UNIVERSITA' DEGLI STUDI DI MILANO

SCUOLA DI DOTTORATO
Scienze Biochimiche, Nutrizionali E Metaboliche

Dipartimento Di Scienze Veterinarie E Sanita' Pubblica

Corso Di Dottorato Di Ricerca In Biochimica, Ciclo XXVII

TESI DI DOTTORATO DI RICERCA

MASS SPECTROMETRY BASED PROFILING OF TWO DIFFERENT BIOLOGICAL SYSTEMS FOR POSSIBLE CLINICAL APPLICATION.

Dott.ssa Nerea CUEVAS POLO
Matricola n° R09606

Docenti guida: Prof.ssa Gabriella TEDESCHI

Coordinatore del dottorato: Prof. Francesco BONOMI

Anno Accademico 2013-2014

In dedication to my mother for making me be who I am and my husband for supporting me all the way!!

TABLE OF CONTENTS

SUMMARY

Several techniques including two-dimensional electrophoresis, imaging, mass spectrometry, and bioinformatics are used in proteomics to identify, quantify, and characterize proteins for clinical applications. The main aim is to identify proteins involved in pathological processes and to understand how illness can lead to altered protein expression in order to develop new diagnostic and prognostic tests, to identify new therapeutic targets, and eventually to allow the design of individualized patient treatment.

For this purpose this dissertation is divided into two main chapters. Chapter one concerns the development of a novel method to isolate exosomes. Normal human urine contains large numbers of exosomes, which are 40 to 100 nm vesicles that originate in multivesicular bodies from every renal epithelial cell type facing the urinary space. Exosomes are rich in potential biomarkers, especially membrane proteins such as transporters and receptors that may be up- or downregulated during disease states. Differential centrifugation methods are commonly used to purify exosomes from urine. Here, we developed a new method to isolate exosomes by solubilizing exosomes in 1 % Sarkosyl, an anphyphilic detergent, followed by a carbon fractionation. We used LC-MS/MS to profile the proteome of human urinary exosomes. Overall, the analysis unambiguously identified 1618 proteins, showing an enrichment of 61.3% in exosomal proteins, after performing a Gene Ontology analysis. Thus, our modified exosome precipitation method is a simple, fast, highly scalable, and effective alternative for the isolation of exosomes. It may facilitate either the identification of exosomal biomarkers from urine or the production of drug delivery devices.

Chapter two concerns the identification of proteins in adhesive material of the capsalid Neobenedenia girellae by a proteomic approach based on de novo sequencing and database search to overcome the lack of information concerning the genome of these parasites. Glandular secretions were obtained by a new method, set up in our laboratory, which allowed collecting a small amount of secretion without any contamination from other tissues either from the parasites as well as from the skin of the host. The proteomic analysis reveals that the

adhesive is mainly composed of cytoskeletal proteins (actin, keratin and tubulin) but contains also ATP-synthase, 78 kDa glucose regulated protein and albumin.

This work reports for the first time the characterization of a novel bioadhesive material used by capsalid parasites to adhere to fish. Such information broadens our knowledge of the molecular mechanisms involved in adhesiveness of parasites to hosts. Moreover, it offers new clues in understanding the mechanism of stickiness and adhesion of cytoskeleton components, often involved in both physiological and pathological processes, including neurodegenerative diseases.

CHAPTER 1

1. INTRODUCTION

1.1 VESICULATION

Vesiculation is a physiological mechanism that is used in cell growth, activation and protection. For example, for mineral formation in cartilage, bone and predentin, calcification is initiated by matrix vesicles released by chondrocytes, osteoblasts and odontoblasts [1]. Vesicles released by activated monocytes expose tissue factor and enhance coagulation.

In resting mammalian cells, the constitutive aminophospholipids, phosphatidylserine and phosphatidylethanolamine, are mainly sequestered in the inner (cytoplasmic) side of the plasma membrane, whereas sphingomyelin and phosphatidylcholine constitute the majority of the outer (exoplasmic) side [2, 3]. This asymmetric distribution is under the control of an inward aminophospholipid translocase (flippase), of as yet elusive nature [4]. For example, when subjected to procoagulant, pro-inflammatory or apoptogenic stimulation, cells of the vascular compartment show specific responses according to lineage and stimulus, but in most of them a spontaneous collapse of their membrane asymmetry happens [3, 4]. One of the resulting universal hallmarks is the occurrence of phosphatidylserine in the exoplasmic leaflet where it expresses two of its properties as a multifunctional membrane effector and the formation of vesicles. However, the mechanisms of packaging and vesicular emission of proteins and nucleic acids are poorly understood.

1.2 EXTRACELLULAR VESICLES IN BODY FLUIDS

Cellular microparticles (MPs), also referred to as microvesicles, are fragments shed almost spontaneously from the plasma membrane blebs of various eukariotyc cell types when submitted to a number of stress conditions, including apoptosis. Microvesicles release is an integral part of the membrane-remodeling process in which the asymmetric distribution of

constitutive phospholipids between the two leaflets is lost. After having long been considered 'cell dust', microvesicles have more recently been shown to reflect *in vitro* cell stimulation, and testify to cellular activation and/or tissue degeneration occurring *in vivo* under a variety of pathophysiologic conditions [5].

Interestingly, this phenomenon seems conserved during evolution, as bacteria are even described to release microvesicles that are important components of biofilms, and is a major signal trafficking system [6].

Vesicle shedding is an important defense mechanism protecting against complement attack, by allowing the removal of the C5b-9 attack complex from the cell surface by a calcium (Ca++)-dependent elimination as shown for many cell types including platelets, polymorphonuclear leucocytes (PMN), erythrocytes and oligodendrocytes [7]. Specific vesiculation is triggered or enhanced in pathological conditions such as inflammation, injury, vascular dysfunction or cancer [8].

A major problem in the microvesicle literature is the confusing nomenclature. Various names have been used, including particles, microparticles, vesicles, microvesicles, nanovesicles, exosomes, dexosomes, argosomes, ectosomes, etc. [3, 5]. Whereas their formation, size and biological function may be different, one common point between all vesicles is the fact that they bud from a membrane, whether this occurs at the cell surface or in a vesicular compartment inside the cell. Johnstone et al. [8] initially named exosomes, vesicles produced by intracellular budding into the late endosomal compartment. Evidently, every vesicle brings many of the specificities of the originating cell; however, there are some general properties characterizing those released directly from the cell surface which justifies a specific name, i.e. ectosomes [9], or 'shedding' microvesicles [10].

1.3. ECTOSOMES

Ectosomes are also known by other names, for example, microparticles, microvesicles, and shedding vesicles. These names emphasize that these vesicles are discharged outside, and not inside the cell, and that they are similar to, but distinct from, exosomes.

Ectosomes are vesicles of various sizes (0.1–1mm in diameter) that bud directly from the plasma membrane and are shed to the extracellular space. Stein and Luzio [9] coined the term 'ectocytosis' for the release of right-side-out-orientated vesicles with cytosolic content (ectosomes) from the surface of PMN attacked by complement. However, ectocytosis corresponded not only to the removal of the C5b-9 complex, but also to a specific sorting of membrane proteins into the shed ectosomes. Enrichment in cholesterol and diacylglycerol in the membrane attested to a specific sorting of lipids.

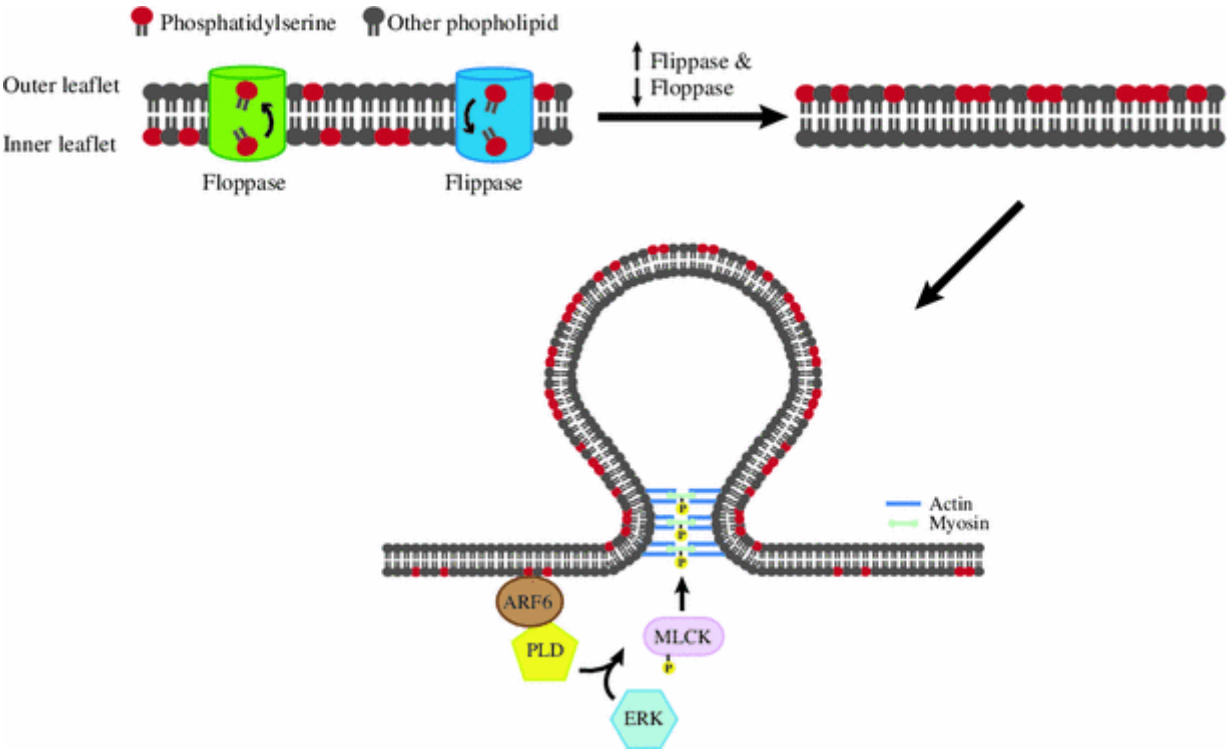

Figure 1. Biogenesis of ectosomes

Microvesicular formation is the result of dynamic interplay between phospholipid redistribution and cytoskeletal protein contraction. The protein and phospholipid distribution within the plasma membrane is far from uniform and forms micro-domains. The exposure of phosphatidylserine in the outer leaflet of the membrane is also a specific characteristic [3]. Although ectocytosis describes the same phenomenon in all cell types, the stimuli inducing cell-membrane budding can differ from one cell to another. Endothelial and circulating blood cells

release ectosomes when exposed to specific stimuli such as complement attack [7].Monocyte ectocytosis is induced by bacterial cell wall components including lipopolysaccharides (LPS), and platelets release ectosomes by activation through thrombin [5]. Fibroblasts release ectosomes in response to stress relaxation when cultured in a three-dimensional collagen matrix [11]. Many cancerous cells have an activated phenotype with highly active ectocytosis in the absence of any stimulus [8],[12],[13]. Background levels of microvesicles originating from circulating and endothelial cells are found in blood, and similarly ectosomes originating from glomerular epithelial cells are found in urine [14],[15].

