, Dept.Biochem.Biophys. Univ. PA) and murine anti-HuR IgG (Gallouzi I.-E. et al. PNAS 97:3073). For flow cytometry platelets were fixed with 0,5% paraformaldehyd, treated with 0,1% saponin, immunolabeled with same specific primary IgG used above and secondary rabbit FITC-F(ab´)2 anti-mouse-IgG (Dako).
Results: The platelet specific mRNA was used to detect and clone HUR. The AUF1 transcript was not detectable in platelets compared to the cloned PCR positive control. The 37 kD Auf1p isoform was detected in platelets, whereas the megakaryocyte cell line CMK used as positive control contains 37, 40, and 42 kD Auf1p isoforms. The 36 kD HuR was detected, compared to the cloned positive control in platelets as well. Other members of the ELAVL protein family (HuC, HuB, HuD) were neither detected in platelets nor in CMK. The detection of Auf1p and HuRp in platelets protein extract was confirmed by flow cytometry using fixed, permeabilized platelets, Bcl3p, vWFp, and CD62P specific antibodies.
Conclusions: This study demonstrates that human platelets possess Auf1p, HuRp and HuR mRNA. Both proteins can compete to bind to ARE and may regulate translation and protein expression. Signals modifying Auf1p, HuRp or HuR mRNA function or concentration modulate ARE containing mRNA stability and platelet function.

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***PLATELET ADP RECEPTOR ANTAGONISTS IN CARDIOVASCULAR DISEASE**

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ADP-induced platelet activation and aggregation is initiated by the P2Y1 receptor and amplified in a synergistic manner by the P2Y12 receptor. The P2Y12 receptor plays an important and central role in amplifying platelet activation, aggregation, granule secretion and procoagulant responses induced by agonists other than ADP. It is now recognised that the active hepatic metabolites of the thienopyridines, ticlopidine and clopidogrel, bind irreversibly to the P2Y12 receptor, thus inhibiting platelet responses to ADP and some other agonists. Ticlopidine and clopidogrel are superior in safety and efficacy to anticoagulant regimens in preventing the thrombotic complications of intracoronary stent implantation. The addition of clopidogrel to standard therapy for patients with unstable angina and non-Q wave myocardial infarction, including aspirin and heparin, reduces the combined incidence of myocardial infarction and death over the course of up to 1 year, albeit at the expense of increased bleeding events. These studies establish the P2Y12 receptor as an important therapeutic target. However, we have found that clopidogrel only yields partial P2Y12 receptor blockade as evidenced by the fact that clopidogrel treatment does not inhibit significantly TRAP-induced platelet aggregation despite the important role of the P2Y12 receptor in this aggregation response. On the other hand, the ATP analogue AR-C69931MX, a highly selective P2Y12 receptor antagonist and an intravenous antithrombotic agent, can yield more complete receptor blockade than clopidogrel. Phase II studies show that AR-C69931MX is well tolerated when administered intravenously to patients as adjunctive therapy in the management of acute coronary syndromes and its pharmacokinetic profile, with ultra-short half-life, is ideally suited to these patients. Orally active ATP analogues are also being developed. ATP analogues potentially may offer further advances in therapy beyond those established by clopidogrel and warrant further study.

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PROGRESS IN PLATELETPHERESIS: PAIRED STUDY OF COBE TRIMA AND COBE SPECTRA LRS (SINGLE NEEDLE)

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Background: The technical improvement of blood cell separators for plateletpheresis has led to platelet concentrates (PC) with high purity, low content of leukocytes (WBC), and high platelet yields. The main advantages of single donor platelet concentrates (SDP) are a reduced frequency of HLA antibody formation and reduced risks of platelet refractoriness and of virus transmission.
Study design and methods: Two cell separators, the Cobe trima and the Cobe Spectra LRS (COBE BCT, Lakewood, CO, USA), were compared under routine conditions. Each of 20 healthy voluntary donors underwent single needle procedures for plateletpheresis in both cell separators. Regarding our standards, a SDP contains less than 10E6 WBC and a minimum of 3×10^{11} platelets in 250 ml plasma. In both cell separators the platelet yield, WBC and RBC contamination of the PCs were analysed; regarding donor safety and comfort, separation time, consumption of ACD-A and processed blood volume were determined. Platelets were counted in a cell counter (Sysmex K 4500, TOA Medicals, Kobe, Japan), WBCs in a Nageotte chamber and RBCs using a Neubauer chamber. Platelet function was assessed by flow cytometry (CD41-FITC and CD62-PE, Ortho Diagnostic Systems Inc., NY, USA).
Results: Comparing the results between both cell separators, separation volume, ACD-A infused, and separation time were significantly lower (about 25%) using the Cobe trima. Platelet yields of all PCs fulfilled our quality standard. The WBC contamination of the PCs was always below 1×10^{6} using both cell separators except for one separation of the Cobe Spectra (1.1×10^{6} WBC per unit). The RBC contamination was significantly different between both separators ($p<0,05$) suggesting better results in the Cobe trima. Platelet function as measured by the expression of baseline CD62P and after activation (ADP) was comparable in both cell separators proposing similar platelet quality (Tab. 1).
Conclusion: 1. Both cell separators using single needle standard procedures fulfilled our quality standards. 2. The Cobe trima showed clear advantages for the donor with respect to separation time and the consumption of ACD-A. 3. The Cobe trima is a very efficient and reliable tool for plateletpheresis. From the technical point of view this cell separator represents a further step in the development of apheresis techniques.

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THE INFLUENCE OF FIBRINOGEN AND HEMATOCRIT ON THE OUTCOME OF REVASCULARISATING CATHETER PROCEDURES IN PATIENTS WITH POAD

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Introduction: There is a known relation between the outcome of intraaterial catheter interventions and the morphology of the treated vessel but till now our knowledge is poor concerning blood composition and outcome of invasive procedures. Therefore we analysed 330 patients with POAD attending a revascularisating catheter intervention in order to examine is also determined by hemorheological parameters as fibrinogen and hematocrit.
Methods: The outcome of catheter interventions (success/failure)is related to the laboratory parameters fibrinogen and hematocrit. 330 patients with known POAD have been recruited (246 men, 84 women), mean age 63,6±10,9 years, men 63,2±10,7 years, women 65,1±11,9 years. 9,1% of the patients showed stage IIa, 63,3% as stage IIb, 8,1% as stage III and 19,5% as stage IV according to Fontaine`s classification.
Results: Diabetics ($p<0,02$) and non diabetics ($p<0,006$) show significantly lower mean values of fibrinogen in the stages IIa-IIb than in the advanced stages of POAD. In the advanced stages diabetics have significantly lower values of hematocrit ($p<0,002$). The success rates of both groups decrease by a factor three and two, respectively. In critical limb ischemia mean values of fibrinogen are significantly lower in patients with a finally successful intervention than in the unsuccessful cases ($p<0,048$).

Conclusion: There are significant age adjusted correlations between fibrinogen values and the stages of POAD according to Fontaine's classification and with the success rate for catheter interventions. The underlying results show that also hemorheological factors as fibrinogen and hematocrit influence the success rate. In the advanced stages of POAD high values of fibrinogen seem to have a negative effect on the primary success rate. Low values of hematocrit however seem to have a compensating influence on plasma viscosity in diabetics in the advanced stages of POAD.

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HOW TO OPTIMIZE TARGET BINDING AND BIOAVAILABILITY FOR ENZYME INHIBITOR-BASED ANTICOAGULANTS

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Thrombo-embolic diseases are a major cause of mortality and morbidity, particularly in the Western world, which has stimulated enormous efforts to develop and introduce new antithrombotic therapies. One strategy is the development of direct inhibitors of thrombin and factor Xa, which hold central positions in blood coagulation. Furthermore, also other clotting factors such as factors VIIa and IXa, but also factor XIIIa are possible target enzymes.
For use in prophylaxis of thrombo-embolic disorders such inhibitors should be orally available, must be safe to avoid bleeding complications and should have an appropriate half-life to maintain adequate, antithrombotically effective blood levels, allowing once or twice-daily dosing.
Many inhibitors of thrombin and factor Xa published during the last decade, are highly effective in *in vitro* coagulation assays, however, most of them had drawbacks in animal studies, especially after oral administration. The main reason for the low oral bioavailability of many inhibitors of thrombin and factor Xa is low intestinal permeation due to their basic nature – the substrate specificity of these enzymes for Arg and Lys requires a basic moiety in the inhibitor molecule.
Therefore, the research effort must not only focus on improvement of affinity and selectivity of the inhibitors, but modifications – affordable with activity – should also modulate their overall physico-chemical properties, i.e. reducing the strongly basic character and optimising lipophilicity to enhance enteral absorption, lower non-specific plasma protein binding, and prolong the half-life in blood. However, to optimize these characteristcs – enteral absorption and elimination from the circulation – a delicate balance of the physico-chemical properties is required: absorption is enhanced by increasing lipophilicity while elimination is delayed by reducing hydrophobicity.

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NOVEL BENZAMIDINE-DERIVED INHIBITORS OF FACTOR XA

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Two clotting cascades – the intrinsic and the extrinsic pathway - are responsible for the control of the blood flow following vascular or tissue injury. Both pathways converge at the activation of Factor X to Xa. Factor Xa (FXa) hydrolyzes and therefore activates prothrombin to thrombin. The major role of thrombin is to convert fibrinogen to fibrin and – ultimately – to create a solid clot. Today, thrombin remains the main therapeutic target for thrombosis prophylaxis. However, the inhibition of FXa may represent a more favorable strategy for anticoagulation because it is suggested to result in less bleeding events.
We have identified a scaffold of small molecule inhibitors of FXa with the general structure R1-D-Ser(R2)-Gly-4-amidino-benzyl-amide. A variety of derivatives have been investigated in terms of inhibition of FXa and related enzymes and for their activity in clotting assays. A first lead with an N-terminal benzylsulfonyl group and D-Ser(tBu) as P3 residue selectively inhibits human FXa with a Ki of 14 nM. The replacement of the P2 Gly by other amino acids, like Ala or Pro, reduces the selectivity but still maintains potent inhibition of FXa. The compounds inhibit coagulation as effective as other known lead inhibitors of thrombin and FXa.

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***MICROPARTICLES IN HEALTH AND DISEASE**

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Microparticles (MP) are small fragments released from the outer membrane of cells undergoing activation or apoptosis. Their cellular oringin can be determined via the exposure of specific antigens exposed on their surface. Originally, platelet-derived MP (PMP) were studied in vitro and their presence in the circulation determined in various diseases prone to thrombo-embolic complications. Presently, it is known that healthy volunteers have circulating MP of especially platelets and erythrocytes. In meningococcal disease, MP from granulocytes and monocytes are also present. Endothelial cell-derived MP have also been reported in several diseases, including SLE.
MP may play a role in coagulation activation in vivo via several mechanisms: 1. Exposure of negatively charged phospholipids, 2. Exposure of tissue factor (TF). TF-exposing MP may be involved in disseminated intravascular coagulation, in excessive coagulation activation in the pericardial cavity of patients undergoing cardiac surgery with cardiopulmonary bypass, and in synovial fluids of inflamed joints. Other functions of MP may involve activation of the inflammatory response (via limited conversion of their phospholipids by secretory PLA_2, ensuing binding of C-reactive protein and complement activation), transport of substances to other cells (for instance PMP providing arachidonic acid to endothelial cells in vitro) or cell activation (for instance P-selectin exposing PMP stimulating monocytes to the production of cytokines and surface exposure of TF).
In conclusion. MP may play various roles in vivo, but their exact importance still has to be established. Therapeutic intervention into their formation, by e.g. GP IIb/IIIa inhibitors, may then prove useful.

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INFLUENCE OF LOW MOLECULAR PLASMINOGEN ON ONSET OF LABOR AND HEMOSTATIC SYSTEM

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Purpose: Both bradykinin and prostaglandin act on the muscles of the uterus causing contraction. Fibrin plays an important role in maintaining the integrity of utero-placental circulation. Therefore, it is necessary to investigate the mechanism of the fibrinolytic parameters related to the hemostatic system during pregnancy and at the onset of labor.
Methods: (1) Fourty-four cases of normal pregnant women and 25 cases of healthy women (neither pregnant nor on oral contraceptive drugs). Plasminogen Activator (u-Pa activity) was measured by Zymography, and fractions were detected using Image-Analyzer. (2) Euglobulin-Lysis-Time (ELT) and Prekallikrein (S-2302) were tested as parameters of the fibrinolytic and the kinin-kallikrein system.
Results: (1) U-Pa activity of low molecular weight (LMW) during the latter half of pregnancy was 0.32+/-0.13 IU/ml, but it decreased to 0.19+/-0.03 IU/ml at the onset of labor ($p<0.05$). (2) ELT during this latter half of pregnancy was prolonged (840+/-26.4 sec), at the onset of labor it shortened (356.6+/-54.3 sec). Prekallikrein is decreased to 90.6+/-16.8% after labor (latter half of pregnancy: 196.8+/-33.6%).
Conclusion: The action of kallikrein upon LMW Plasminogen Activator causes it to change into plasmin at the onset of labor. They have an intimate relationship and affect the contraction of the uterus and the hemostatic system.