Exosomes are highly heterogeneous, both in size and in composition. Ectosomes are made up of components (proteins, mRNAs, and miRNAs) that are typical of their cell of origin and are therefore distinct from those of other cell types. They can vary depending on the cellular state (e.g. resting, stimulated) and depending on the agent employed for stimulation.

Generation of ectosomes is a complex but efficient process. Specific domains are assembled in the plane of the plasma membrane; proteins destined to appear in the ectosomes are sorted to these domains, and proteins destined to remain in the cell are excluded. Concomitantly, specific cytosolic proteins and nucleic acids (mRNAs and miRNAs) accumulate in contact with the plasma membrane domains. Budding of the plasma membrane domains with their associated protein/RNA packages requires the local disassembly of the cytoskeleton, possibly by activation of caspase 2. Finally, ectosome discharge occurs by an actomyosin-based abscission process. The mechanisms of its regulation (perhaps involving the ESCRT machinery and/or the small GTPase ARF6) are just beginning to emerge.

Ectosomes are discharged even by resting cells, but the rate of release is increased considerably upon appropriate stimulation. Impressive responses, mimicking eruptive gun shooting from warships of the early 19th century, have been reported from macrophages and microglia. Weaker responses do occur from many cell types. The local signal that triggers the response is the increase of cytosolic free Ca2+([Ca2+]i) inducing the disassembly of the cytoskeleton and membrane abscission. The p38 MAPK, acid sphingomyelinase and the Rho–ROCK axis also appear to be involved. [Ca2+]i increase and MAPK activation can be induced by a variety of

agents, for example, ATP-mediated activation of the P2X7 receptor in macrophages and TNFa signalling in endothelia (figure 1).

Their function depends on their site of discharge and on the properties of their membrane. Ectosomes from platelets, endothelia and leukocytes are discharged directly into the blood where they can release their content rapidly or persist in the circulation for quite some time. Ectosomes discharged to the tissue intercellular space can also release their content there, remain trapped locally or diffuse some distance. Effects are triggered when ectosomes (or their released molecules) reach their targets, often in cell types distinct from the cells of origin. Released molecules activate key cell-surface molecules, such as receptors and enzymes. Intact ectosomes can either fuse with target cells (with the ensuing incorporation of their membrane in the plasma membrane and release of the segregated package to the cytosol) or be taken up by endocytosis. The fate of the latter is variable: fusion with lysosomes; release of contents in the cytosol; or discharge to the extracellular space by transcytosis (Figure 2).

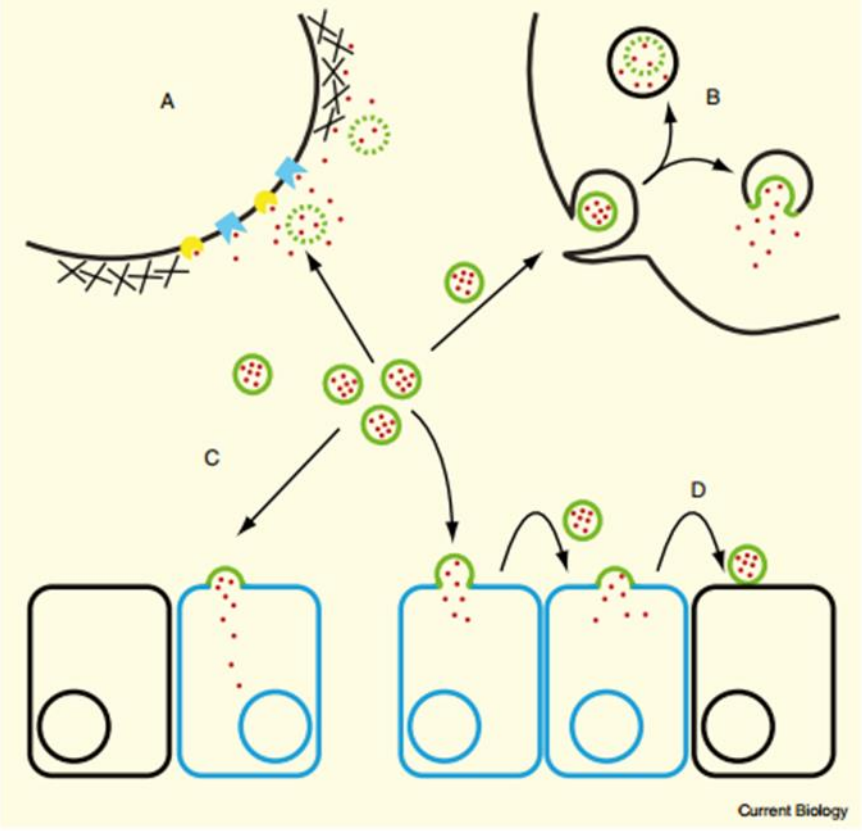

Figure 2. Life cycle of ectosomes.

Generation of ectosomes requires the segregation of membrane domains with associated packages of proteins and RNAs. After their release, ectosomes can (A) diffuse into the extracellular space where they can release their content, (B) be endocytosed by cells, ending up in lysosomes or releasing their content to the cytoplasm, or (C) fuse with a target cell, with the release of the segregated proteins/RNAs to the cytosol. These processes can lead to changes to the target cell phenotype, with generation and budding of new vesicles that establish a horizontal transfer of their proteins/RNAs to adjacent cells (D).

Ectosomes are specific, multi-purpose carriers that expand the borders of cells away from the plasma membrane, establishing communication networks by which specific properties and information can be shared among cells. By delivering their molecules at distance without dilution or degradation they reproduce effects otherwise induced by direct cell–cell contact, playing major roles in the integrated functioning of tissues and organs. Fusion of ectosomes at the surface of target cells delivers exogenous antigens, enzymes and other proteins to discrete sites of the plasma membrane. Concomitantly, release of the segregated protein/RNA packages to target cells can alter gene expression. This might explain, among other events, the functional and phenotypic changes taking place in stem cells without transdifferentiation, sustained by genetic information transferred from tissue cells via ectosomes.

The heterogeneity of ectosomes can play different, even opposing roles. Ectosomes containing cytokines, in particular interleukin 1b, are pro-inflammatory; others, however, are anti-inflammatory. Monocyte and endothelial ectosomes are often rich in tissue factor, a potent activator of the coagulation cascade, and can therefore trigger coagulation, thrombosis and also angiogenesis. Platelet ectosomes, however, contain low levels of tissue factor, and therefore work differently. Ectosomes derived from leukocytes and platelets have profound effects on innate immunity and also on the induction of adaptive immunity, reprogramming macrophages and dendritic cells toward immunosuppression.

Ectosomes of specific origin are also being studied as a target of new therapies for rheumatoid arthritis and multiple sclerosis, where ectosomes are believed to promote inflammation and cell death, and for cancer, in which ectosomes play a role in invasion and metastasis. The

mechanisms of the effects on cancer are multiple. In addition to the above-mentioned roles in digestion of the intercellular matrix and immunosuppression, ectosomes can induce the horizontal transfer among tumor cells of critical molecules such as proteins (e.g. P-glycoprotein (which confers multidrug resistance to the cells), glutaminase, and fibronectin), mRNAs and miRNAs. This transfer is considered to be greatly important for cancer progression.

1.4. APOPTOTIC BLEBS

Apoptosis is a major mechanism of cell death for both normal and cancerous cells [16]. A cell dying by apoptosis progresses through several stages, initiating with condensation of the nuclear chromatin, followed by membrane blebbing, progressing to disintegration of the cellular content into distinct membrane enclosed vesicles termed apoptotic bodies or apoptosomes [16].

These membrane vesicles, which are condensed remnants of the shrinking apoptotic cell [17], are referred to as apoptotic blebs or apoptotic bodies. Apoptotic bodies are released during the late stages of cell death [18], they are generally larger in size (500–4,000 nm) [19], and are characterized by the presence of organelles within the vesicles (figure 3) [20].

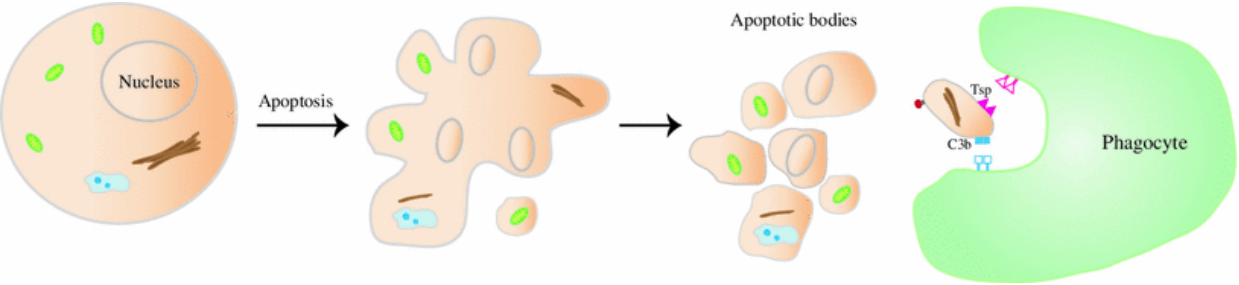

Figure 3. Biogenesis and destiny of apoptotic bodies during apoptosis

During normal development, most apoptotic bodies are phagocytosed by macrophages [20] and are cleared locally. This clearance is mediated by specific interactions between recognition receptors on the phagocytes and the specific changes in the composition of the apoptotic cells

membrane [21]. Among these changes, the best characterized involves the translocation of phosphatidylserine to the outer leaflet of the lipid bilayer. These translocated phosphatidylserines bind to Annexin V, which is recognized by phagocytes [22]. Another well-characterized membrane alteration involves oxidation of surface molecules. These changes create sites for binding of thrombospondin [23] or the complement protein C3b [21]. Thrombospondin and C3b are, in turn, recognized by phagocyte receptors [24], [25]. Annexin V, thrombospondin, and C3b thus, serve as three well-accepted markers of apoptotic bodies [26].
The ability to transfer genetic content intercellularly does not appear to be unique to one class of extracellular vesicles. In mice bearing tumor xenografts, apoptotic bodies can also be detected in the blood of the organism [27]. Importantly, uptake of apoptosomes derived from H-rasV12- or human c-myc-transfected cells by murine fibroblasts resulted in loss of contact inhibition in vitro and a tumorigenic phenotype in vivo [28]. These results suggest that genetic information can also be transferred by uptake of apoptotic bodies.

1.5. EXOSOMES

1.5.1. Biogenesis

Eukaryotic cells stay in contact with the environment by receiving signals such as cytokines or chemokines, the uptake of nutrients and the secretion of proteins into the extracellular space. For uptake and secretion, each cell has a complex network of membranes inside the cell. Using these compartments, cells not only take up macromolecules from the exterior environment (endocytosis) but also release newly-synthesized proteins or carbohydrates (exocytosis) (Figure 4). Both make use of membrane vesicles for the packaging and trafficking of molecules. While endocytosis is the process in which the extracellular substances enter into a cell without directly passing through the cell membrane, exocytosis is the primary means of cellular secretion. During both constitutive and regulated exocytosis the secretory-vesicles dock and/or fuse with the plasma membrane. Endocytic pathway, which is primarily responsible for the uptake, trafficking and sorting of internalized proteins has a role in vesicle secretion too [29]. In the endocytic pathway, transmembrane proteins are sorted into lumenal vesicles of

multivesicular bodies (MVBs). MVBs can have different destinies: they can fuse or mature with lysosomes where the degradation of their protein cargo takes place, or can fuse with the cell membrane to secrete the intraluminal vesicles (ILVs) into the extracellular space. These extracellularly released ILVs are called exosomes [30],[31]. During this process, the second inward budding of the endosome membrane results in a positive orientation of the ILVs lipid membrane. Thus when the ILVs are released to the extracellular environment, they have the same orientation as the cell membrane and have been shown to display many of the surface markers from their cell of origin [29]. The sorting process of membrane proteins during ILV formation is considered to be an active process and thus, exosomal surface proteins seem not to be a plain one-to-one presentation of the surface markers for the cell of origin.

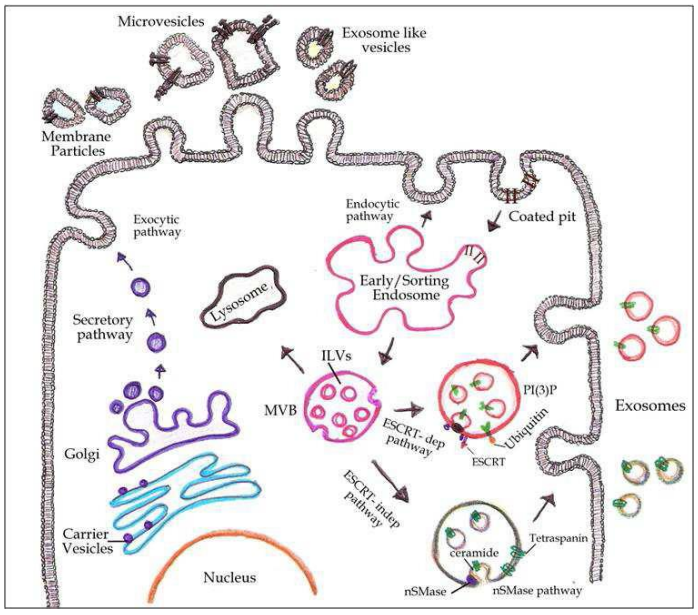

Figure 4. Representation of extracellular vesicles biogenesis.

While the regulation of endocytic cargo sorting and its delivery to lysosomes have been extensively studied [32] relatively less is known about the factors which regulate the formation, the release and the cargo sorting into vesicles destined to be exosomes. One hypothesis is the possible role of the ubiquitinization (mono- or oligoubiquitinization) and ESCRT (endosomal sorting complex required for transport) protein complex in this process[33],[34]. ESCRT is a protein machinery consisting of ESCRT-0, -I, -II, and -III[11]. ESCRTs are predominantly cytosolic

proteins that become recruited by endosomes. It was reported that ESCRT-0, -I, and -II have ubiquitin binding domains and play a central role in cargo sorting. It was shown that the depletion of the tumor susceptibility gene-101 (TSG101), a component of the ESCRT-I complex, has an inhibitory effect on receptor degradation and causes different endosomal morphology [35]. However, some data suggest that certain molecules, such as epidermal growth factor receptor (EGFR) and EGF, could be involved in the ESCRT-II–independent sorting pathway [32]. ESCRT-III has no ubiquitin binding domains, and it is probably important in the recruitment of deubiquitinizing enzymes that should remove ubiquitin before cargo incorporation into ILV. Another important role of ESCRT-III is to activate the molecular machinery that will facilitate the disassembly of the ESCRTs from the endosomal membrane [32], [36]. The dissociation and recycling of the ESCRT complex is dependent on interaction with AAA-ATP-ase Vps4 [37].

The mechanisms involved in the budding of vesicles from the limiting membrane of MVB are also poorly understood. It is known that ESCRT-III forms a lattice-like structure on the surface of the endosomal membrane [33]. Activation for assembly into lattices may be the consequence of interaction with the membrane itself, ALIX (accessory protein of the ESCRT-II), and some other ESCRT-III–related proteins. The presumption is that these lattices spatially restrict membrane curvature–inducing factors to initiate budding away from the cytoplasm. However, some authors point to the crucial role of the lipids in this process, such as phosphatidylinositol-3-phosphate (PI3,5P2) and lysobisphosphatidic acid (LBPA) [38], [39] .

The tetraspan protein family is abundant in exosomes and may be responsible for recruitment of membrane proteins into ILV [37]. It is also possible that there could be differences in the mechanism of

exosome secretion between the different cell types [40]. Therefore, several different mechanisms may be involved in the process of ILV formation.

A ESCRT-independent mechanisms by means of ceramide-mediated budding of exosomes into ILVs within the MVBs have also been identified [41, 42]. Further evidence of ESCRT-independent pathway of ILV formation has come from studying the protein Pmel17, a main component of the c fibrils of pre-melanosomes, which is targeted to intraluminal vesicles of MVBs independently of ubiquitination, ESCRT0 and ESCRTI [43].

Protein contents of exosomes from different cells have been mapped by proteomics and the most of the data obtained has been catalogued in Exocarta database [44].

1.5.2. Composition

Exosomes purified from the cell culture supernatants are usually heterogeneous in size and contain functional mRNA translatable to proteins, mature microRNAs, lipids and proteins. Protein composition analysis of exosomes shows a rather limited sub-cellular localization for the exosomal proteins. Indeed, usually the preparations of exosomes are mostly enriched in cytosolic and membrane proteins and contain less proteins of nuclear, mitochondrial, endoplasmic-reticulum or Golgi-apparatus origin. Secondly, exosomes express a common set of proteins (figure 5). These are structural components and proteins with a role in exosome biogenesis and trafficking. Cell type specific components which presumably reflect the biological function of the parent cell on the other hand could also be identified in exosome preparations [37].

Proteins of exosomes have been analyzed both by proteomics and targeted immunochemical methods, like Western-blot, FACS with immunolabeling, and immunoelectron microscopy. Exosomes contain a distinct set of proteins such as the Alix, TSG101, HSP70 and the tetraspanins CD63, CD81 and CD9. The protein content of exosomes has been extensively analyzed from various cell types and body fluids by mass spectrometry, Western blotting, fluorescence-activated cell sorting and immunoelectron microscopy. A detailed analysis of 19 proteomic studies revealed a more generic outlook of exosomal proteins (Figure. 5). The 19 exosomal studies used for this analysis were derived from dendritic cells [45], melanoma cells [46], urine [47, 48], microglia [49], mast cells [50], colorectal cancer cells [51, 52], mesothelioma cells [53], brain tumor [54], oligodendrocytes [55], tracheobronchial cells [56], hepatocytes [57], neuroglial cell [58], plasma [59], breast milk [60], breast cancer cells [61], saliva [62] and embryonic fibroblast cells [63]. Exosome protein composition varies depending on the cell type of origin and a unique tissue/cell type signature for exosomes was revealed

[52]. In addition, a conserved set of proteins were identified in exosomes in spite of their cellular origin.