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***WHAT IS THE PROHYLACTIC DOSE OF ACETYLSALICYLIC ACID (ASA) FOR THE INHIBITION OF THE PLATELET FUNCTION? INVESTIGATION USING BORN'S METHOD OF PLATELET AGGREGOMETRY**

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The Platelet Aggregation test (PA) can measure the individual platelet function and may also be ordered for antiplatelet medications to monitor the effects of a treatment.
ASA is widely used in the primary and secondary prevention of cardiovascular diseases. In our study we used Born's method für the platelet aggregation ex vivo in platelet rich plasma (PRP) induced with arachidonic acid (AA) and also with adenosine diphosphate (ADP), adrenalin and collagen to determinate the individual dose of ASA which inhibits platelet aggregation.
A dose of 30 mg ASA per day was sufficient to block the AA-induced PA nearly completely in 40% of the patients studied. A dose of 100 mg ASA

per day was necessary in further 50% of the patients. In 10 % of all patients, the dose had to be further increased to 300 mg/d. Only one of 108 patients tested had to be treated with 500 mg ASA per day. An inhibition of more than 70% and a slope of the aggregation traces of less than 10 were parameters indicating a sufficient effect of ASA. There was a dose-response relationship especially in the slope of the collagen- and adrenaline-induced PA concerning the dose of ASA.
One can, therefore, conclude that each patient had a individual response in ASA treatment. A low dose of 30 mg ASA per day was sufficient in some patients. In those patients, who are not investigated in the PA, a daily dose of 300 mg ASA should be applicated.

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***RISKS AND BENEFIT OF ANTICOAGULANT THERAPY**
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Even when respecting all contraindications due to an increased bleeding diathesis, the risk of bleeding remains the most common and potentially the most serious complication. This risk is increased in a number of medical conditions – in particular in malignant disease – and in old patients. However, this does not preclude the use of anticoagulant drugs in these patients, because the risk of thrombotic events is also increased in most of these conditions. Therefore, in addition to following the established general guidelines for the initiation and the duration of anticoagulant therapy, a careful assessment of the individual benefit and the individual risk must be made for every patient taking into consideration the particular circumstances of the case. These may also influence the choice of the anticoagulant drug and the intensity of anticoagulation aimed at.
Heparin-induced thrombocytopenia and coumadine necrosis are discussed in detail in separate lectures. Other complications of anticoagulant therapy are either relatively benign conditions or they are very rare. They include osteoporosis, loss of hair, coumadine embryopathy, icteric and non-icteric hepatitis, thrombocytopenic and non-thrombcytopenic purpura, and,allergic reactions.

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ANALYSIS OF THE THROMBOMODULIN GENE IN PATIENTS WHO SUFFERED FROM VENOUS THROMBOSIS
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Introduction: The protein C anticoagulant pathway comprises protein C (PC), protein S (PS), and thrombomodulin. Thrombomodulin represents the starting point of the PC anticoagulant reaction cascade by acting as high affinity receptor for thrombin. Patients carrying mutations within PC and/or PS as well as factor V leiden mutation or antithrombin III (ATIII) mutations are prone to develop venous thromboembolic disease. Whether mutations within the thrombomodulin gene are involved in the molecular events leading to venous thromboembolic disease is not clear. To this end the thrombomodulin gene was analyzed for mutations in two different patient collectives.
Patients and Methods: One collective (n=34; group I) of patients suffered from thromboembolic disease without factor V leiden mutation and exhibited normal biological activities for PC, PS, and ATIII. A second collective of patients (n=55; group II) was analyzed characterized by impaired PS, and/or PC, and/or ATIII activity and/or factor V leiden mutation. A combination of mutations in these genes increases the risk for venous thromboembolic disease. The thrombomodulin gene was screened for mutations by polymerase chain reaction-single stranded conformation polymorphism (PCR-SSCP) analysis.
Results: The previousily described neutral polymorphism [nt 1418: C–>T, (Ala 455–>Val)] was found in patients tested herein exhibiting allele frequencies in the range of 0.85 to 0.87 (Ala) and 0.13 to 0.15 (Val). Further mutations within the thrombomodulin gene were not present. Conclusion: These data indicate that thrombomodulin gene mutations are not majorly involved in thromboembolic disease in the two patients' groups. Since an association between thrombomodulin gene mutation and thromboembolic disease was not found in our study we suggest that a routine check-up of the thrombomodulin gene in thrombosis patients is not justified.

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GENERATION AND CHARACTERIZATION OF HUMAN HEMATOPOIETIC CELL LINES EXPRESSING FACTOR VIII
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Considering the plasticity of hematopoietic stem cells (HSC), they would be ideal targets for gene therapy of hemophilia A by virtue of their progeny providing immediate access to the blood stream. However, several attempts to show expression of recombinant factor VIII (rFVIII) by primary hematopoietic cells and cell lines have failed, which was attributed to the inability of HSC to secrete rFVIII. Here we describe the generation of stable, FVIII-secreting hematopoietic cell lines representing different blood-cell types using a bicistronic lentiviral vector encoding for a B-domain deleted FVIII (FVIII*f´*B) and enhanced green fluorescence protein (EGFP). Transduced cell lines with erythroid and/or megakaryocytic background, (K562-F8 and TF-1-F8), secrete high levels of FVIII in the order of 76,4 and 41,6 ng FVIII:C/ml, while moderate and low levels are observed in B-lymphoblastoid Raji-F8 cells and the T leukemia line Jurkat-F8 which secrete 6,73 and 1,83 ng FVIII:C/ml, respectively. The capacity to secrete rFVIII appeared to depend on factors related to the cell lineage, rather than on the transduction efficacy. Stimulation of transduced cells with the PKC-activator PMA resulted in a marked augmentation of rFVIII secretion. Incubation with 0,1 and 1 ng/ml PMA resulted in an up to 2,8-fold (K562-F8, Raji-F8) and 1,7-fold (293T-F8) increase in rFVIII. The established cell lines should be helpful in further elucidating mechanisms which are able to improve FVIII secretion in hematopoietic cells on a post-translational level and suggest reanalysis of hematopoietic cells as target for gene therapy of the hemophilias.

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HYPERPROLACTINEMIA ACTIVATES PLATELETS AND IS A RISK FACTOR FOR THROMBOEMBOLIC DISEASES
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Hyperprolactinemia is a newly recognized platelet coactivator due to potentiation of ADP-induced platelet activation (1). However, until now an association between hyperprolactinemia and venous thromboembolism (VTE) has not been systematically investigated and prolactin signalling mechanisms in platelets still need to be elucidated.
In this study, plasma prolactin levels in healthy subjects and patients with VTE were determined, demonstrating that patients with VTE and no other congenital risk factors had significantly increased plasma prolactin levels. Moreover, patients with prolactinomas demonstrated a higher incidence of VTE than the general population. To elucidate the molecular mechanisms for the development of venous thrombosis, prolactin receptor signalling during platelet activation was investigated with a focus on ADP-stimulated G-protein-regulated signalling pathways. The short isoform of prolactin receptors was detected on platelets. Signalling through this receptor, although not directly linked to Gq-proteins, substitutes for Gq-protein regulated signalling pathways involved in platelet activation. We identified protein kinase C, a well established signalling molecule in platelet activation, as a target molecule for prolactin signalling pathways in human platelets. Our findings indicate that hyperprolactinemia may be an important novel risk factor for VTE suggesting that its thrombogenic effect is mediated through enhanced platelet reactivity. Revealing the molecular mechanisms of prolactin signalling will allow the design of new anti-thrombotic therapies.

1) Wallaschofski, H., Donné, M., Eigenthaler, M., Hentschel, B., Faber, R., Stepan, H., Koksch, M., and Lohmann, T. 2001. Prolactin as a novel potent cofactor for platelet aggregation. J Clin Endocrinology and Metabolism, December 2001, in press.

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DEVELOPMENT OF A NON-HUMAN PRIMATE SUB-CLINICAL MODEL OF HEPARIN-INDUCED THROMBOCYTOPENIA: PLATELET ACTIVATION AND IMMUNOGENIC RESPONSE

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The purpose of this study was to compare the responses of human and non-human primate platelets (Macaca mulatta) to anti-heparin-platelet factor 4 (AHPF4) antibodies. Due to the variations observed in the functionality and immunoglobulin isotypes in patients with heparin-induced thrombocytopenia (HIT), we used highly characterized anti-heparin-platelet factor 4 antibodies to study platelet activation. Three HIT patients' plasmapheresis fluid, with similar responses in ELISA and SRA systems, were pooled. This pool was then used to study platelet activation responses of human and primate platelets by platelet aggregometry and flow cytometery in the presence and absence of glycoprotein IIb/IIIa inhibitors. The most prevalent immunoglobulin isotype present in the plasmapheresis fluid was IgG. Using this pool in the platelet aggregation assay, without any heparin present, the percent platelet aggregation with human platelets (11.8±2.35, n=5) was much less compared with the primate platelets (54.3±10.2, n=9, $p<0.001$). In the presence of 0.4 U/ml heparin, the two platelet types had similar percent aggregations ($p>0.05$). This same pattern was observed using a flow cytometry assay, in which microparticle generation and P-selectin expression were used as markers of platelet activation. Purified AHPF4 IgG also produced similar activation responses with platelets from both species. In a modified platelet aggregation assay, three glycoprotein IIb/IIIa receptor inhibitors were used to further evaluate similarities in platelet activation. Eptifibatide was found to be a strong inhibitor of both species' platelet types at concentrations greater than 0.01 mcg/ml. This was not the case with tirofiban which inhibited both platelet types at 0.025, 0.05, and 0.1 mcg/ml. Abciximab inhibited aggregation at 6.25, 12.5, and 25 mcg/ml. These data indicate that phylogenetic similarities in platelets of humans and primates allow primate platelets to be used to further characterize the pathophysiology of HIT syndrome. Repeated challenge of heparins triggered the generation of AHPF4 antibodies in primates (as measured by ELISA). The activation of primate platelets together with the generation of AHPF4 antibodies strongly supports the hypothesis that primates can be employed to investigate the pathogenesis of HIT.

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A NEW ASSAY FOR MONITORING THE PHARMACOKINETIC OF FVIII-BYPASSING AGENTS

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Hemophilia A patients with inhibitors are treated with preparations containing activated coagulation factors to achieve hemostasis by inducing activation of the clotting cascade independently from and, thus, bypassing factor VIII (FVIII). The decisive step is the conversion of prothrombin into thrombin, which then induces clot-formation by transforming fibrinogen into fibrin.
Our aim was to 1) investigate the kinetic of thrombin generation firstly in FVIII inhibitor plasma reconstituted in vitro with FEIBA or with recombinant activated FVII (rFVIIa) and secondly in plasma samples from model animals and hemophilia A patients who develop inhibitors after FEIBA and rFVIIa treatment, and 2) develop an assay for routine laboratory use as a diagnostic tool in inhibitor-bypassing therapy.
In our experiments coagulation was triggered in citrated plasma by adding a very low concentration of tissue factor/phospholipid complex and $CaCl_2$ in the presence of a fluorogenic thrombin substrate. We continuously monitored the increasing fluorogenic intensity using a fluorimeter and converted it to thrombin concentration from a reference curve constructed with purified human thrombin. Under the assay conditions practically no thrombin generation was observed in the FVIII inhibitor plasma but in normal plasma a 140-220 nM maximum thrombin concentration was detected within 30 minutes. Thus, our system mimics small vessel damage that can be repaired by intact hemostasis but cannot be repaired in severe FVIII deficiency.
When plasma was spiked with either FEIBA or rFVIIa, the rate and maximum thrombin generation increased dose-dependently. In different animal models, treatment with FEIBA and rFVIIa induced a statistically significant increase in thrombin generation. Within an hour of treatment plasma samples from FVIII inhibitor patients treated with FEIBA or rFVIIa showed an increased thrombin maximum, which thereafter gradually returned to baseline values.
This assay enables treatment of bypassing therapeutics to be monitored, thus, helping to optimize treatment dosage and to avoid thrombotic complications due to overdosing.