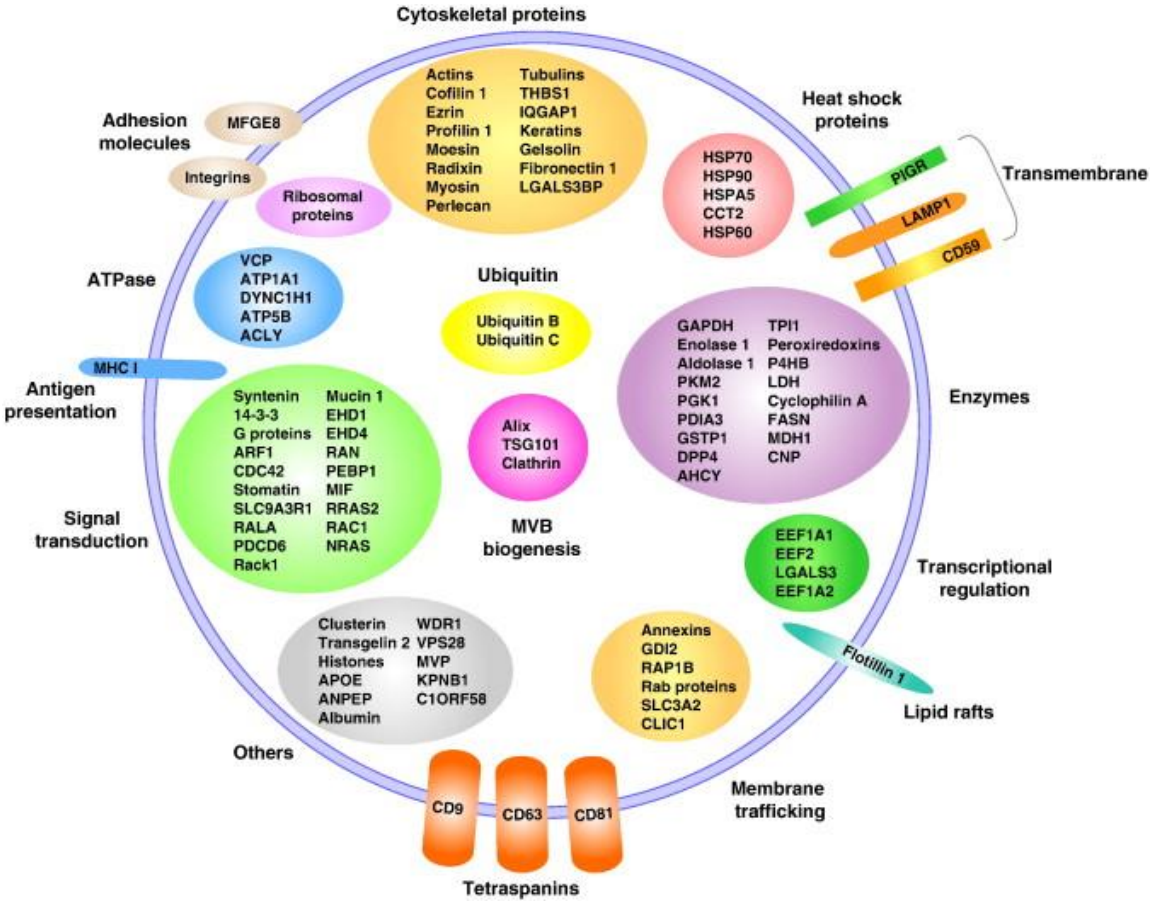

Figure 5. A graphical representation of the protein composition of exosomes categorized as per the function performed [64].

One class of cytosolic proteins commonly seen in exosomes includes the Rabs, the largest family of small GTPases, which regulate exosome docking and membrane fusion [46]. Active Rabs interact with proteins involved in vesicular transport and protein complexes that regulate vesicle fusion with acceptor membranes [65]. RAB5 localized in early endosomes mediates endocytosis and endosome fusion of clathrin-coated vesicles [66]. Interestingly, studies using green fluorescent protein-tagged endosomal GTPases showed the existence of mechanisms for segregating Rab GTPases into membrane domains with distinct functions [66]. Such studies revealed the presence of multiple combinations of RAB4, RAB5 and RAB11 membrane domains

in early and recycling endosomes in contrast to RAB7 and RAB9 membrane domains presence in late endosomes.

In addition to Rabs, exosomes are rich in annexins (annexins I, II, IV, V, VI, VII and X1) which aid in membrane trafficking and fusion events [67]. Exosomes are also enriched with tetraspanins (CD63, CD81 and CD9) [52] and heat-shock proteins (HSP60, HSP70, HSPA5, CCT2 and HSP90), and contain cell-type-specific proteins such as A33 (colon epithelial-derived) [35], MHC II (antigen presenting cells-derived) [68] and [69], CD86 (antigen-presenting cells) [70] and [71] and MFG-E8/lactadherin (immature dendritic cells) [72]. Interestingly, colorectal cancer-derived exosomes showed a significant enrichment of coiled-coil, RAS and MIRO domain containing proteins [35]. Coiled coil motifs play a vital role in localization of proteins to early endosomes [73] and vesicular transport [74] while RAS and MIRO domains are found in small GTPases (Rabs) and Rho GTPases.

Other exosomal proteins include the metabolic enzymes (GAPDH, enolase 1, aldolase 1, PKM2, PGK1, PDIA3, GSTP1, DPP4, AHCY, TPL1, peroxiredoxins, P4HB, LDH, cyclophilin A, FASN, MDH1 and CNP), ribosomal proteins (RPS3), transmembrane (PIGR, LAMP1 and CD59), signal transduction (syntenin, 14-3-3, G proteins, ARF1, CDC42, stomatin, SLC9A3R1, RALA, PDCD6, rack1, mucin 1, EHD1, RAN, PEBP1, MIF, RRAS2, RAC1, NRAS and EHD4), adhesion (MFGE8 and integrins), ATPases (VCP, ATP1A1, DYNC1H1, ATP5B and ACLY), cytoskeletal (actins, tubulins, cofilin 1, ezrin, profilin 1, moesin, radixin, myosin, perlecan, THBS1, IQGAP1, keratins, gelsolin, fibronectin 1 and LGALS3BP) and ubiquitin molecules (ubiquitins B and C) [35].

1.5.3. Biological and pathological role

Despite their role in immune system modulation [75], the biological role of exosome secretion remained largely elusive until recent years when Lötvall's group demonstrated that exosomes can transfer genetic information from one cell to another [50]. Since then several mechanisms have been proposed to describe exosome-cell interactions: (i) cellular binding via conventional receptor–ligand interactions, similar to cell–cell communication. (ii) attaching/fusing with target

cell membrane and (iii) internalization by recipient cells by endocytosis in a transcytotic manner (figure 6).

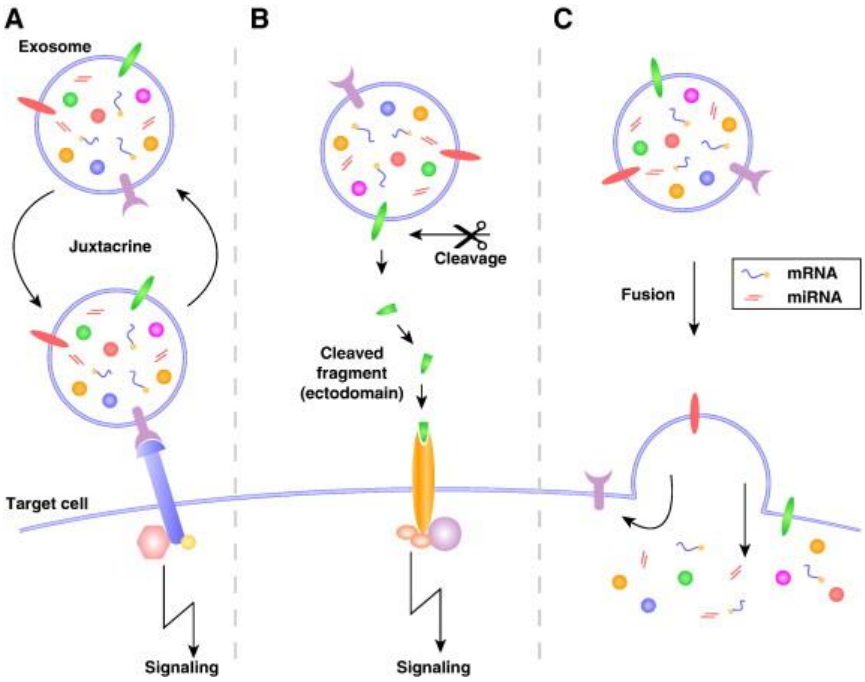

Figure 6. Possible mechanisms of intracellular communication by exosomes [64].

Besides the physiological roles of exosomes to remove the unwanted cellular debris, recent findings uncover an entirely new and exciting modes of cell–cell communication and paracrine signalling mediated by exosomes [29, 76].

Many mammalian cells like dendritic, mast, epithelial, neural, stem and hematopoietic cells, reticulocytes, astrocytes, adipocytes, and tumor cells have been reported to release exosomes [37, 77]

Emerging data shows their involvement in different diseases including inflammation, renal diseases, Alzheimer diseases, aging, bacterial and viral infections, allergies and cancer. Using different sources of tumor-derived exosomes, several groups claim that exosomes can prevent tumor development, induce tumor specific immunity, and provide a possible strategy for therapeutic tumor vaccination reviewed by van Niel et al.[37].

1.5.4. Proteomic approach

Compared to the techniques required for other protein enrichment for proteome-based analysis, the isolation of exosomes may be fairly straightforward. Their protein and lipid contents could be identified and characterized unaffected by isolation and purification procedures [40]. Currently, the most effective method for exosome isolation is ultracentrifugation [40], [47]. To further purify the exosomes, methods such as sedimentation via sucrose gradient ultracentrifugation and immunoisolation via antibody-based derivatized Dynabeads have proven effective [40], [48]. The purified exosomes could be verified onwards usually using electron microscopy techniques or western blotting using antibodies against known exosomal biomarkers, such as hsp70, hsp90, and annexins I, II, V, VI [40], [48]. The purified exosomes for proteomic analysis could be submitted to various protein or peptide separation approaches in combination with different types of mass spectrometry (MS) instruments[40]. Alternative approaches using ultrafiltration show some promise, but the main obstacle is a tendency to retain and concentrate soluble proteins in urine in addition to exosomes. These contaminating proteins are able to compete with exosomal proteins for identification by liquid chromatography (LC)-MS/MS and therefore may reduce the sensitivity of the discovery process [78].

1.5.5. Lipidomic approach

Rapid technological advancements in MS and chromatographic techniques have led to expansion of lipidomics research. The increasing importance of this research field reflects the wealth of information accumulated in the past decade, which resulted in online resources such as Lipidomics Expertise platform (http://www.lipidomics-expertise.de), Nature Lipidomics Gateway (http://www.lipidmaps.org), Lipid Bank (http://lipidbank.jp), Lipid Library (http://www.lipidlibrary.co.uk), Lipid Data Bank (http://www.caffreylabs.ul.ie), Kyoto Encyclopedia of Genes and Genomes (KEGG) http://www.genome.jp/kegg/), European Federation for the Science and Technology of Lipids (http://www.eurofedlipid.org). Exosomes

seem to be vehicles of bioactive lipids and lipolytic enzymes [79], [80]. Advent soft ionization technologies, such as matrix-assisted laser desorption/ionization (MALDI), electrospray ionization (ESI), and atmospheric pressure chemical ionization (APCI) for MS, possibly coupled to LC, provided powerful tools for rapid and sensitive analysis of the majority or a substantial fraction of lipids possible in one analysis [81]. It seems that lipids involved in exosome composition differ according to the cells of origin and their function. Reticulocyte-derived exosomes showed no increase in cholesterol/phospholipids ratio, opposite to the MHC II–enriched exosomes derived from B cells [82], [83]. Mast cell (RBL-2H3)–and dendritic cell–derived exosomes showed a high amount of disaturated phosphatidylcholine and phosphatidylethanolamine classes. This, together with a limited amount of diglycerides, suggests elevated membrane rigidity and an elevated transmembrane movement of lipids compared to the plasma membrane [82]. Most of the exosomes studied showed enrichment in raft lipids, such as cholesterol, sphingolipids, ceramide, and glycerophospholipids with long and saturated fatty-acyl chains [42, 80, 82, 84] . Exosome formation could be a way to secrete enzymes involved in lipid signaling [80, 82]. It was shown that cross-linking of sphingomyelin triggers calcium influx and ERK phosphorylation, indicating that these domains could be a specific signaling platform [82]. Recent studies provided evidence that the ESCRT-independent pathway of exosomal biogenesis requires ceramide and that release of exosomes was reduced after the inhibition of neutral sphingomyelinases [42, 84]. The lipidomic approach would not only provide insight into the roles of specific lipids in exosome biogenesis and functions, but also holds promise in the biomarker discovery field, shoulder to shoulder with other powerful “omics” technologies.