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CLOPIDOGREL IN COMBINATION WITH PHENPROCOUMON FOLLOWING CORONARY STENT IMPLANTATION AND SEVERE PULMONARY EMBOLISM? A CASE REPORT

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Background: In order to avoid subacute coronary stent thrombosis the combined use of aspirin (A) and clopidogrel (C) is recommended for several weeks following stent implantation. In some cases, there is an additional indication to use phenprocoumon (P; e.g. severe pulmonary embolism). The combined use of C and P is, however, neither approved nor well documented. This report describes a patient in whom a severe pulmonary embolism occured following coronary stent implantation. It is essential to document such cases for clinical decision making since randomized studies are not to be expected (on ethical grounds).
Case report: A 68 years old male was admitted for invasive evaluation of angina pectoris following anterior myocardial infarction. A coronary angiogram revealed relevant stenoses of the second left obtuse marginal and the proximal LAD. Stents were implanted successfully in both vessels. At this time LVEF measured 35%. The patient was treated with A (100 mg/day) and C (75 mg/day). The next day he reported a sudden onset of dyspnea and chest pain. A spiral CT confirmed a severe pulmonary embolism (right upper and lower as well as left basal pulmonary artery). Asymptomatic right sided femoral deep venous thrombosis was documented by Doppler venous ultrasound. Weight-adapted LMWH and overlapping P therapy was initiated and both A and C were discontinued after reaching an INR of 2.0. The patient was discharged home in stable conditions. Three days later he was rehospitalized at another hospital because of recurrent angina. Since an acute re-infarction was suspected the patient was initially treated with streptokinase. Because of refractory chest pain the patient was transferred to our hospital and a coronary angiogram was performed showing subacute LAD stent thrombosis. A Rescue percutaneous thrombectomy catheter (Boston Scientific) was applied and thrombus material was removed. An additional in-stent angioplasty resulted in a TIMI 2 flow. The anterior wall was, however, dyskinetic with a LVEF of 20%. The patient died the next day in cardiogenic shock.
Discussion: The single use of P, even though highly indicated, is not sufficient to prevent subacute coronary stent thrombosis. The combination of P with C (and A) might be an alternative in such cases.

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PLATELET-LEUKOCYTE INTERACTION IN PATIENTS WITH MYELOPROLIFERATIVE SYNDROMES

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Introduction: myeloproliferative disorders are associated with an increased risk of both thromboembolic and bleeding complications. This has been attributed to altered platelet number and function. We used flow cytometry to study platelet activation in patients with chronic myeloproliferative disorders. Last year we reported the findings of 20 patients, this is an update with results from 47 patients and 16 controls.
Methods: Whole blood from patients with essential thrombocythemia (ET, n=12), P. vera (PV, n=13), myelofibrosis (MF, n=12), chronic myeloid leukemia (CML, n=10), and from healthy volunteers (n=16) was analysed with flow cytometry. Platelet microparticles and platelet microaggregates were identified with anti-CD42b and forward scatter, activated platelets with anti-CD62p. Anti-CD42b, anti-CD14, and anti-CD45 were used to study platelet-leukocyte conjugates.
Results: The percentage of CD62p-positive platelets was significantly elevated in all myeloproliferative subgroups (PV 11.7%, ET 13.1%, MF 17.6%, CML 14.6%, controls 10.1%). Platelet microparticles were increased in patients with PV, ET, MF, but not with CML (PV 11.2%, ET 10.4%, MF 10.7%, CML 6.4%, controls 5.4%). Patients with PV and ET had a significantly higher percentage of platelet-neutrophil (PV 9,1%, ET 10.7%, controls 6.6%) and platelet-monocyte conjugates (PV 16,6%, ET 19.1%, controls 8.8%), there was no difference between CML or MF patients and controls. The number of patients with thromboembolic or haemorrhagic complications was too low to perform a separate analysis in each subgroup.
Conclusion: Patients with myeloproliferative syndromes show evidence of platelet activation and increased platelet-leukocyte interaction. Myeloproliferative syndromes are clonal proliferations of all blood cells and of leukocytes in particular. These results suggest that changes in leukocyte-platelet interaction contribute to coagulation system alterations and tests on this function should be incorporated into future studies in patients with myeloproliferative syndromes.

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***REFACTO®: CLINICAL EVALUATION IN PTPS AND PUPS**

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ReFacto® (Wyeth/Genetic Institute) is a B-domain deleted (BDD) form of human coagulation factor VIII. The rationale for the design was the observation that the B-domain is not required for expression of biologic activity. A lyophilized formulation has been developed that does not require the addition of human serum albumin (HSA) or other macromolecular stabilizers. This first albumin-free formulated rFVIII was approved 1999 in the EU and 2000 in the US.
ReFacto® has been extensively evaluated in clinical studies since 1993. In an initial 12-month study with subsequent yearly extensions over a 5-year period, 113 PTPs received on-demand and/or prophylactic treatment including treatment during surgery if required. A total of 7.310 haemorrhages occurred in patients with on-demand treatment; with 71% resolving after a single infusion. Of the 11.655 rated infusions given for haemorrhages, 92% were rated by investigators and patients as providing an “excellent” or “good” response. During the prophylactic period, 12% of patients experienced no bleeding episodes and 17% of the patients had no on-demand treatment. The mean dose was 28 IU/kg for prophylactic and 30 IU/kg for on-demand treatment. The efficacy in conjunction with surgery was assessed to be “very useful” or “useful” in all cases. One patient developed an inhibitor after 107 EDs and approximately 3 years after entry into the study; this was concomitantly diagnosed with a monoclonal gammopathy.
In the Phase III open-label multicentre study in PUPs treated with ReFacto® 101 patients had been enrolled. They received routine prophylactic and/or on demand treatment as well as treatment related to surgery for 50 exposure days or for up to five years. A total of 1.362 haemorrhages occurred. 92% of bleeding episodes resolved after three infusions or fewer. Of the 2.375 infusions rated by investigators, 93% were rated as providing an “excellent” or “good” response. 27 patients received routine prophylaxis, which significantly reduced breakthrough bleeding episodes by twofold when compared with patients receiving on-demand treatment. Administration in conjunction with 40 surgical procedures showed no adverse effects and the overall assessment was “very useful” or “useful.” The mean dose was 56 IU/kg for routine primary prophylaxis and 53 IU/kg for on-demand therapy. 32 of the patients (32/101; 31.7%) developed inhibitors within a median of 12 exposure days. 16 out of these patients were high responders (4 patients ≥5 BU/ml to <10 BU/ml; 12 patients ≥10 BU/ml) and 16 were low responders (<5 BU/ml). The inhibitor risk was comparable to that seen with full-length recombinant products.
The efficacy of ReFacto® has been proven in multicentre, international studies. From these studies and the clinical experiences since then it can be concluded that BDD rFVIII has biological activity comparable to that of other recombinant FVIII products or plasma factor VIII. ReFacto® has been shown to be well-tolerated and effective for the treatment and the prevention of bleeding episodes, as well as in routine and surgical prophylaxis, for PTPs and PUPs with haemophilia A. ReFacto® should therefore be considered as an excellent treatment for patients with haemophilia.

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DETERMINATION OF B-DOMAIN DELETED RECOMBINANT FACTOR VIII WITH AN ONE-STAGE CLOTTING ASSAY IN ROUTINE PRACTICE

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Background: In patients treated with recombinant factor VIII (rFVIII) concentrates considerable discrepant FVIII plasma activities were found between chromogenic and one-stage clotting methods. After therapy with the B-domain deleted rFVIII concentrate ReFacto®, the FVIII plasma activities with some one-stage assays were only 50 percent of the values recorded by the chromogenic reference method. Nevertheless, most clinical laboratories for practical reasons use an one-stage assay. Utilizing a product specific standard we developed a reliable one-stage FVIII assay for the monitoring of rFVIII treatment.
Methods: The one-stage FVIII assay was performed with Dapttin® (Progen Immuno) as aPTT reagent and the ReFacto® laboratory standard (RLS, Wyeth/GI) diluted in FVIII deficient plasma (Progen Immuno) on a Sysmex CA-6000 (Dade Behring). As chromogenic procedure the Coamatic® FVIII assay (Chromogenix) was adapted on a Hitachi 917 (Roche). Stability of FVIII activity in diluted RLS (100 µl RLS plus 900 µl FVIII deficient plasma; expected FVIII activity 94%) was tested with both methods over a period from one hour to three months at room temperature, 6±2º C, -18º C, and -40º C. In six haemophilia A patients treated with ReFacto® we measured the FVIII activity with both methods in samples obtained before, and from 10 minutes up to 4 hours after infusion.
Results: With both assays FVIII activities in diluted RLS were stable for 4 and 24 hours at room temperature and at 6±2º C, and for 3 months when stored at -18º C and -40º C, respectively. Analysis of samples (n=36) from patients treated with rFVIII showed that the one-stage FVIII assay with aPTT reagent Dapttin® and RLS calibration yields nearly corresponding results with the chromogenic method (no significant difference in the paired t-test).
Conclusions: The diluted rFVIII standard RLS shows a stable FVIII activity for 24 hours at 6±2º C, and can be stored for at least three months at -18º C without a significant loss of activity. Results of the one-stage FVIII assay combined with RLS correspond well to those obtained by the chromogenic method. This one-stage FVIII assay is well suited to monitor the treatment with B-domain deleted recombinant FVIII in routine practice.

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***IS IT POSSIBLE TO DETECT AN IN VIVO ACTIVATION OF PLATELETS?**

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Ischaemic syndromes are due to activation of platelets at unstable atherosclerotic plaques. Detection of this in vivo activation of platelets has been attempted by several methods: release products (β-TG, PF4, TX-metabolites), spontaneous platelet aggregation, flowcytometry etc. Results of these studies have been disappointing and up to now none of these methods has been used in clinical routine.
In peripheral venous blood, we could not detect an increase in the absolute count of CD62p-positive single platelets in different settings (after coronary angiography or angioplasty). Even in blood drawn from the coronary sinus, the absolute count of CD62p-positive platelets did not significantly increase after coronary angioplasty, only the percentage of CD62p-positive platelets increased. There was a drop in platelet count due to a substantial increase in the number of platelet aggregates. This drop in platelet count and increase in aggregates disappeared after one to two minutes. Even the peak number of platelet aggregates in the blood from the coronary sinus was so small that one cannot detect increased numbers of circulating platelet aggregates in samples from the systemic circulation.
From our data we conclude that platelets form aggregates when activated in vivo and that secretion of their granules only occurs after aggregate formation has already taken place. Aggregate formation can only be detected for a short time in the blood probably due to the fast retention of the aggregates in the microcirculation.

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***EXTRACORPOREAL CIRCULATION IN THE YEAR 2001 – IMPORTANCE AND LIMITATIONS**

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In the last 20 years cardiac surgery showed an impressive growth concerning the numbers of patients operated upon as well as regarding the social and economic impact in general.
In the year 2000 20 centers in the federal republic of germany performed about 98.000 cardiac operations. Out of these 73.000 were done for coronary artery disease, while 16.000 were necessary for valve reconstruction or replacement. In contrast only 4000 operations were performed in a minimal invasive setup without extracorporeal circulation.
Facing this situation, the application of extracorporeal circulation is still a major and important tool in the armentarium of the cardiac surgeon. However, nowadays, the usage can be performed with an extremely high standard due to the incorporation of modern hollow or membrane oxygenators, the application of coated bypass equipment as well as online blood gas monitoring of the patient. Based on this quality standard equipment related procedure complications are extremely rare.
Despite these achievements, changes in the coagulatory system of the patient, changed flow conditions throughout the procedure as well as the necessary anticoagulation of heparin with protamin still results in an measurable interaction with the patient leeding to neurocognitive dysfunction in some aside from the so called systemic inflammatory response syndrom.
To optimise these limitations, we currently investigate inflammatory reactions (cytokins, activation of the coagulatory cascade) of the metabolism to reduce complex reactions in the body throughout open heart procedures to further enhance the safety of this type of patient treatment.

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***BIOCHEMICAL AND PHARMACOLOGIC LIMITATIONS OF HEPARIN-DERIVED OLIGOSACCHARIDES**

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Several ultra low molecular weight, heparin-derived oligosaccharide (HDO) mixtures (MW <2500) and a synthetic pentasaccharide are in advanced clinical development for various thrombotic indications. OP 2000 is a porcine mucosal HDO that is undergoing clinical trials for the treatment of inflammatory bowel disease. C3, an HDO derived from gamma radiation cleavage, is undergoing clinical trials for the management of vascular dementia and Alzheimer's disease. Pentasaccharide (fondaparinux, Arixtra®) has been found to be effective for the management of post-orthopedic surgery thrombosis. Both fondaparinux (Fond) and OP 2000 have also been reported to be effective in the management of acute coronary syndrome (unstable angina). In comparison to heparin and low molecular weight heparins, HDOs have a different pharmacologic spectrum. Fond has a unique biochemical profile and, unlike other HDOs, it is homogeneous with a high affinity to ATIII. All HDOs have a narrower mechanism of action than heparin targeting the inhibition of Xa and thrombin generation. They are devoid of other heparin-like pharmacologic actions such as the interaction with HCII and endothelial release of antithrombotic mediators such as TFPI. HDOs are capable of passing through the placenta and blood brain barrier. Despite a very low or no anti-IIa action HDOs produce a proportionately higher than expected hemorraghic effect, the mechanism of which is unknown. There is no known antagonist to neutralize these bleeding effects. Furthermore, HDOs are cleared through the kidneys and may accumulate in patients with renal dysfunction necessitating dosage adjustment in patients on the basis of creatinine clearance rates. While parallel dose-response curves can be obtained in *in vitro* and *in vivo* studies with OP 2000 and C3, a similar parallel response was not observed with Fond such that a clear dose-response (efficacy or safety) was not obtained in human trials (post-orthopedic surgery and in unstable angina). The hemorrhagic effects at slightly higher dosages, lack of dose-response and the recommendation for no monitoring makes it difficult to compare the observed clinical efficacy of these agents to heparin. The characteristics of HDOs taken together suggest that the recommended fixed dosing may not be an optimal approach for these drugs. Additional preclinical pharmacologic data is needed to validate the claims regarding the therapeutic safety and efficacy of these agents.

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HEMATOLOGIC RESPONSE TO INTRACORONARY RADIATION FOR THE PREVENTION OF POST-PTCA RESTENOSIS

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Restenosis limits the long-term success of coronary angioplasty. Intracoronary radiation is efficacious in limiting restenosis, though late thrombotic stent occlusion occurs in a significant proportion of patients. We hypothesized that leukocyte and platelet activation are associated with these adverse reactions. Blood samples were collected at baseline (BL) and 12-24 hrs and 4-6 weeks post-procedure from patients undergoing PTCA with stent placement (n=41), PTCA of stent restenosis+beta-irradiation (BETA; n=14) or PTCA of stent restenosis+gamma-irradiation (GAMMA; n=12). All patients were treated with heparin and a GPIIb/IIIa antagonist prior to PTCA and clopidogrel post-procedurally. At 12-24 hrs post-procedure, neutrophil CD11b expression was increased ($p<0.05$) in patients receiving stents (mean fluorescence intensity: 77.2±3.2 vs. 122.0±9.5 at BL and 12-24 hours, respectively) and BETA (88.6±11.2 vs. 163.6±19.1). CD11b expression 12-24 hrs post-procedure in the BETA group was significantly higher compared to the stent-only and GAMMA groups ($p<0.05$) and returned to BL levels in all groups by 4-6 weeks. Platelet-monocyte aggregates were increased in the BETA group (15.9±4.7 vs. 56.1±5.9%; $p<0.001$), GAMMA group (16.7±3.3 vs. 30.7±5.6%; $p=0.007$) and the stent only group (16.9±3.1 vs. 36.1±3.2%; $p<0.001$). The level of platelet-monocyte aggregates was significantly higher in the BETA group compared to the other two groups ($p<0.05$) and returned to BL levels by 4-6 weeks. Platelet-neutrophil aggregates were significantly elevated at 12-24 hrs post-procedure in the BETA group (5.4±0.5 vs. 8.9±1.7%; $p=0.03$) and in the stent-only group (4.9±0.3 vs. 6.3±0.4%; $p=0.001$). There was a non-significant increase in platelet CD62 expression at 12-24 hrs relative to BL in all treatment groups. These results demonstrate that leukocyte activation occurs in PTCA patients with or without radiation, and despite anticoagulation with heparin and anti-platelet therapy, platelet-leukocyte aggregates are formed. The current approach to antiplatelet therapy is not sufficient to protect patients undergoing PTCA from hemostatic activation. The differential ability of beta and gamma irradiation to induce alterations in the hemostatic system may point to a mechanism by which brachytherapy promotes late stent thrombosis in some patients.

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PROLACTIN AS A NOVEL POTENT COFACTOR FOR PLATELET AGGREGATION

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Pregnancy (including puerperium) is a period of hypercoagulability and seems to be an independent major risk factor for venous thromboembolism (VTE). However, the basis of the increased risk of VTE in pregnancy and around delivery is unkown. We hypothesized that changes of prolactine (PRL) which is the most prominent increasing hormone during pregnancy and lactation might be involved in the activation of platelets. To investigate platelet functional abnormalities in pregnancy, we assessed the ADP-stimulated and nonstimulated P-selectin expression of platelets in 42 consecutive pregnant women, normo (n=11)- and hyperprolactinemic (n=11) patients with pituitary tumours, and 100 controls. In addition, the in vitro stimulation of platelets by human PRL was studied. We show a significant correlation between PRL values and ADP stimulation of platelets in pregnant women ($r=0.56$;$p<0.0001$) and patients with pituitary tumours ($r=0.57$;$p=0.006$). Hyperprolactinemic pregnant women or hyperprolactinemic patients with pituitary tumours revealed significant higher ADP stimulation of platelets ($p<0.0001$) than healthy controls or normoprolactinemic patients with pituitary tumours. Furthermore, during TRH test or dopamine agonist therapy we detected concordant short term changes of PRL and platelet stimulation. These results were reconciled by an increased in vitro stimulation and aggregation of platelets using human PRL.Our absolutely novel findings lead to the conclusion that PRL may be a physiological regulator of the delicate coagulation balance during pregnancy and puerperium. Moreover, our data indicate that hyperprolactinemia causes increased ADP stimulation of platelets both in vitro and in vivo what might explain the increased risk of VTE in pregnant women around delivery. Further studies of the interaction between PRL and platelets will clarify the clinical relevance of hyperprolactinemia as a potential risk factor for VTE.

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HYPERPROLACTINEMIA IN PATIENTS ON ANTIPSYCHOTIC DRUGS CAUSES PLATELET STIMULATION – WHAT MIGHT EXPLAIN THE INCREASED RISK FOR VENOUS THROMBOEMBOLISM

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In a previous study we demonstrated that hyperprolactinemia causes increased ADP stimulation of platelets both in vitro and in vivo what might explain the increased risk of venous thrombembolism (VTE) in pregnant women around delivery (1). The molecular aetiology of increased risk of VTE in patients on antipsychotic drugs is unkown (2). Most antipsychotic drugs act as dopamine antagonists and some of them cause hyperprolactinemia. We assessed the PRL values as well as the P- selectin expression of ADP- and thrombin receptor activator 6 (TRAP-6)- stimulated and non- stimulated platelets of patients prescribed antipsychotic drugs. Twenty consecutive patients (mean age: 41±10 years, 14 females, 6 males) on antipsychotic drugs without history of VTE were investigated. Eleven of these 20 patients showed normoprolactinemia or only a slight increase of PRL values (PRL<700 mU/l), whereas 9 patients revealed hyperprolactinemic values (PRL>1000 mU/l). In these 20 patients, the PRL values were significantly correlated with the ADP stimulated P-selectin expression of platelets ($r=0.5$; $p=0.006$). Furthermore, the patients with high PRL values (PRL>1000 mU/l) revealed significant higher ADP stimulated P-selectin expression than did the 100 healthy controls ($p<0.0001$) and the patients with normoprolactinemia ($p<0.001$). In contrast to ADP-stimulated P-selectin expression, the TRAP-6 stimulation of platelets was not influenced by PRL values. Therefore, hyperprolactinemia might be the yet unknown acquired risk factor for VTE in patients on antipsychotic drugs explaining the increased risk for VTE in these patients.

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***GENTHERAPIE DER HÄMOPHILIE**

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Die Hämophilie ist eine X-chromosomal vererbte Störung der Blutgerinnung und beruht auf einem Defekt im Gen für Faktor VIII (Hämophilie A) oder Faktor IX (Hämophilie B). Die gegenwärtige Therapie dieser Erkrankung besteht in der intravenösen Verabreichung des fehlenden Proteins, das aus dem Plasma von Spendern gewonnen wird oder mit gentechnologischen Verfahren hergestellt wird. Diese Therapie wird entweder nur beim auftreten von Blutungen vorgenommen (Bedarfstherapie) oder aber in Form einer ständigen Prophylaxe zur Verhinderung von Blutungen gegeben. Nachteil der Bedarfstherapie ist, dass unvermindert häufig Blutungen auftreten, die mit entsprechenden Spätschäden vor allem der großen Gelenke einhergehen. Nichtsdestotrotz hat diese Therapie, durch das vergleichsweise raschere Sistieren der Blutungen, zu einer dramatischen Verbesserung der Mortalität und Morbidität der Erkrankung geführt. Die prophylaktische Gabe von Faktor VIII oder Faktor IX ist zwar in der Lage Spontanblutungen fast vollständig zu verhindern und somit - wenn sie bereits im Kindesalter begonnen wird - auch die blutungsassoziierte Morbidität zu verhindern, muss aber wegen der kurzen Halbwertszeit der Faktoren mehrmals wöchentlich mittels intravenöser Infusion vorgenommen werden und ist vor allem im Kindesalter auf Grund der Schwierigkeit eines venösen Zugangs oft problematisch. Diese und andere Probleme, wie etwa die Gefahr von Infektionsübertragung durch Plasmaprodukte oder die begrenzte Verfügbarkeit von Plasma haben die Suche nach alternativen Therapieformen stimuliert. Dabei zeigt sich, dass die Hämophilie günstige Voraussetzungen für die Durchführung einer Gentherapie mit sich bringt. So ist etwa die therapeutische Breite des Genproduktes Faktor VIII oder Faktor IX sehr groß, unterliegen weder die FVIII noch die FIX -Spiegel einer engen Genregulation und können als ausgezeichnete Surrogatmarker für die Blutungsneigung und damit die Effizienz einer Gentherapie leicht bestimmt werden. Darüber hinaus gibt es eine Reihe von natürlichen Tiermodellen der Hämophilie, die die präklinische Einschätzung der Nutzen Risiko Relation vor den ersten klinischen Studien erleichtert haben. Vor diesem Hintergrund sind vier klinische Studien durchgeführt worden, von denen bereits Ergebnisse vorliegen und ist eine Reihe weiterer klinischer Studien in Entstehung begriffen.

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THE PREKALLIKREIN REAGENT PROMOTED BY THE EUROPEAN PHARMACOPOEIA ONLY CONTAINS MINUTE QUANTITIES OF PREKALLIKREIN

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Background: The European Pharmacopoeia defines limits for the concentration of prekallikrein activator (PKA) in albumin and IgG preparations and also recommends a specific method for the preparation of the prekallikrein (PK) reagent required for the assay. Here we present the results of the biochemical characterization of PK reagents used for quality control testing.
Methods: We characterized several reagent batches and one purified prekallikrein preparation using general methods like SDS-PAGE and size exclusion chromatography (SEC). In addition we used ELISA systems to quantify selected proteins, e.g. prekallikrein, factor XII, factor XI, high molecular weight kininogen, immunoglobulin G, albumin, C1-inhibitor and antithrombin. Furthermore, the levels of factor XIIa as well as the amidolytic activities towards the substrates PK-1 and S-2302 were determined with and without activation by PKA.
Results and discussion: The result of the immunoassay showed that prekallikrein constituted less than 1% of the total protein. This was further supported by the data obtained on SDS-PAGE and SEC. The main constituent in all the PK reagents proved to be IgG. Other proteins involved in the process of contact activation like high molecular weight kininogen, factor XI and factor XII, when detectable, were present in very low concentrations. C1-Inhibitor and antithrombin showed levels below 1.5 µg/ml corresponding to less than 0.006 U/ml and 0.01 IU/ml, respectively. The presence of high amounts of alpha2-macroglobulin could be excluded due to the molecular weight profiles obtained on SEC. Almost identical amidolytic activities were found using both of the chromogenic substrates and factor XIIa was found at concentrations below 10 ng/ml in all preparations.
Our results show PK reagents have a low purity. This could have an effect on the measurement of PKA levels in biopharmaceutical products. In particular, the presence of inhibitors in the PK preparation, which is not checked on a routine basis, could be the reason for non-valid test results. Therefore, the construction of a well-defined high purity PK reagent should be considered.

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***PLATELET-INDUCED MITOGENESIS**

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Experimental and clinical evidence indicates an involvement of platelets in the pathology of atherosclerosis and restenosis. However, the precise role of platelet-derived growth factors for smooth muscle cell (SMC) proliferation is not clear and many questions remain unresolved. Platelet-dependent mitogenesis is determined by a coordinate action of several classes of mitogenic factors which are either released from storage pools or generated upon platelet activation. Although platelet-derived growth factor (PDGF) is considered to be the most important platelet mitogen it is very likely that yet uncharacterized mechanisms (e.g. platelet surface membranes, platelet microparticles, CD40 ligand) are involved. In addition, differential (stimulatory or inhibitory) effects on SMC growth have been reported for some platelet-derived growth factors. Thus, for the overall response, complex interactions between multiple factors need to be considered.