1.5.6. Urinary exosomes

Urinary exosomes are delivered to the urine when the outer membranes of multivesiculat bodies (MVB) fuse with the apical plasma membrane of the cell. Proteomic analysis of urinary vesicles through nanospray LC-tandem MS identified numerous protein components of MVB, suggesting their similar biogenesis as in other cell types [29], [47], [48]. Approximately 75% of

the proteins that constitute ESCRT-0, -I, -II, -III, and ATP-ase complexes involved in the MVB formation are identified in urinary exosomes, as well as ALIX [48]. Urinary exosomes are normally secreted into the urine from all cell types that face the urinary space and account for 3% of the total urinary protein [48], [85]. Proteomic analysis identified specific membrane proteins starting from podocytes through transitional epithelial cells lining the urinary drainage system. First, tandem mass spectrometry proteomic analysis of urinary exosomes from normal human subjects revealed 295 unique proteins. At least 20 of these proteins were already established as highly deregulated in various renal diseases and hypertension [47]. A recent study of urinary exosomes from normal human urine using highly sensitive LC-MS/MS based on an ion trap mass spectrometer (LTQ; Thermo-Finigan; ThermoElectron, San Jose, CA) identified 1412 unique proteins. Of these proteins, 927 were not previously identified, including 14 phosphoproteins. The identified phosphoproteins are known to be involved in serine/threonine and tyrosine kinase signaling pathways, as well as in some other signaling pathways that occur during cytokine and growth factor receptor activation and numerous other cell regulatory processes. Further analysis of all obtained data identified 1132 proteins unambiguously. A large number of identified proteins were soluble cytoplasmatic proteins and integral membrane proteins. Integral membrane proteins predominantly represent apical transporters present in every renal tubule segment. Multiple small GTP binding proteins, including proteins in the Rab, ARF, Rho, and Ral families, were identified, as well as cytoskeletal motor and peripheral-membrane proteins [48]. Ubiquitin was identified throughout the molecular weight range of the analysis, although the published data still do not distinguish between mono- and polyubiquitinated proteins [48].

1.5.7. Clinical relevance

a) Exosomes as biomarkers

In the last years, the literature was overwhelmed with papers proving the role of exosomes (especially tumor and APC exosomes) in the transfer similar receptors to homologous and heterologous cells. Their specific structure probably made them a more advantageous carrier of

"distant" signal delivery compared to soluble molecules [79]. The lipid bilayer highly enriched with cholesterol, sphingomyelin, and other characteristic molecules may significantly contribute to the preservation of stabile conformation conditions, including post-translational modification of the proteins they carry, allowing their detection by powerful proteomic-based strategies. A number of the novel putative markers for the clinically important states were proven to be proteins that have undergone disease-specific post-translational modification [86]. One of the most important post-translational modifications is phosphorylation that regulates cellular signaling processes and may determine protein structure, function, and subcellular localization. Phosphoproteins and even the specific phosphorylation sites were identified in urinary exosomes [48]. Thus, urinary exosomes may become a very interesting source for biomarker discovery. The valuable information that could be obtained from the proteomic, lipidomic, and other powerful molecular analyses also could provide further insight into the biogenesis and function of exosomes. One of the possible applications in clinical research is large-scale biomarker discovery, such as those currently being pursued in blood, tissue homogenates, and liquor [78]. Another promising approach could be choosing a combination of proteins whose identification can be hypothesized to provide the required specificity (lack of false-positives) and sensitivity (lack of false-negatives). Urine has evolved as one of the most attractive body fluids in the biomarker discovery field, with a potentially rapid application in the clinic. Considering their origin, urinary exosomes may provide a novel noninvasive method of acquiring unique information about the physiological or pathophysiological state of the renal cells of their origin. For the proteomics-based approach, exosome isolation could be helpful in minimizing highly abundant proteins in urine. This could also provide a significant enrichment of low-abundance urinary proteins that have potential pathophysiological significance and enhance the detectability of rare proteins that may have diagnostic value.

b) Exosomes as vectors for stem cell therapy

Stem cell therapies exhibit great potential for the treatment of various diseases. The therapeutic effects of adult stem cells remain to be fully elucidated. However, exosomes

carrying a cargo of packaged signals in the form of RNA, miRNA, or protein, among others, may be a key mechanism and the vehicle of their action to reduce inflammation, alter cellular signaling, and result in tissue repair. Mesenchymal Stem Cells (MSC) therapy, for example, is being tested in animal models and in multiple clinical trials for the treatment of disorders including acute lung injury, myocardial infarction, diabetes, sepsis, graft-versus-host disease, and hepatic and acute renal failure [87]. The therapeutic effect has been recapitulated in several preclinical models with administration of cell-free media from MSC cultures that contain exosomes. Research on the effect of exosomes in vitro has focused mostly on the interaction with immune system [6]. Moreover, exosomes from MSCs originating from almost all sources, including human embryonic stem cells have been characterized and proposed as the alternative therapeutic vehicle is many diseases. For example, MSCs may activate kinase pathways that are critical for ischemic preconditionig by increasing extracellular ATP levels and decreasing oxidative stress and inflamation [88].

c) **Exosome as vehicle of drug delivery**

The recognized function of exosomes as vehicles of intercellular communication has been further explored in the delivery of therapeutic signals such as small interfering RNAs (siRNAs) for drug delivery. The development of an exosome-based drug delivery system may improve targeting of specific cargos to treat disease. Types of therapeutic cargo include interfering RNAs, such as siRNAs and miRNAs [89]. Because MSCs are efficient and high producers of exosomes, they can be engineered to overexpress specific miRNAs that are incorporated into the exosomal cargo and delivered in vivo for the specific targeting of disease [90]. In addition to the therapeutic transfer of interfering RNAs, chemotherapeutics such as doxorubicin have been loaded onto exosomes and used to inhibit growth of breast and colon cancers [91]. The use of exosomes as drug delivery systems is just beginning to be explored.

1.6. Common methods for the isolation of extracellular vesicles.

1.6.1. differential centrifugation

Differential centrifugation is considered a gold standard, and is the most common method to isolate extracellular vesicles and is widely used to isolate them from body fluids and conditioned media. Although various protocols are available, generally it consists of multiple steps: first, a low speed spin (300 g for 10 min), which eliminates dead cells and bulky apoptotic debris, followed by higher speed spins, which varies among laboratories, from 1000 g to 20 000 g and eliminates larger vesicles and debris. A final high speed spin at 200,000 g precipitates exosomes (Figure 7). For more purified exosomes and eliminating contaminations, the pellet can be washed again in a large volume of PBS and centrifuged one last time at 200 000 g.

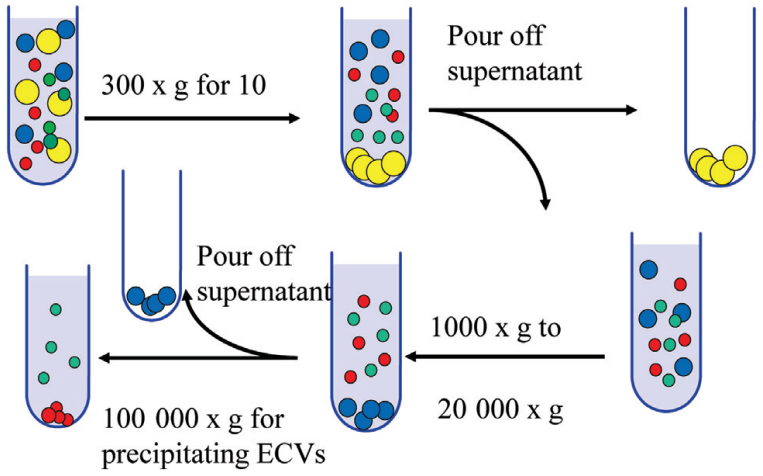

Figure 7. Summary of a differential centrifugation method.

One of the most important factors in the determination of sedimentation efficiency of exosomes in a differential centrifugation protocol is the clearing factor or k-factor of the rotor. The k-factor is a scale of the time taken for a particle to sediment through a particular medium. The value of the k-factor is determined by the maximum angular velocity (ω) of a centrifuge (in rad/s) and the minimum and maximum radius r of the rotor (Langer et al., 2003). It represents the relative sedimentation efficiency of a given centrifuge rotor at maximum rotation speed. K-factor can be utilized to predict the time t (in hours) required for sedimentation of exosomes

using different rotors. The following formula represents the correlation between t, time (in hours), k-factor, and s, sedimentation coefficient (in Svedbergs):

t= k/s

Admittedly, the most efficient rotors have the lowest k-factor value and operate at a relatively high centrifugal force (RCF) or g, and have a low sedimentation path length.

Another factor that should also be taken into account for increasing sedimentation stability is streaming, which affects both accuracy and resolution of sedimented exosomes.

1.6.2. Advantages and disadvantages

The main advantages of the differential centrifugation approach are the simplicity and requires simple laboratory equipment. However, is lengthy (4–5 h), requires an ultracentrifuge and results in a relatively low recovery of exosomes [92], ranging from 5% to 25% of the starting concentration. Another limitation in using differential centrifugation for isolating extracellular vesicles is co-precipitation of protein aggregates, apoptotic bodies, or nucleosomal fragments, which may lead to less sample purity and less correctly bound proteins.

Although exosome like vesicles are abundant in human urine, they are difficult to purify due to the presence of the most common urinary protein, Tamm–Horsfall protein (THP), also known as uromodulin. THP is a glycoprotein secreted by the epithelial cells lining the thick ascending limb of the loop of Henle, which can reach concentrations of 1.5 mg/mL, it has a role in protecting the urinary tract from pathogens by acting as a decoy receptor, and it may also inhibit stone formation in supersaturated urine. The protein has a signal peptide cleaved mass of 67.1 kDa and a pI of 4.9. THP contains a disulfide cross-linked zone pellucida (ZP) domain that is responsible for its self-associating into long, double-helical fibrils. Under high g force, THP precipitates, contaminating the exosome like vesicle pellet. Further, when the pellet is resuspended, the THP forms a hydrated three-dimensional gel that traps the vesicles. To recover the exosomes, dithiothreitol can be used to reduce the disulfides in the ZP domain, break up the fibrils, and release the vesicles [93]. However, this reduces all proteins, including

exosomal proteins, inactivating them and exposing their extended polypeptide backbones to proteolytic assault by endogenous proteases.

1.6.3. Sucrose gradient centrifugation

One way to address the limitations of the differential centrifugation approach and increase the purity of isolated exosomes is to use a sucrose gradient, which separates vesicles based on their different flotation densities [94] from THP. Exosomes, the finest sub-fraction of extracellular vesicles, have floatation densities of 1.08–1.22 g/ml on sucrose gradients [70]. In comparison, vesicles purified from the endoplasmic reticulum float at 1.18–1.25 g/ml, and vesicles from the Golgi at 1.05–1.12 g/ml [95].

Chen Y et al.' s protocol [96] uses the THP precipitation to sweep exosome like vesicles from the chilled urine into the pellet. The resuspended pellet is then loaded onto a heavy-water 5–30% sucrose gradient. The heavy water has a density of 1.1 g/ml, denser than normal water, and so is a sucrose-sparing solvent that is dense but not osmotically active. It allows the exosome like vesicles to band at lower sucrose concentrations than in normal water and to some extent prevents alterations in exosome like vesicles density. This allows the THP to separate from the exosome like vesicles. THP is normally secreted from the thick ascending loop of Henle. In contrast, exosome like vesicles are secreted from all major segments of the nephron [47]. As such, there are multiple populations of ELVs in urine depending on their origin, and these populations separate into individual bands following heavy-water centrifugation. The uppermost band expresses high levels of aquaporin-2, indicating that they derive from the collecting duct. The middle band is heavily enriched for the polycystin proteins, which origin from the proximal tubules. These exosome like vesicles are most likely shed from the proximal tubule as they are also megalin and aquaporin-1 positive. The most dense bottom band contains ELVs with podocin, providing evidence of glomerular origin.

1.6.4. Microfiltration

Attempts to isolate exosomes using filtration-based methods, omitting ultracentrifugation, have shown discrepant results. This may be due to the fact that protein quickly accumulates on the filters blocking further flow. One example of a commercially available filter is a nanomembrane concentrator with 13 nm pore size, which requires only 0.5 mL of urine as starting volume. While some proteins, such as annexin V, podocalyxin and neuron specific enolase do not adhere to the membrane and are easily recovered, other proteins are more adherent, such as aquaporin-2 and TSG-101 [78]. Compared to other isolation methods, the purity of recovered protein in urinary extracellular vesicles remains low [97] and this method therefore seems less suitable for nephrotic urine [98] and to isolate RNA from urinary extracellular vesicles [97].

Recently Merchant et al. [99] proposed a microfiltration isolation method, using low protein-binding size exclusion filters for isolation of urinary biomarkers. They utilized hydrophilized polyvinylidene difluoride membrane, which have a 100 nm pore size, to easily isolate extracellular vesicles from fresh urine samples. Liquid chromatography-mass spectrometry immuno-blot analysis, and electron microscopy were used for validation and assessing the efficacy of the microfiltration method. They reported equivalent enrichment of extracellular vesicles proteomes with reduced co-purification of abundant urinary proteins in comparison with other standard methods of extracellular vesicles isolation, including ultracentrifugation and nano-filtration. Although filtration technologies are improving quickly, they face several challenges, such as the lack of extracellular vesicles condensation, co-purifying abundant proteins with extracellular vesicles isolation, contamination of isolated extracellular vesicles, and trapping of extracellular vesicles in nano- or micro-pores. Therefore, isolation conditions must be optimized for maximal recovery of extracellular vesicles and a more pure isolation/enrichment. Another limiting factor for high throughput application of these microfilters is the need for a stirred cell apparatus (a positive pressure filtration based concentrating device). Incorporation of the microfilter into a commercial spin filtration device may alleviate this limitation.

1.6.5. Precipitation

ExoQuick™ is a commercially available precipitation kit to isolate microvesicles (System Biosciences, Mountain View, CA, USA). For urine, its current protocol results in a very low yield of both protein and RNA [97]. Modification of the sample work-up resulted in the highest quantities of mRNA and miRNA and an acceptable protein yield compared to the ultracentrifugation methods [97]. This method is relatively easy, omits the need for ultracentrifugation and uses less sample material (5 mL urine). However, the overnight incubation step limits its use for immediate diagnostic use, making it less suitable for disorders such as acute kidney injury.

A standardized method to isolate exosomes doesn't exist yet. With this work our aim was to develop a novel method to purify exosomes from urine. The new method is fast, easy and allow a high enrichment in exosomal proteins. The great advantage is that it could be used for several biomedical applications.

2. MATERIALS AND METHODS

2.1. Urine collection and storage

Urine samples were either taken from ongoing internal review board (IRB)-approved research studies, or they are discarded, de-identified urine specimens. The urine is collected as clean-catch, midstream samples. Subsequently, the samples are stored at -20°C until further aliquoting and processing. Informed consent was obtained from all subjects and the research conformed to the principles set out in the WMA Declaration of Helsinki and the NIH Belmont Report [100].

In order to isolate exosomes from urine, the methods described below were used.

2.2. Ultracentrifugation

2.2.1. Single ultracentrifugation and DTT reduction

Protease inhibitors were added (complete Protease Inhibitor Cocktail Tablets, Roche). 50 ml) of urine samples were centrifuged at 17,000 x g for 10 min at 37^{o}C (Beckman L8-70M ultracentrifuge; Beckman Coulter, Fullerton, CA) . The supernatant was saved and the pellets were resuspended in an isolation solution (250mM sucrose, 10mM triethanolamine (pH 7.6)) followed by incubation with DTT (final concentration of 200 mg/ml corresponding to 1.3 M; Sigma-Aldrich, St Luois, MO) at 37 ^{0}C for 10 min to denature and reduce the disulfide bonds of zona pellucida domains in uromodulin. During the DTT incubation, samples were vortexed every 2 min, transferred to clean centrifuge tubes (Beckman polycarbonate) and more isolation solution was added to a final volume of 10 ml. The samples were centrifuged again at 17,000 x g for 10 min at 37 ^{o}C. The two supernatants from the 17,000 x g spins were pooled and ultracentrifuged at 200,000 x g for 1 h at 37 ^{o}C. Pellets were solubilized in 1% SDS and TBS (pH 7.6) [93].

2.2.2. Double ultracentrifugation and DTT reduction

Protease inhibitors were added (complete Protease Inhibitor Cocktail Tablets, Roche). 50 ml of urine were centrifuged at 17,000 x g for 10 min at 4°C. The 17,000 x g supernatant was ultracentrifuged at 200,000 x g for 1 h at 4°C. The abundant urinary protein uromodulin forms very high molecular weight complexes through disulfide linkages. These complexes sediment in the 200,000 x g spin unless denatured. To denature the zona pellucida domains in the Tamm-Horsfall protein, also known as uromodulin, we mixed the resuspended pellet with 200 mg/ml (1.3 M) dithiothreitol (DTT) at 95°C for 2 min.The resuspended pellet was added to an ultracentrifuge tube, and isolation solution (10mM triethanolamine and 250 mM sucrose) was added to increase the volume to 10 ml. The sample was centrifuged at 200,000 x g for 1 h at 4°C. The pellet was suspended in 1% SDS, TBS pH (7.6) [48].

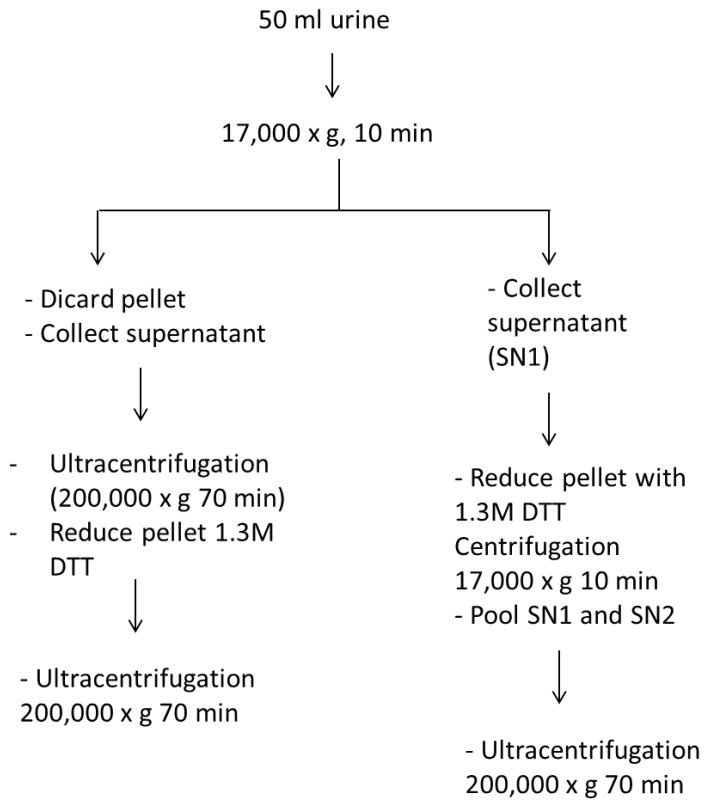

Figure 8. Single(right) and double(left) ultracentrifugation workflow.

2.3. Sarkosyl fractionation: rationale

Sodium lauroyl sarcosinate (INCI), also known as sarkosyl, is an ionic detergent derived from sarcosine. This surfactant is amphiphilic due to the hydrophobic 14-carbon chain (lauroyl) and the hydrophilic carboxylate. Addition of an mixture of equal parts of sodium lauroyl sarcosinate and the non-ionic surfactant sorbitan monolaurate (S20) to water led to the formation of micelle-like aggregates, even though neither surfactant formed micelles when present alone. Such aggregates can help carry other small molecules, such as drugs, through the skin [101].
The Sarkosyl solubilization method facilitated separation of the inner and outer membranes, making the procedure amenable for effective probing of the subcellular proteome. It has been commonly used to fractionate bacterial proteome [102] or to solubilize overexpressed proteins in bacteria without denaturing them [103].