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FLOW CYTOMETRY ANALYSIS OF PLATELET CYCLOOXYGENASE 2 EXPRESSION

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There are conflicting reports regarding the expression of cyclooxygenase (COX)-2 in human platelets. The present study describes a flow cytometry method for the measurement of platelet COX. Using this method, COX-2 in addition to COX-1 was shown to be expressed in platelets. This method has subsequently been applied to measure the expression of platelet COX in patients undergoing coronary artery bypass grafting. There was a significant increase in COX-2 expression at day 5 as compared to pre-surgery values (mean fluorescence 12.31±0.88 versus 9.15±0.88; means±SEM, n=7, $p<0.05$), while COX-1 levels did not change (mean fluorescence 13.45±1.11 versus 12.38±1.41; means± SEM, n=7, $p>0.05$).
These data confirm the expression of COX-2 in human platelets and provide evidence for an up-regulation of COX-2 expression levels under certain clinical conditions.

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TOWARDS A DEFINITION OF ASPIRIN RESISTANCE – A TYPOLOGICAL APPROACH

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By determination of collagen (1 µg/ml)-induced platelet aggregation and thromboxane formation (measured as thromboxane B2) in citrated platelet-rich plasma, this study demonstrates that “aspirin resistance” of platelets can be classified into three distinct types. In aspirin responders, both, collagen-induced platelet aggregation and thromboxane formation is completely (>95%) inhibited by oral aspirin treatment (100 mg/d). In type I resistance (pharmacokinetic type), oral treatment with aspirin is ineffective but addition of aspirin (100 µM) in vitro results in a complete inhibition of collagen-induced platelet aggregation and thromboxane formation. In type II resistance (pharmacodynamic type), neither oral treatment with aspirin nor addition of aspirin in vitro inhibits collagen-induced platelet aggregation or thromboxane formation. In type III resistance (pseudo-resistance), platelet aggregation can be induced by a low concentration of collagen (1 µg/ml) despite of a complete inhibition of thromboxane formation by oral aspirin treatment. This typology of aspirin resistance should help to clarify the mechanisms, the actual rate, and the possible clinical consequences of this phenomenon.

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IN-VITRO MEASUREMENT OF THE HEMOSTATIC EFFECT OF RECOMBINANT FACTOR VIIA (NOVOSEVEN) USING A THROMBIN GENERATION ASSAY

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Recombinant factor VIIa treatment is now well established as a therapy for inhibitory antibody hemophilia, though efficacy monitoring is still a problem, as e.g. the PT standard clotting assay is not sensitive to FVIIa in therapeutic ranges. Thrombin generation as a parameter of procoagulant activity under rFVIIa treatment in individuals has a large degree of variation, probably caused by molecular differences in activated platelets. Furthermore, standardisation of hemostatic effect measurement is not feasible due to large variations of platelet procoagulant activity between individuals (Summer et al., Thromb Res 1996; 81:533-543).
Hence, to monitor the hemostatic response of FVIIa in individuals before and after treatment, it is necessary to use the patient's own platelet rich plasma (PRP) as a standard. A semiautomatic assay of thrombin generation (Hemker et al., Thromb Haemost 2000; 83:589-591) was used to monitor the hemostatic potential of rFVIIa. A standard curve using concentrations from 50-1000 U/ml was taken to create a dose-response profile for each individual. For each profile, the amounts of thrombin (ETP), their maximum generation velocity (peak) and the time to reach the peak (lag time) were calculated. The ETP showed a sigmoid progression with dose increase, whereas the peak and the time to peak showed an exponential increase resp. decrease. Between 100 and 500 U/ml the signal increased strongly, whereas the supratherapeutical concentrations describe the beginning of a plateau phase with an ETP signal of about 800 relative fluorescent units in our testing system, comparable to that generated from a maximum effective concentration of TF (Dade Behring), also in healthy individuals. A plateau phase was also reached by the decreasing lag-time for concentrations above 200 U/ml. There seems to be a maximum thrombin generating potential, resulting in plateau levels differing from patient to patient in the assay. When a plateau is reached, further application of FVIIa has no stronger hemostatic effect therefore.
The results indicate that the thrombin generation assay is a suitable test to monitor hemostatic effect of rFVIIa when ETPs or lag-times of dose response profiles are used. Whether these results, obtained in a research laboratory in vitro, are transferable to clinical routine use, has to be clarified in further studies.

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TISSUE FACTOR LIGAND INTERACTION ACTIVATES STRESS-ACTIVATED PROTEIN KINASE P38 INDEPENDENTLY OF THE PROTEOLYTIC ACTIVITY OF FVIIA

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The complex of Tissue Factor (TF) - Factor VIIa (FVIIa) modifies intracellular signal transduction pathways. Dependent upon its proteolytic activity FVIIa stimulates protease-activated receptors. In addition, TF ligand interactions induce recruitment of the Actin-binding Protein-280 to the cytoplasmic domain of TF independently of the proteolytic activity of FVIIa and thereby support cell spreading and migration.
Stress-activated kinase-2 (SAPK/2) p38 enhances intracellular F-actin concentrations by phosphorylation of the F-actin polymerization factor heat shock protein (HSP) 27. Activation of this pathway by oxidants and growth factors results in an increased cell migration.
To investigate TF-mediated activation of SAPK-2 p38, smooth muscle cells were incubated with FVIIa and proteolytically inactive FFR-FVIIa. Dose-dependent activation of SAPK-2 p38 kinase activity was found for both ligands to a similar extent. In vitro kinase assays showed an increase in kinase activity by 88+3% after incubation with FVIIa and by 75+4% after incubation with FFR-FVIIa compared to unstimulated controls. In experiments with the human bladder carcinoma cell line J82, that express high levels of TF, similar results were obtained. The FFR-FVIIa-induced activation of SAPK-2 p38 was specific for TF, because it was inhibited by the soluble TF extracellular domain.
In addition to the interaction of the cytoplasmic domain of TF with the actin-binding protein-280, TF-mediated activation of SAPK-2 p38 may enhance cell motility.

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LOW MOLECULAR WEIGHT HEPARIN REDUCES TUMOR CELL ATTACHMENT TO ENDOTHELIAL CELLS BY DECREASING CYTOKINE INDUCED EXPRESSION OF ADHESION MOLECULES

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Heparins have many actions that may affect the malignant process and studies show an improved outcome in cancer patients treated with heparin. Low molecular weight heparins (LMWH) seem to be superior to unfractionated heparins (UFH) and reduction in death is not correlated with preventing thromboembolism. The reason for the more favorable profile of LMWH particularly in cancer patients is still not understood.
We focused on the effect of heparins on tumor cell adhesion to endothelial cells, as this is an early and important step in metastasis.
After pretreatment of human umbilical vein endothelial cells (HUVEC) monolayers with LMWH (10 to 500 U/ml) for 2 hours and subsequent stimulation with interleukin-1beta (IL-1beta;1ng/ml/4h), there was decreased adhesion of 3H-radiolabeled malignant pancreas tumor cells (PaTu-8902 and ELAM-98, a pancreatic adenocarcinoma established in our laboratory) as compared to non-heparin-pretreated HUVEC. Significantly superior results were observed as compared to pretreatment with UFH.
Quantitative assessment of E-selectin (ELAM-1, CD62E) expression on HUVEC by flow cytometry is suited for adhesion molecule studies, as E-selectin is not expressed constitutively, it is quickly regulated, well understood and of particular importance in pancreatic cancer. Expression of E-selectin was induced by proinflammatory cytokines IL-1beta and tumor necrosis factor alfa (TNFalfa) and correlated with dose (0,1-1 ng/ml) and time (1-12 h) of stimulation. Pretreatment with LMWH (10 to 500 U/ml) significantly decreased E-selectin expression dose dependently. Increasing time of pretreatment (1 to 3 hours) correlated with a more pronounced decrease in expression of E-selectin. No such effect could be seen with the use of unfractionated heparin.
In addition we could show significantly decreased transcriptional levels of E-selectin mRNA by Nothern Blot analysis in LMWH-pretreated stimulated HUVEC as compared to non-heparin-pretreatment.
These results suggest an interaction of LMWH with cytokine induced protein biosynthesis of adhesion molecules and may explain the favorable effect of LMWH on the malignant process.

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DETECTION OF PLATELET ADHESION TO ARTIFICIAL SURFACES USED FOR EXTRACORPOREAL CIRCULATION PROCEDURES BY CD 41 WESTERN-BLOTTING

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Background: Platelet adhesion and aggregation at the inner surfaces of the extracorporeal circulation (ECC) devices is still an unsolved phenomenon. Till now the detection of the platelet sticking was only possible by inaccurate microscopically methods. We have developed a reproducible technique for quantification of platelet adhesion by using a non-blocking monoclonal antibody against the glycoprotein receptor GP IIb/IIIa of the platelet membrane.
Materials and Methods: Two ECC models were used: A simulated cardiopulmonary bypass model using membrane oxygenators, and a modified Chandler Loop model with tubings. We compared non-coated with biopassive (polypeptides) or bioactive (heparin) coated devices. After 120 minutes of recirculation with fresh human whole blood the devices were rinsed off extensively with physiological saline followed by washing steps with PBS containing 20 mM EDTA (pH 7.4). Then the surface adsorbed proteins were eluted with lithiumdodecylsulfate (1% [w/v] in PBS), concen-trated by ultrafiltration, assayed for protein concentration, separated by SDS-PAGE, and transferred by semidry-electroblotting onto nitrocellulose membranes. Subsequently the blots were incubated with a monoclonal antibody for CD 41 (GP IIb/IIIa, Immunotech, Marseille, France) and visualized with the alkaline phosphatase substrate BCIP (Biorad, Hercules, CA, USA).
Results: The Western-Blotting experiments with the CD 41 antibody indicated a major band with a molecular mass of approx. 115 kDa. The devices without surface coatings showed significantly higher concentrations of CD 41 compared to polypeptide- and heparin-coated surfaces. These results corresponded with lower total platelet count, elevated b-thromboglobulin levels and higher fibrinogen adsorption within the uncoated devices.
Conclusion: The thrombogenicity of ECC devices is highly dependent on the degree of platelet adhesion and loss of functional reactivity. Our newly developed immunological technique for quantification of platelet sticking enables us to evaluate the affinity of platelets to the artificial surfaces used for ECC. This criterion may be useful to choose less platelet activating materials for ECC procedures which may ameliorate the various post-pump syndromes, including blood loss.

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PATHOLOGICAL PFA-100 TEST IN CHILDREN WITH LUPUS ANTICOAGULANTS

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Background: Childhood lupus anticoagulants (CLA) normally are transient and do not lead to clinical symptoms, but a small number of the patients (pts.) are at risk for bleeding or thrombosis, especially those with thrombocytopenia. We evaluated the role of PFA-100 test (measurement of platelet adhesion and aggregation) in these children.
Patients and Methods: We examined 71 children (40 males, median age 6 years, range 0.9-15.9) with CLA after exclusion of other bleeding disorders. 73 healthy children (46 boys, median age 4.6 years, range 0.5-15) served as controls and had no underlying diseases and neither bleedings nor thromboses in their history. Diagnosis of CLA was made if: 1) aPTT or dRVV time were prolonged and 2) mixing-test was positive and/or 3) antibody titers were elevated and/or 4) coagulation factors were decreased in lower dilutions and normalised in higher dilutions.
Results: PFA was pathological in 46/71 pts. with CLA (64.8%) and in 8/73 controls (11%) ($p<0.0001$). No cases of thrombocytopenia were observed. Collagen/Epinephrine alone was pathological in 14 pts., Collagen/ADP in 15 and both tests in 17 cases. In 18 pts. (39%) the closure times (CT) were prolonged >40 sec. In 23/46 pts. PFA was repeated during the following months. The disappearance of the lupus antibodies corresponded to a normalisation of PFA in 14/23 pts. (median 6.5 months; 1 week–24 months) and a partial normalisation in another 7/23 children (median 2 months; 2 weeks–14 months). In two out of 23 children the lupus antibodies persisted and the test remained pathological.
A mild bleeding tendency was observed in 22/46 pts. with pathological PFA (48%) and in 11/25 pts. with normal PFA (44%). 2/46 (7.3%) with prolonged CT had severe bleedings. In 6/46 pts. with pathological PFA, no bleeding complications occured during adenoid surgery. The CT of these children was only slightly prolonged. The other elective operations were either accomplished after normalisation of the test or were cancelled.
Conclusion: Although our pts. had a normal number of thrombocytes, 64.8% of them showed transiently abnormal platelet function analysis. Since it is not clear if these children will have a higher bleeding tendency during surgical procedures, prospective evaluations are required. In addition, CLA diagnosis has to be taken into consideration in children with pathological PFA-100 test.