2.3.1. Sarkosyl fractionation (Method 1)

Urine (50 ml) was centrifuge at 17,000 x g 15 minutes at 4^{o}C. The Supernatant, containing free exosomes, was ultracentrifuged at 200,000 x g 1 hour at 4^{o}C whereas the pellet, composed by exosomes trapped by uromodulin, was resuspended in 1% Sarkosyl in Tris Buffered Saline (TBS) pH 7.6 (Biorad Laboratories) and incubated 1 hour, under costant rotation, at 4^{o}C. The pellet solubilized in Sarkosyl was ultracentrifuged at 200,000 x g 1 hour at 4^{o}C. The supernatant was collected in a new vial and the pellet was resuspended in 1% SDS in TBS pH 7.6 (Biorad Laboratories).

2.3.2. Sarkosyl fractionation using different volumes of urine (Method 2)

Different urine volumes (50 ml, 20 ml and 5 ml) from 2 different donors were used. A technical repeat was also performed. The samples were ultracentrifuge at 200,000 x g 1 hour at 4^{o}C. The supernatant was discard and the pellet containing exosomes and uromodulin was resuspended

in 1% Sarkosyl in Tris Buffered Saline (TBS) pH 7.6 (Biorad Laboratories). The solution was incubated 1 hour at 4°C under rotation and centrifuged at 20,000 x g 1 hour at 4°C. The supernatant was transferred to a new tube (Sarkosyl soluble fraction), whereas the pellet was resuspended in 1% SDS in TBS (Sarkosyl insoluble fraction). The 2 fractions were processed separately using Filter Aided Sample Prepatration (FASP) and analyzed by LC-MS/MS.

2.3.3. Sarkosyl and carbon fractionation (Method 3)

Urine (50 ml) of donor 1 was processed according to the protocol described in 3.1. After proteolitic digestion and recovery of the peptides, Sarkosyl soluble and Sarkosyl insoluble fractions were fractionate using Carbon packed TopTips (Glygen corporation, Columbia, MD). A double pre-wetting step with 200µl of 95% ACN and 0.1% TFA was performed. The carbon tips were equilibrated twice by adding 200µl of 0.1% TFA. The peptides were acidified using 20% TFA to a final concentration of 0.5% TFA. The sample was loaded to the carbon tip and wash 3 times with 200µl of 0.1% TFA. The elution buffers (20% ACN (Acetonitrile) in 0.1% TFA (Trifloroacetic acid), 25% ACN in 0.1% TFA, 30% ACN in 0.1% TFA, 35% ACN in 0.1% TFA, 40% ACN in 0.1% TFA, 50% ACN in 0.1% TFA, 60% ACN in 0.1% TFA, 95% ACN in 0.1% TFA) were added and the flow through was collected in separate tubes. Each fraction was dried completely using a SpeedVac (Eppendorf).

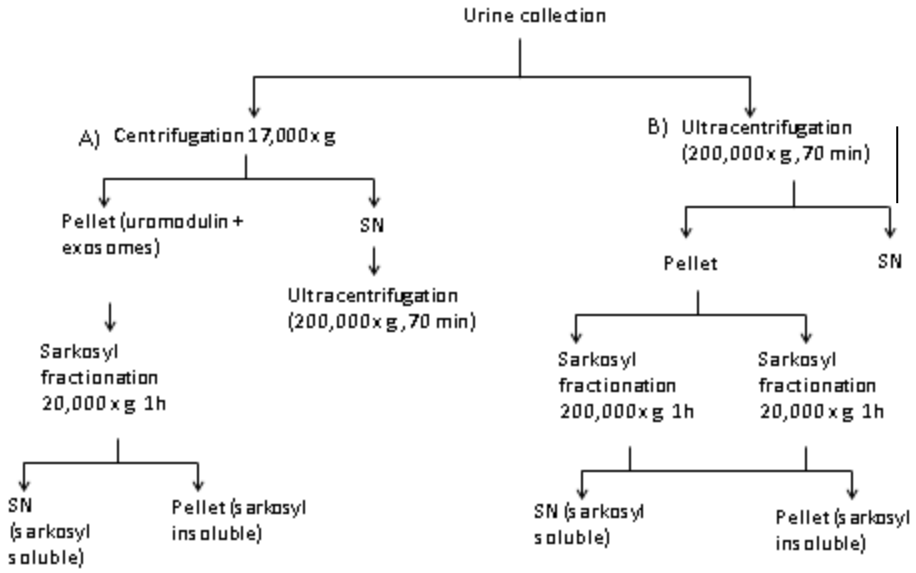

Figure 9. Summary of Sarkosyl fractionation optimization. Exosomes were purify from 50 ml of urine for the method A) and from different volumes (50 ml, 20 ml, 5 ml) of urine for the method B). In the last method 2 biological repeats were performed.

2.4. Filter Aided Sample Preparation (FASP)

Each fraction (200 µl) was reduced in 0.1M DTT for 15 minutes at 37°C, and filtered twice with 200µL of 8M urea in 100mM Tris-Hydrochloride Solution (pH 8.5) through a 30kDa spin filter (Millipore, MA) for 15 minutes at 14,000g. Samples were alkylated with 1% acrylamide solution for 20 minutes at room temperature. They were then centrifuged for 10 minutes at 14,000g, and washed three times with 100µL of 8M urea solution followed by three washes with 100µL of 50mM ammonium biocarbonate solution. The samples were digested overnight with 2µg trypsin (Promega, WI) at 37°C in a humidify chamber 1:50 (protein:protease).The peptides were eluted with 40µL of 50mM ammonium bicarbonate, followed by 50µL of 0.5 M sodium chloride solution. The proteins were then acidified with TFA to a final concentration of 0.2% prior to desalting with C18 Silica columns (The Nest Group, Inc., MA) and then dried in a vacuum centrifuge before reconstitution.

2.5. LC-MS/MS

Samples were analyzed by a nanoLC system (Eksigent) equipped with LC-chip system (cHiPLC nanoflex, Eksigent, trapping column: Nano cHiPLC Trap column 200 µm x 0.5 mm ChromXP C18-CL 3 µm 120 Å, analytical column: Nano cHiPLC column 75 µm x 15 cm ChromXP C18-CL 3 µm 120 Å) coupled online either to a Q-Exactive mass spectrometer (Thermo Scientific) or a TripleTOF 5600 (AB SCIEX, Concord, ON). Peptides were separated by linear 30, 60 or 120 minutes gradients from 95 % buffer A (0.2 % FA in water)/5 % buffer B (0.2 % FA in ACN) to 65 % buffer A/35 % buffer B.

The mass spectrometers acquire *m/z* ratios of both the precursor ions (MS1) and fragment ions (MS2) in positive ion mode; the instruments were operated in the data dependent mode. In the Q-Exactive, scans were acquired in the Orbitrap mass analyzer with resolution 70,000 at 200 Th for MS1 and with resolution 17,500 at 200 Th for MS2. For the full scans, 3E6 ions in the mass range 300-1500 m/z were accumulated within a maximum injection time of 20 ms in the C trap and detected in the Orbitrap analyzer. The 30 most intense ions with charge states ≥ 2 were sequentially isolated to a target value of 2E5 with a maximum injection time of 250 ms with an isolation window of 2.0 m/z and fragmented in the collision by higher-energy collisional induced dissociation (HCD) with normalized collision energy of 27%. In the TripleTOF 5600, scans were acquired in the TOF and detected in the accelerator TOF mass analyzer. For TOF MS, the mass range 300-1300 m/z ions were accumulated within a maximum injection time of 20 ms and detected in the accelerator TOF mass analyzer. The 35 most intense ions with charge states ≥ 2 were sequentially isolated with a maximum injection time of 50 ms with an isolation window of 2.0 m/z and fragmented in the collision by collisional induced dissociation (CID).

2.6. Data processing and analysis

The Thermo ".raw" files were converted into the Mascot generic format (MGF-files) using the proteoWizard software tool. All MS/MS data were searched against a concatenated target-decoy uniprot-based human protein sequence database including protein sequences of

common contaminants by the Protein Pilot Software (AB SCIEX, Foster City, CA), using the Paragon algorithm. For the searches, trypsin was defined as the protease. The search included propionamide of cysteine as a fixed modification and N-acetylation of protein and oxidation of methionine as variable modifications. Up to two missed cleavages were allowed for protease digestion and peptide had to be fully tryptic. A filter identifications of 1% False Discovery Rate (FDR) was used at peptide and protein level.

The "wiff" data acquired with the TTOF 5600 were processed with the Protein Pilot Software (AB SCIEX) utilizing the Paragon algorith. All MS/MS data were searched against a concatenated target-decoy uniprot-based human protein sequence database including protein sequences of common contaminants. For the searches, trypsin was defined as the protease. The search included propionamide of cysteine as a fixed modification and N-acetylation of protein and oxidation of methionine as variable modifications. Up to two missed cleavages were allowed for protease digestion and peptide had to be fully tryptic. A filter for identification of 1% False Discovery Rate (FDR) was used at peptide and protein level.

3. Gene Ontology and exosomal databases.

To establish a comprehensive set of functional annotations and enrichments of different proteins we used The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (National Institute of Allergy and Infectious disease (NIAID), NIH),[104] and Functional Enrichment Analysis Tool (FunRich) version 1.1. The total proteome was annotated. Annotations for biological processes were reduced to nine categories, including amino acid biosynthesis, cytoskeletal, development, homeostasis-related, localization, metabolism, neurotransmitter-related, synaptic and cell death-related. For the cellular component analysis, categories included exosomes, lysosomes, extracellular region, extracellular space, extracellular matrix, plasma membrane, cytoskeleton. In addition, to validate the data from gene ontology annotations, 2 different databases containing exosomal proteins were used: Exocarta (http://exocarta.org/) and the Urinary Exosome Protein Database (http://dir.nhlbi.nih.gov/papers/lkem/exosome/Default.aspx?protein).

A comparison between the different approaches was performed using Venny program. (http://bioinfogp.cnb.csic.es/tools/venny/).