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FUNCTIONAL ASSESSMENT OF FIBRINOLYTIC RESISTANCE IN WHOLE BLOOD USING MODIFIED THROMBELASTOGRAPHY

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Introduction: In theory alterations of the fibrinolytic system should be predisposing factors for thrombosis and embolism. However recent studies have shown a limited predictive value of the classical fibrinolysis parameters in respect to the thrombophilic risk of the individual patient. In a pilot examination we evaluated the individual fibrinolytic response to a standardized urokinase stimulus analyzed in whole blood.
Methods: Citrated blood was drawn from 51 thrombophilia patients and 29 healthy volunteers. Whole blood coagulation and lysis was assessed in dublicate on the ROTEG coagulation analyzer (Pentapharm, Munich), a novel thrombelastographic system with 4 channels and computer analysis. Coagulation was triggered by the addition of 0,15 ng recombinant tissue factor/ml blood. Fibrinolysis was triggered by the addition of 30 or 60 U of urokinase/ml blood (Medac). Fibrinolysis was characterized by the lysis time (time form the onset of clotting until the lysis of the clot).
Results: The lysis times of the two groups showed no significant difference under low urokinase activation. Using the high urokinase dose a significant difference of the lysis times was observed between healthy volunteers and patients ($p<0.05$ ANOVA).
Conclusion: Using the high fibrinolytic stimulus approximately 10% of the analysed thrombophilia patients showed longer lysis times than any of the analyzed healthy volunteers. The test system we applied evaluated the fibrinolytic system in whole blood using a standardized stimulus by a defined dose of urokinase. Urokinase was applied because of the better stability when compared to t-PA. This is a promising approach for an functional testing of the fibrinolytic system.

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***RFVIIA (NOVOSEVEN®) AND PLATELETS FROM EX-VIVO MODELS TO CLINICAL USE**

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Patients with Glanzmann´s thrombasthenia (GT) often suffer from severe bleeding complications in case of injury or surgery. Prolonged bleeding time is caused by the diminished or lacking glycoprotein (GP) IIb-IIIa, which mediates platelet aggregation via fibrinogen binding, while platelet count and coagulation are normal. As yet, platelet transfusion remain the sole life-saving treatment in this situations, with the risk of developing a refractory state due to anti-HLA or anti-GPIIb-IIIa allo-immunization. Recombinant factor VIIa (rFVIIa, NovoSeven®) has been shown to be effective in the treatment of severe bleeding episodes and for coverage of surgical procedures in patients with thrombocytopathies and a previous history of ineffective platelet transfusion, which will be reviewed in the current presentation. However, there is still the question in which way rFVIIa compensates platelet dysfunction and whether the heterogen population of GT patients shows differences in the hemostatic response to rFVIIa. In a current study we investigated the hemostatic effect of rFVIIa in vitro on platelets of five members of a well characterized familiy with GT (2 homozygot, 3 heterozygot GT patients). Moreover we investigated the in vivo effect of rFVIIa treatment during a surgical intervention of a homozygot GT patient.
In the in vitro experiments with platelets of two homozygote sisters (type I) and three heterozygot familiy members with no fibrinogen binding capacity respectively 60-70% fiibrinogen binding capacity we measured simultanously expression of the activation marker CD62 and Phosphatidyserin exposition (annexin V binding). Thrombin generation (Hemker et al., Thromb Haemost 2000; 83:589-591) was estimated in response to 50-200 U/ml rFVIIa (20-200 nM) using platelet rich plasma. The homozygot sisters showed a diminished thrombin generation compared to the heterozygote persons and annexin V binding, respectively phopshatidylserin (PS) exposition on the platelet surface was initated only at higher concentrations of rFVIIa. The results suggest, that in case of homozygote GT (type I) the hemostatic effect of rFVIIa on thrombin generation is diminished, may be caused by decreased platelet activation and delayed exposition of PS on the platelet surface.
Despite the reduced response of homozygote GT patients to rFVIIa treatment in vitro we observed a good benefit of rFVIIa treatment in a surgery intervention. The observation in case of a homozygot GT patient treated with rFVIIa showed that rFVIIa enhanced the diminished PS exposition of this patient. This probably increased the thrombin generation on the platelet surface causing strong elevated fibrin generation. The generated “unphysiological” fibrin clot stopped bleeding without participation of platelet aggregation.
Taken together, rFVIIa is increasingly recognised an an effective haemostatic agent and as an alternative to platelet transfusion in the management of severe bleeding secondary to thrombocytopenia or thrombocytopathy.

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MEASUREMENT OF THROMBIN-GENERATION, ANNEXIN V BINDING, FIBRINOGEN BINDING AND CD62 EXPRESSION IN CASE OF A FAMILIY WITH HETEROZYGOTE AND HOMOZYGOTE GLANZMANN’S THROMBASTHENIA SPIKED IN VITRO WITH RFVIIA (NOVOSEVEN)

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Patients with Glanzmann’s thrombasthenia (GT) often suffer from severe bleeding complications in case of injury or surgery. Prolonged bleeding time is caused by the diminished or lacking glycoprotein (GP) IIb-IIIa, which mediates platelet aggregation via fibrinogen binding. Platelet transfusions remain the sole life-saving treatment in this situations, with the risk of developing anti-HLA or anti-GPIIb-IIIa allo-immunization to reapeted platelet transfusion. Recombinant factor VIIa (rFVIIa, NovoSeven) has been used in a few patients with GT with some success for surgical procedures. An interesting question is whether different patients with GT have differences in the hemostatic response to rFVIIa. We describe here the effect of rFVIIa on platelets of heterozygote (father and mother) and homozygote members (two sons of 14 and 16 years) of a familiy with GT.
Genetic analysis of cDNA revealed a deletion of GPIIb exon 29, the putative transmembrane region of GPIIb (Frankfurt III). Flowcytometric analysis of GPIIb-IIIa on the patients platelets showed an amount of <3%

in case of the two homozygote sons and about 60% GPIIb-IIIa in case of the heterozygote parents. Platelets of the two homozygote sons had almost no fibrinogen binding capacity in response to 0.1-10 µM ADP or 1-10 µM TRAP, while the fibrinogen binding capacity of parents platelets was about 60-70%. Simultanously we measured expression of the activation marker CD62 and platelets of the two homozygote sons had a significant reduced expression of CD62 in response to ADP and TRAP, while the parents had an almost normal platelet activation. Thrombin generation (Hemker et al., Thromb Haemost 2000; 83:589-591) in response to 50-200 U/ml rFVIIa (20-200 nM) using platelet rich plasma showed a diminished amount and prolonged starting point of thrombin generation in case of the two homozygote sons compared to the heterozygote parents, which react almost normal. Annexin V binding, respectively phopshatidylserin exposition was initated by lower concentrations of rFVIIa on the platelet surface of the heterozygote parents than the homozygote sons.
The results suggest, that in case of homozygote GT (type I) the hemostatic effect of rFVIIa on thrombin generation is diminished, may be caused by decreased platelet activation and delayed exposition of phosphatidylserine on the platelet surface.

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RETENTION TEST HOMBURG (RTH) IN MONITORING OF VWF-MEDIATED PLATELET ADHESION AFTER DESMOPRESSIN ADMINISTRATION

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Background: The Retention Test Homburg (RTH) is based on retention of platelets by a polyurethane filter which is athrombogenic in contrast to other test systems. This platelet retention is mainly the result of an activation of platelets and/or v.Willebrand factor (vWF) induced by the shear stress when whole blood is pressed through the filter. The aim of this evaluation was to examine the RTH in monitoring platelet adhesiveness by vWF.
Methods: Healthy volunteers (n=12, pre-treated with 500 mg aspirin) received 0.4 ug/kg BW DDAVP by infusion over 0.5 h; blood was taken 0, 0.5, 1, 2, 2.5, 3, 4 and 6 h after starting DDAVP. Further data were obtained from substitution therapy of a patient with vWF disease. The RTH was performed by applying 90 g centrifugal force to the whole blood specimen given onto the disposable filter from Eppendorf (Germany).
Results: The RTH platelet retention values were not significantly reduced by the aspirin pre-medication (p>0.05). The infusion of 0.4 µg/kg DDAVP led to an immediate and distinct increment of retention values from 34±9 to 71±4% (p<0.00001). Subsequently, platelet retention gradually declined to pre-DDAVP levels (56±16, 50±13, 44±8, 35±9, 33±7 and 30±7% retention in above mentioned samples). When applying twofold 0.2 µg/kg DDAVP with a delay of 2h, the 2nd infusion induced only a slight but significant increase from 44±9 to 48±10% (p<0.05). The kinetics of these RTH values did not correlate either with vWF/FVIII concentrations or with in-vitro bleeding values (measured by PFA from Dade-Behring) or with data from the platelet adhesion assay (PADA) as described by G.Nowak.
Further insight into RTH specificity was gained from therapy of a patient with the Miami subtype of v.Willebrand disease 2A. This patient received an infusion of 0.4 µg/kg DDAVP and 4h later 50 U/kg of a vWF-enriched FVIII concentrate (Haemate HS with 2.2U vWF-RiCoF per unit FVIII). DDAVP resulted in an increment of plt. retention from 20 to 47% and improved it for roughly 2 h. vWF/FVIII infusion led to an increase from 23 to 42% declining to 30% within next 4 h. Furthermore, the in-vitro addition of 2 FVIII units of Haemate to 1ml whole blood (n=5 healthy donors) led to an increment in plt. retention from 25.6±7 to 61.6±5% (p<0.0001).
In conclusion, RTH platelet retention seems to sensitively measure vWF-mediated adhesion of platelets.

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TWOFOLD ADMINISTRATION OF 0.2 µG/kg BW CAN IMPROVE THE EFFICACY OF 0.4 µG/KG DESMOPRESSIN ON PRIMARY HEMOSTASIS

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Desmopressin (DDAVP) is used in patients with platelet disorders to shorten bleeding time. Prior studies demonstrated that bleeding time normalization lasted only approx. 2-3 h and showed a different kinetics than the vWF/FVIII increase. Thus a second dosage might be reasonable to avoid a delayed bleeding episode after initial hemostasis. It was the aim of this study to compare different infusion regimens of 0.4 µg/kg DDAVP to improve primary hemostasis.
In this randomized cross-over study n=12 healthy volunteers (pre-treated with 500 mg aspirin) received 0.4 µg/kg BW DDAVP in total (regimen B) and in two aliquots á 0.2 µg/kg with a delay of 2 h (A); each DDAVP infusion lasted 0.5 h. Blood samples were taken before, 0.5, 1, 2, 2.5, 3, 4 and 6 h after the first infusion. Platelet retention by a polyurethane filter was measured by the Retention Test Homburg (RTH) according to E.Wenzel; bleeding time was determined in-vitro with the PFA from Dade-Behring.
Due to aspirin pre-medication the PFA bleeding was markedly prolonged before DDAVP infusion, but without normal distribution. Thus, bleeding volume BVp, that passed the epinephrine/collagen cartridge within 30 to 120 s was calculated. Initially this bleeding volume BVp was markedly increased (194±30 µl) but normalized immediately after DDAVP (81±45 µl, p<0.001). Up to 2 h after the first infusion BVp did not significantly differ between both regimens (p>0.02). After the 2nd infusion i.e. 2.5 and 3 h after 1st infusion BVp was distinctly lower (p<0.01) when using DDAVP regimen A (84±35 and 95±45 µl) than regimen B (127±40 and 150±53 µl). This difference was the result of the second DDAVP administration once more leading to a reduction of in-vitro bleeding (p<0.05) which, in contrast, gradually increased when regimen B was applied containing only one DDAVP dosage. Both regimens led to a distinct increase of FVIII and vWF. The additional effect of the 2nd infusion was slight and could not explain the corresponding in-vitro bleedings. The RTH platelet retention did obviously correlate with the initial release of large vWF multimers but not with the bleeding values.
In conclusion, a second administration of 0.2 µg/kg DDAVP might be advisable as early as 2 to 6 h after the first infusion for treating a platelet function disorder which seems to require other DDAVP regimens than FVIII or vWF deficiency.

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AN AMINOACID SUBSTITUTION GLY140GLU IN THE CANINE FVII GENE OF DOGS WITH SEVERE FVII DEFICIENCY

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The genetic defect for severe FVII deficiency in Beagle dogs is unknown. We measured the PT of totally 42 Beagle dogs, 36 healthy and 6 FVII deficiency dogs. The activity of the defcient FVII is about 10%. Therefore, we isolated DNA from EDTA blood of 19 healthy and 5 FVII deficieny dogs. In a litter of seven puppies two dogs had a FVII deficiency, the other five puppies were healthy. Additionally, we got the DNA of other FVII deficiency dogs (n=4) unrelated to these animals like the other controls (n=15). We sequenced both strands of the complete coding region for the factor FVII gene of all 24 dogs. The canine FVII cDNA comprises 1444 with an open reaging frame of 1350 bp. The open reading frame of the RNA codes for a 450 amino acid protein.
The cDNA alignment analysis of all six FVII deficiency sequences showed a single base pair substitution (Nt.443) G>A leading to an amino acid exchange Gly140Glu in the FVII protein sequence. All five FVII deficiency dogs are homozygous carriers of the mutated allele and had a FVII activity of about 10%. None of the 19 healthy dogs showed the Gly140Glu mutation. All animals were homozygous for the normal allele. We could not detect any further nucleotide exchange in the FVII gene. The FVII gene of the dog is a highly conserved gene and codes for an 450 amino acid protein. We detected a single nucleotide exchange in the FVII cDNA sequence (Nt.443) G>A amino acid leading to an amino acid exchange Gly140Glu of the FVII deficiency protein in comparison to the wild-type FVII. The Gly140Glu is localized in the EGF-1 domain of the FVII gene. Mutations in the EGF-1 region of the human gene are known to lead to multifaceted symptoms, including severe bleedings. We suppose that this amino acid substitution is responsible for the FVII deficiency. The reduced FVII activity could be caused by conformational changes of the protein in the EGF-1 domain due to the mutation.

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P-SELECTIN EXPRESSION OF PLATELETS IN PATIENTS WITH MYELOPROLIFERATIVE DISEASES

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In patients with myeloproliferative syndromes bleeding complications may be seen despite elevated platetelet count. P-selectin released of platelets alpha granula is known to be essential for platelet adhesion to neutrophils and monocytes. In order to characterize platelet function we looked for expression of P-selectin by flow cytometry both in resting and activated platelets.

Methods: After standardized venous punctures we analysed blood samples of 20 patients with myeloproliferative syndrom (essential thrombocythemia n=7, osteomyelofibrosis n=4, chronic myelogenous leukemia n=7, polycythemia vera n=2) as well as 20 healthy volunteers. Platelet activation was analysed by CD 62 antibody (Becton Dickenson, PE labeled) using FACS-Calibur (Becton Dickenson). The expression of P-selectin both in combination with PAC 1 (antiglycoprotein IIb/IIIa epitop; Becton Dickenson FITC labeled) and CD 42a antibody (antiglycoprotein IX; Becton Dickenson, FITC labeled) were done in resting (rpl) and activated platelets (apl) according to the manufacturers recommendation.
Results: In patients with MPS we found significantly lower P-Selectin expression of activated platelets with both antibody combinations compared to healthy volunteers. After CD 62 and PAC1 incubation as well as after incubation with CD62 and CD 42b resting platelets of MPS patients present significantly higher P-selectin expression than resting platelets of healthy volunteers. This finding means higher activation in MPS patients compared to healthy volunteers.
Conclusions: Our data indicate higher spontaneous platelet activation in patients with MPS compared to the control group but lower activation after stimulation by ADP, respectively. These findings respond to the different types of hemostaseologic complications in patients with MPS and should be proven in further studies.

	cd62rpl(PAC1)		cd62apl(PAC1)		cd62rpl(cd 42a)		cd62apl(cd 42a)	
	mean	rank	mean	rank	mean	rank	mean	rank
healthy volunteers	1,4	16,02	58,6	25,85	1,71	11,85	75,25	26,3
myelo-proliferative disorders	2,35	25,74	46,37	15,15	5,68	29,71	65,87	15,95
significance		0,009		0,003		<0,0001		0,006

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***MODULATION OF ENDOTHELIAL BARRIER FUNCTION BY FACTOR XIII: EXPERIMENTAL AND CLINICAL RESULTS**

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Surgeons may be confronted with breakdown of endothelial barrier function in different critical situations as in systemic inflammatory response syndrome or sepsis, during ischemia and reperfusion, in inflammatory bowel disease, in myocardial and generalized edema formation, and in wound healing.
Recently, we demonstrated that thrombin-activated factor XIII reduces endothelial permeability (P) and prevents hyperpermeability in cultured endothelial cells (EC) [in EC-monolayer reduction of P by 34±5% within 20 min (1U/mL)] and in isolated-perfused rat hearts (significant reduction of myocardial water content) and may thus confer a protective effect against edema formation.
The following prospective, randomized and blinded clinical study on the effect of a single dose (250 U FXIII, Fibrogammin HS) preoperative substitution in young children (n=40) undergoing open heart surgery revealed a significant reduction of the incidence of myocardial edema formation usually leading to a delayed sternal closure.
All our studies give evidence that FXIII may stabilize endothelial barrier function in cultured EC-monolayer as well as under clinical conditions with the risk of a capillary leakage.

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FACTOR XII DEFICIENCY – MOLECULAR GENETIC ANALYSIS

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Factor XII (Hagemann factor) is an importent element in plasma protease cascades such as the blood coagulation system, the kinin system and fibrinolysis.
In our study the factor XII gene was analysed from more than 95 unrelated probands with FXII deficiency or reduced FXII activities from different haemostasiological centres of Germany for variation in the FXII gene at the genomic level.
The FXII gene mutations *-13C>T, -8G>C, IVS13-1G>A, *Gly372 Asp (* are novel mutations) and the FXII reduced polymorphism 46C>T of the 5′ untranslated region were investigated in different genetic conditions. 10 patients are homozygous for a causative mutation, 5 patients were compound heterozygous for two different FXII lesions and 39 are heterozygous. 20 probands have one FXII mutation in combination with the mutant form of the 46C>T polymorphism in the 5′ untranslated region and in 44 probands with reduced FXII levels (x=40,8%) the 46T allele of the 46C>T polymorphism was detected homozygous.
The results of the genomic diagnosis in FXII deficiency in 95 unrelated probands were reported.

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A PROGESTERONE ENHANCE NON-VIRAL GENE DELIVERY SYSTEM FOR TREATMENT OF HAEMOPHILIA B

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Here we present a progesterone enhanced non-viral gene delivery system. The system comprises of three major elements which are administered as a premixed cocktail: A DNA vector, containing a gene expression cassette and several progesterone-responsive-elements (PRE), the A-form of the human progesterone receptor protein (hPRA) and progesterone in a lipid matrix. Depending on the progesterone carrying lipid matrix this gene transfer system can either be administered orally or used in cell culture for in vitro gene transfection. In vitro studies indicate that the presence of the progesterone component enhances the transfer of the ternary complex into the target cells. Our current concept is that within the cell the DNA-hPRA complex is then transported to the nucleus following the physiological steroid transduction pathway. We are aiming to develop an oral gene therapy for hemophilia B on the basis of the here described gene delivery system. Preliminary analysis of hemophilic male factor IX knock out mice (FIX0/-) ingested with our gene delivery system containing the gene encoding the full length recombinant human clotting factor IX (hFIX) have shown the presence of the expression vector in several organs and a considerable decrease in blood coagulation time, neither of which were detectable in the mock treated hemophilic FIX0/- control animals.

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COAGULATION-FIBRINOLYSIS IMBALANCE IN SEPSIS PROMOTES MULTIPLE ORGAN FAILURE AND WORSENS OUTCOME

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Background: Multiple organ dysfunction syndrome (MODS) is a frequent complication of severe sepsis and septic shock and has a high mortality. Though the precise mechanisms of MODS are unclear, coagulation activation and inadequate fibrinolysis might be critically involved.
Methods: We assessed markers of coagulation (thrombin-antithrombin [TAT] complexes, fibrin monomers [FM]), fibrinolysis (tissue-type plasminogen activator [t-PA], plasminogen activator inhibitor type-1 [PAI-1], plasmin-alpha2-antiplasmin [PAP] complexes, D-Dimers [DD]), neutrophil activation and cytokine levels in 32 normotensive and 8 hypotensive sepsis patients. Moreover, logistic organ dysfunction (LOD) and sepsis related organ failure assessment (SOFA) scores were calculated.
Findings: Coagulation and fibrinolysis activation was found in the majority of the patients. TAT was significantly ($p<0.05$) higher (12.7 µg/L [3.2-54.4]) and FM tended to be higher (143 µg/ml [69.8-296.7]) in non-survivors (n=13) as compared to survivors (n=27; 7.1 µg/L [0.7-117] and 74.2 [0.4-393.3], respectively), whereas no such difference was found for fibrinolysis parameters. The TAT/PAP ratio was significantly higher (0.71 [0.27-6.07]) in non-survivors as compared to survivors (0.51 [0.08-4.33]). Hypotensive patients had significantly higher t-PA (15.5 ng/ml [<4-44]) and PAI-1 levels (705.5 [131-5788]) as compared to normotensive patients (9 ng/ml [<4-28] and 316.5 ng/ml [53-1311], respectively) whereas no such relation was observed for the other parameters. Patients with elevated TAT complexes (n=32) showed significantly worse LOD (5.0 [1.0-14.0]), SOFA (8.0 [5.0-18.0]) and creatinine levels (151.5 umol/l [67.0-717.0]) as compared to patients with normal TAT (n=8; 3.0 [2.0-4.0], 6.0 [4.0-11.0] and 86 µmol/l [73.0-295.0], respectively), whereas no differ-

ence was found in patients with elevated (n=33) and normal (n=7) PAP. Patients with a TAT/PAP ratio>1 (n=11) showed significantly higher SOFA scores, platelet counts, cytokine levels (IL-8, IL-10) and a significantly stronger neutrophil activation as compared to patients with a ratio <1 (n=29).
Interpretation: Coagulation activation is associated with a more intense inflammatory response, increased organ dysfunction and poor outcome. Inhibition of fibrinolysis promotes inadequate fibrin removal in the microvasculature, thereby worsening the procoagulatory state and organ function.

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TWO DIFFERENT INTERFERON DOSAGE REGIMENS IN THE TREATMENT OF CHRONIC HEPATITIS C IN PATIENTS WITH HEMOPHILIA

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Alpha-interferon 2a (IFN alpha-2a)is very effective in eliminating HBV-DNA whereas in the treatment of chronic hepatitis C a sustained response can only be achieved in 15-30% of cases. It has been shown, that the higher dosage regimens are more effective. In a first study we treated 14 patients, n=12 with Hemophilia A and n=2 with von Willebrand disease with the following dosage regimen: 6 MU s.c. daily for 4 weeks, followed by 6 MU three times per week for 22 weeks; then the dose was reduced to 3 MU three times per week for further 6 months. Inclusion criteria were the presence of anti-HCV antibodies, HCV-RNA (detected by nested PCR) and elevated transaminases (ALT). The clinical course, transaminases, quantitative HCV-RNA, HCV genotype and immunological parameters were evaluated before, 2, 4, 8, 12, 38, 52 weeks after start of treatment and 6 months after discontinuation of therapy. Transaminases normalized in 10/14 patients within 6 weeks of treatment. HCV-RNA levels were reduced by 80% of the initial value, in 6/14 patients HCV-RNA was undetectable. However, only 2/14 patients showed a sustained response after 18 months. The main reason for the low response rates is the predominant genotype 1a and 1b in our patients and the administration of IFN alpha-2a as a single drug. Therefore we initiated a new treatment protocol which was designed also prospectively. Patients received IFN alpha-2a in combination with ribavirin and amantadine. The latest analysis of the data showed a negative HCV-RNA status in 7/19 patients after 12 months of treatment. These results appear to be more promising with regard to the high prevalence of genotype 1.

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EFFECTS OF HEPARIN, R-HIRUDIN AND MELAGATRAN ON THROMBIN GENERATION AND PLATELET ACTIVATION QUANTIFIED BY AN EXPERIMENTAL HUMAN THROMBOSIS MODEL

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Background: The present experimental human thrombosis model distinguishes the in vitro effects of different antithrombotics on the plasmatic coagulation system by measuring thrombin-antithrombin (TAT) complexes and on platelets by the release of platelet factor 4 (PF4). Reduced levels of ionised calcium may modify the interaction between thrombocytes and serine proteases of the coagulation process. We quantified the antithrombotic potential of unfractionated heparin (UFH) in relation to r-hirudin and melagatran in non-citrated human whole blood with physiological calcium levels.
Methods and material: A sample of free flowing blood was taken without tourniquet from the cubital vein from healthy persons. Thereafter, it was aliquoted into plastic tubes containing an antithrombotic (v/v:9/1). After 30 min. of incubation at 37°C blood adding 0.9 ml of an anticoagulant solution according to Files stopped coagulation. TAT and PF4 concentrations were analysed with enzyme linked immunosorbent assays (ELISA) within the following two weeks. Final concentrations of the antithrombotics were: UFH (n=10) (0.01 to 3 IU/ml), r-hirudin (n=10) (0.1 to 10 µg/ml), melagatran (n=8) (0.0001 to 10 µg/ml).
Results: Melagatran showed at very low doses (0.1 µg/ml) a higher inhibitory effect on thrombin generation as well as on platelet activation (TAT: 1408+/-801; PF4: 106+/-11) compared to UFH (0.1 IU/ml) (TAT: 3508+/-686; PF4: 226+/-26) and r-hirudin (0.1 µg/ml) (TAT: 8258+/-523; PF4: 1958+/-65). At clinical relevant heparin dosages (1 IU/ml) UFH (TAT: 71+/-16) were more effective in staunching TAT levels than r-hirudin (1 µg/ml) (TAT: 1292+/-219) and melagatran (1 µg/ml) (TAT: 438+/-68). In relation to the reduction of the PF4 release melagatran (0.1 µg/ml) (PF4: 106+/-9) leaded to similar PF4 levels as UFH (1 IU/ml) (PF4: 99+/-11) but with lower doses. R-hirudin demonstrated definitively in all concentrations the lowest inhibiting influence of both detected parameters. All values of TAT and PF4 are given as mean+/-SD and in ng/ml.
Conclusions: The anticoagulant effect of all compounds is mediated through an inhibition of blood coagulation as well as platelet function at physiological concentrations. The broader therapeutic range of melagatran is reflected by the inhibition of platelet function.

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PARADOXICAL PLATELET FACTOR 4 RELEASE IN PRESENCE OF THROMBIN INHIBITORS AND ABCIXIMAB

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Background: Thrombus formation can be influenced by differently acting antithrombotics. The combined treatment of GP IIb/IIIa antagonists (abciximab) and thrombin inhibitors (TI) plays a leading role especially in treating acute coronary syndromes. The description of immediately occurring occlusions after coronary interventions may be caused through dosing problems of the anticoagulants.
Methods and material: We quantified the interaction of abciximab and unfractionated heparin (UFH), low molecular weight heparin (LMWH) dalteparin or r-hirudin with regard to thrombin generation and platelet activation by measuring thrombin-antithrombin (TAT) complexes and platelet factor 4 (PF4) levels in an experimental human thrombosis model using human whole blood with physiological calcium levels. Free flowing blood was taken without tourniquet from the cubital vein from healthy persons (n=8). It was aliquoted into plastic tubes containing a TI alone or in combination with abciximab (v/v:9/1:blood/anticoagulants). After 30 min. of incubation at 37°C blood adding 0.9 ml of an anticoagulant solution according to Files stopped coagulation. TAT and PF4 concentrations were determined by means of enzyme linked immunosorbent assays (ELISA). Final concentrations of antithrombotics were: UFH (0.03 to 1 IU/ml), dalteparin (0.03 to 1 IU/ml), r-hirudin (0.1 to 10 µg/ml) alone or combined with 0,1 µg/ml abciximab.
Results: With regard to the staunching effect of TAT values there were no significant additive effects of the combination of TI with 0.1 µg abciximab (UFH (0.03 IU/ml): 4874±401; LMWH (0.03 IU/ml): 8667±819; r-hirudin (0.1 µg/ml): 7997±318) compared to the dosing of TI alone (UFH (0.03 IU/ml): 5838±384; LMWH (0.03 IU/ml): 6881±662; r-hirudin (0.1 µg/ml): 8192±350). Concerning the PF4 levels we detected a paradoxical release of PF4 with low dosages of each examined TI (UFH (0.03 IU/ml): 1963±143; LMWH (0.03 IU/ml): 2049±167; r-hirudin (0.1 µg/ml): 2010±75) in presence of 0.1 µg abciximab compared to abciximab alone (114±11). All values of TAT and PF4 are given as mean ± SD and in ng/ml.
Conclusions: The inhibition of platelet function was abolished by low concentration of UFH, LMWH or r-hirudin in the presence of abciximab. This effect may be of relevance for dosing of thrombin inhibitors in coronary interventions.

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INCIDENCE OF RECURRENT VENOUS THROMBOEMBOLISM DEPENDING ON SINGLE AND COMBINED RISK DETERMINANTS

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The yearly incidence of a recurrent spontaneous venous thromboembolism (VTE) is the most important criterion for a prolonged anticoagulant therapy. To date, there are only few prospective studies with a low number of patients with recurrent events (n=38 to 92) and a short follow-up (2-4 years) available which have evaluated some risk factors of recurrent VTE. We therefore performed a retrospective study covering more than 20 years after a first venous thromboembolic event in a large patient group of 812 individuals to characterize the long-term effect of single or combined risk determinants on the annual incidence of spontaneous recurrent VTE. We analyzed the family history and determined the activity of the coagulation factors I, II, V, VII, VIII:C, IX, X, XI, XII and XIII, vWF:Ag, lupus anticoagulant, antithrombin, protein C, protein S and performed a genetic analysis of factor V G1691A (FVL), prothrombin G20210A, and methylenetetrahydrofolate reductase polymorphism (MTHFR C677T) in 644 patients with first VTE and 168 patients with spontaneous recurrent VTE. Comparisons were performed using the Cox proportional hazards model for multivariate analysis of single and combined risk factors. The mean incidence per year of all recurrent VTE without any risk factor was: 0-2 y: 2%, 2-4 y: 2%, 4-7 y: 2%, 7-10 y: 2%. A higher VTE incidence was found in various subgroups: In patients with a first spontaneous VTE as compared with a non spontaneous first VTE,

the incidence of a recurrent spontaneous VTE was 0-2 y: 8%, 2-4 y: 6%, 4-7 y: 4%, 7-10 y: 4%, for FVL 0-2 y: 3%, 2-4 y: 3%, 4-7 y: 3%, 7-10 y: 3%, for increased vWF:Ag 0-2 y: 4%, 2-4 y: 4%, 4-7 y: 4%, 7-10 y: 5%, for positive family history 0-2 y: 2%, 2-4 y: 2%, 4-7 y: 2%, 7-10 y: 3%. The mean annual VTE incidence for combined predictors were: first spontaneous VTE and FVL 0-2 y: 11%, 2-4 y: 9%, 4-7 y: 6%, 7-10 y: 7%; first spontaneous VTE and increased vWF:Ag 0-2 y: 15%, 2-4 y: 14%, 4-7 y: 10%, 7-10 y: 10%; first spontaneous VTE and positive family history 0-2 y: 9%, 2-4 y: 8%, 4-7 y: 5%, 7-10 y: 7%; first spontaneous VTE and FVL and increased vWF:Ag 0-2 y: 19%, 2-4 y: 21%, 4-7 y: 16%, 7-10 y: 16%. In conclusion, for the first time, this large study presents statistically based estimates of the annual risk of recurrent spontaneous VTE depending on the presence of single or combined risk factors.

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POLYMORPHISM OF PLATELET MEMBRANE INTEGRINS αIIB-β3 (HPA-1B/PLA2) and α2–β1 (GLYCOPROTEIN IA 807TT) AND THE RISK OF PREMATURE MYOCARDIAL INFARCTION

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Conflicting results of an association of the human platelet antigen 1b (HPA-1b or PlA2) localized on beta3 of alphaIIb-beta3 (GPIIb-IIIa) and the alpha2 807TT genotype of alpha2-beta1 (GPIa-IIa) regarding the risk of myocardial infarction and coronary artery disease have been reported. To assess the reason for this discrepancy, we determined the genotype of both platelet receptor polymorphisms in 3,081 patients who had undergone coronary angiography, including 1,175 individuals with myocardial infarction, 1,211 individuals with coronary artery disease but no history of myocardial infarction, and 571 control patients. Using a case-control design, no significant difference between the three patient groups and the patient controls were demonstrated. However, using a multivariate case-only design patients with recent onset myocardial infarction and a lower degree of coronary artery disease (1- or 2-vessel disease), the median age at onset of myocardial infarction was 5.2 years earlier in carriers of the HPA-1b allel (risk ratio 1.47; 95 percent confidence interval, 1.12-1.94; P=0.006) and 6.3 years earlier in individuals with the alpha2 807TT genotype (risk ratio 1.66; 95 percent confidence interval, 1.15-2.38; P=0.006). The relative risks for premature myocardial infarction associated with both platelet receptor polymorphisms were higher than those for conventional risk determinants, including smoking, hypercholesterolemia, diabetes, hypertension, and hyperfibrinogenemia. There was no significant interaction of both platelet receptor polymorphisms and the conventional risk factors. The combined effect of HPA-1b and alpha2 807TT resulted in a multiplicative increase of the risk ratios. In conclusion, HPA-1b and alpha2 807TT genotype are associated with premature myocardial infarction but not with coronary artery disease suggesting a role of receptor polymorphisms for increased platelet thrombogenicity. In this study of conventional risk factors and platelet receptor polymorphisms, HPA-1b and alpha2 807TT genotypes proved to be the most important predictors of the risk of myocardial infarction in already existing coronary artery disease. Thus, these data raise the possibility that the analysis of HPA-1 and alpha2 807 in addition to standard diagnostic screening procedures will generate an improved approach for identifying subjects with already existing coronary artery disease at high risk for future cardiovascular events.

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MIXCON-LA – A PRECISE, SENSITIVE AND SPECIFID APTT-BASED ASSAY FOR DETECTION OF LUPUS ANTICOAGULANT

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Purpose: Lupus anticoagulants (LA) are associated with an increased risk of thrombosis and laboratory detection is of major importance. Multiple tests are available for screening and confirmation, but they differ in sensitivity and specificity, frequently lacking the ability to discriminate between the presence of LA, heparin and oral anticoagulants. Based on the test-principle of the Lupus Ratio-test we have developed an automated, sensitive APTT-based assay, using mixtures of a lupus-sensitive and a lupusinsensitive APTT-reagent with normal plasma for detection of lupus anticoagulants.
Patients and methods: 99 healthy volunteers, 10 patients treated with unfractionated heparin intravenously, 19 patients on stable oral anticoagulation, 5 patients with haemophilia A and 15 patients with antiphospholipid-antibody-syndrome (APS) were investigated. In all patients two APTTs were performed, one with each reagent, on 1:1 mixtures of test plasma and normal plasma (MIXCON-LA-assay). The ratio between the two clotting times was divided by the corresponding ratio for the normal plasma. This final lupus ratio (LR) was used for evaluation.
Results: The within-series imprecision and the between-series imprecision were excellent with coefficients of variation between 1.5 and 1.9%. The mean±2 SD of the LR of the 99 healthy volunteers was used as reference range (LR: 0.95-1.07).
All patients treated either with heparin or oral anticoagulants remained negative in the MIXCON-LA-assay (specificity: 100%), while 1/5 patients with haemophilia A, who had solely developed a factor VIII-inhibitor, showed a false positive result. In 13/15 patients with APS an increased ratio was observed (sensitivity: 87%).
Conclusions: This assay system allows precise, specific and sensitive detection of lupus anticoagulants.

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HEMOSTATIC RISK FACTORS, METABOLIC CONTROL, AND MICROALBUMINURIA IN DIABETES MELLITUS TYPE I

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Diabetic angiopathy is a major complication of diabetes mellitus type I. Microvascular complications are associated with endothelial cell alterations and hypercoagulability. Therefore, we investigated a possible correlation between microalbimunuria, markers of endothelial cell alteration (von Willebrand factor-Ag, P-Selectin-Ag), hemostatic risk factors (VIIc, VIIIc, tPA.Ag, PAI-1-Ag), and metabolic control (HbA1c) in 81 patients with diabetes mellitus type I. Median age of patients was 15.7 years (6.2-53.6), median duration of diabetes was 8.2 years (1.4-33.2), microalbuminuria was 5.8 µg/min (0.24-66), median HbA1c calculated from last 10 visits was 8.0 % (6.4-12.5). There was no correlation between microalbuminuria and markers of endothelial cell alteration or hemostatic risk factors. In contrast, we found significant correlations between HbA1c and vWf-Ag ($r=0.31$, $p<0.005$), P-Selectin-Ag ($r=0.25$, $p<0.05$), Factor VIIc ($r=0.27$, $p<0.01$), Factor VIIIc ($r=0.37$, $p<0.001$), and tPA-Ag ($r=0.33$, $p<0.005$). These correlations stayed significant after adjustment for age and duration of diabetes. Patients with HbA1c values < 8 % showed significant lower plasma levels of P-Selectin ($p<0.01$), Factor VIIc ($p<0.05$), Factor VIIIc ($p<0.005$), and PAI-1-Ag ($p<0.05$) compared to patients with HbA1c values > 8 %. Summing up type I diabetes patients with poor metabolic control showed higher plasma levels of hemostatic risk factors. Markers of endothelial cell activation did not correlate with microalbuminuria.