3. RESULTS

3.1. Exosome isolation by ultracentrifugation and DTT reduction

3.1.1. Single ultracentrifugation

In this study, we carried out a proteomic profiling of a low-density membrane vesicles from human urine consisting mainly of exosomes, using a highly sensitive LC-MS/MS system, based on a TripleTOF 5600 (AB SCIEX). To purify extracellular vesicles (EV), 50 ml of urine was centrifuge at 17,000 x g for 10 minutes to pellet cellular debris and uromodulin. The initial supernatant containing suspended exosomes was kept (SN1) and the pellet was reduced with 1.3 M DTT to free the exosomes trapped in the uromodulin aggregates. The following centrifugation at 17,000 x g removed any cellular debris. The supernatants (SN1 and SN2) were combined and ultracentrifuged at 200,000 x g 1 hour at 4^{o}C to sediment exosomes [93]. The ultracentrifuged supernatant, containing exosomes, and the pellet containing debris were analyzed separately, separating the peptides with a linear 30 minutes gradient.

We identified 251 proteins in the combined supernatant (SN1+SN2; exosome fraction) and 363 proteins in the pellet (cellular debris). The majority of proteins (70.3% in the supernatant and 64.2% in the pellet) derive from exosomes as determined by GO annotation (figure 10). Further GO annotation reveals significant enrichment in lysosomal (49.1% in the supernatant and 42.1% in the pellet) and plasma membrane proteins (49.1% in the supernatant and 41.1% in the pellet. Proteins deriving from extracellular region (22.6% in the supernatant and 21.7% in the pellet), extracellular space (19.3% in the supernatant and 20.4% in the pellet), extracellular matrix (7.5% in the supernatant and 9% in the pellet) and cytoskeleton (10.5% in the supernatant and 10% in the pellet) were less abundant.

The results suggest that proteins found in the supernatant and the pellet do not show the expected differences, possibly because the cellular debris still contained sizeable amounts of uromodulin aggregates (figure 10).

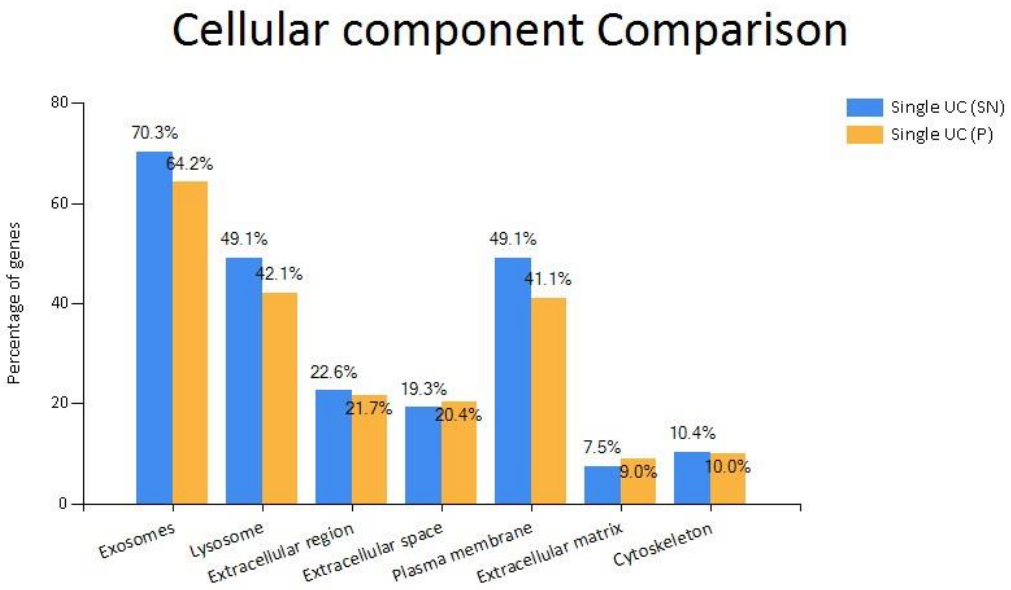

Figure 10. Cellular component comparison between supernatant (SN) and pellet (P) after a single ultracentrifugation (UC) step.

3.1.2. Double ultracentrifugation

To identify proteins deriving from exosomes, a second isolation protocol was followed: 50 ml of urine was centrifuge at 17,000 x g for 10 minutes. The supernatant, containing uromodulin and exosomes, was ultracentrifuge at 200,000 x g for 1 hour at 4^{o}C and subsequently the pellet was reduced with 1.3 M DTT. After adding isolation buffer (250mM sucrose, 10mM triethanolamine, pH 7.6) to a final volume of 10 ml, the sample was ultracentrifuged for the second time to sediment exosomes [48]. The ultracentrifuged supernatant (containing exosomes) and the discarded pellet (containing cellular debris and uromodulin) were analyzed individually, separating the peptides with a linear 30 minutes gradient on a TripleTOF 5600.

We identified 283 proteins in the exosome fraction (SN) and 326 proteins in the cellular debris pellet (P). A large number of the proteins (74.3% in SN and 69.5% in P) that were identified derived from exosomes. As for the single ultracentrifugation protocol, proteins derived from lysosomes (49.8%, 47.4%) and plasma membrane (40.1%, 39.3%) showed similar high abundances in both the supernatant and the pellet. Lower percentages were seen for proteins

originating from extracellular region (19.4%, 19.1%), extracellular space (19.4%, 18.8%), extracellular matrix (8%, 9.6%) and cytoskeleton (9.7%, 10.6%) in the supernatant and pellet, respectively (figure 11). Similarly to what was observed in the single ultracentrifugation method, the supernatant (SN) and the pellet (P) did not show a significant difference in their proteome composition.

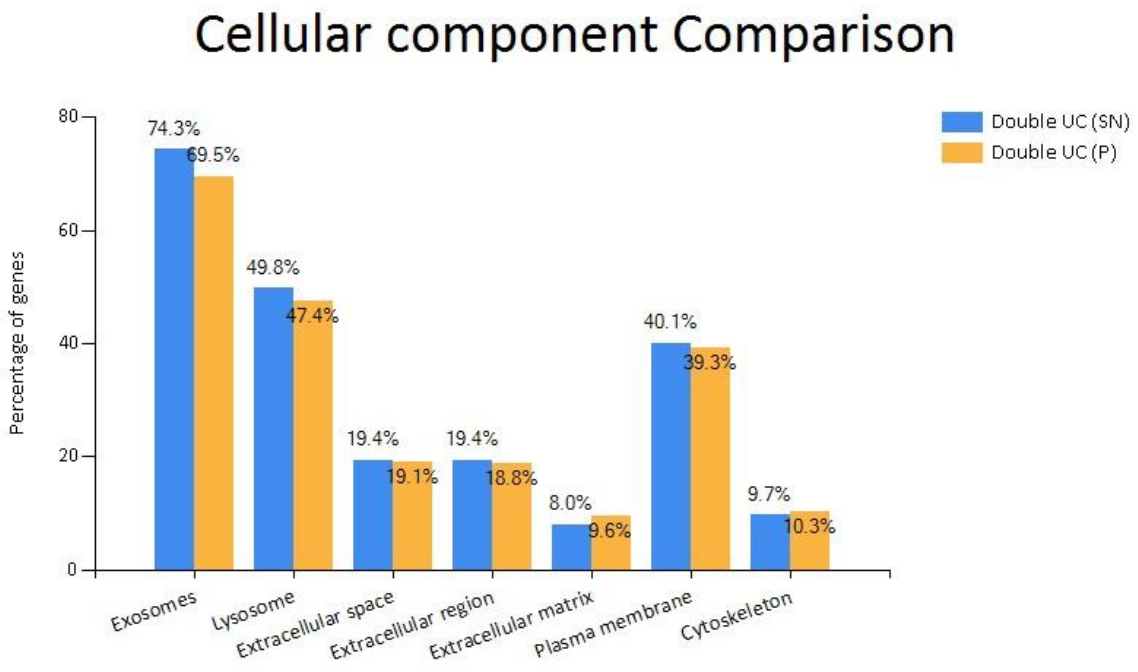

Figure 11. Cellular component comparison between supernatant (SN) and pellet (P) after a double ultracentrifugation (UC) step.

To compare the 2 different approaches, a Venn diagram was created using Venny (http://bioinfogp.cnb.csic.es/tools/venny/), as shown in Figure 12. The greater number of proteins (125) was common to both approaches and appeared both in the supernatant and in the pellet. This result suggests that there is not a significant difference between the single and double ultracentrifugation methods.

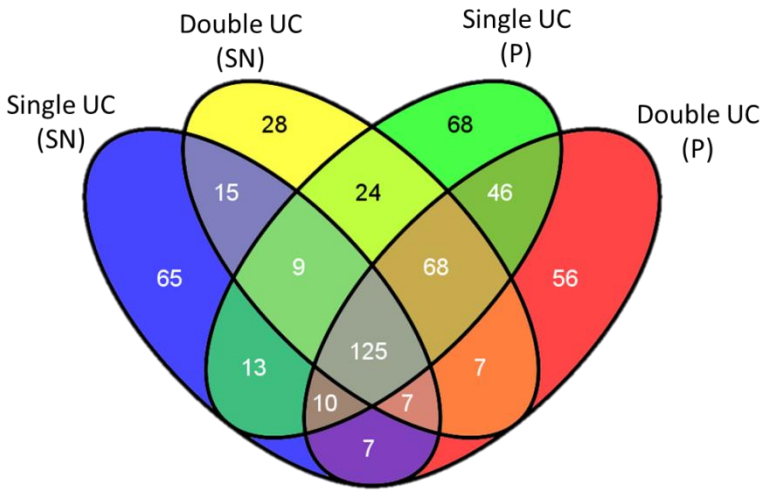

Figure 12. Comparison between the supernatant (SN) and pellet (P) of the single and double ultracentrifugation methods.

3.1.3. Combination of double and single ultracentrifugation.

The samples analized by the methods described in paragraph 1.1 and 1.2 of this section were combined and the peptides were separated using a TTOF 5600 mass spectrometer with a linear 60 minutes gradient. 647 proteins were identified and compared with Exocarta (only proteins from urinary exosomes; http://exocarta.org/) (which is a database, containing exosomal proteins deriving from all biological fluids), and Gonzalez P.A. et al's study [48] (figure 13 A). From the comparison we saw that 124 proteins were common to the 3 datasets, whereas Exocarta and Gonzalez *et al.* [48] shared 1029 proteins. while the combined ultracentrifuged samples shared only 135 proteins with Exocarta.

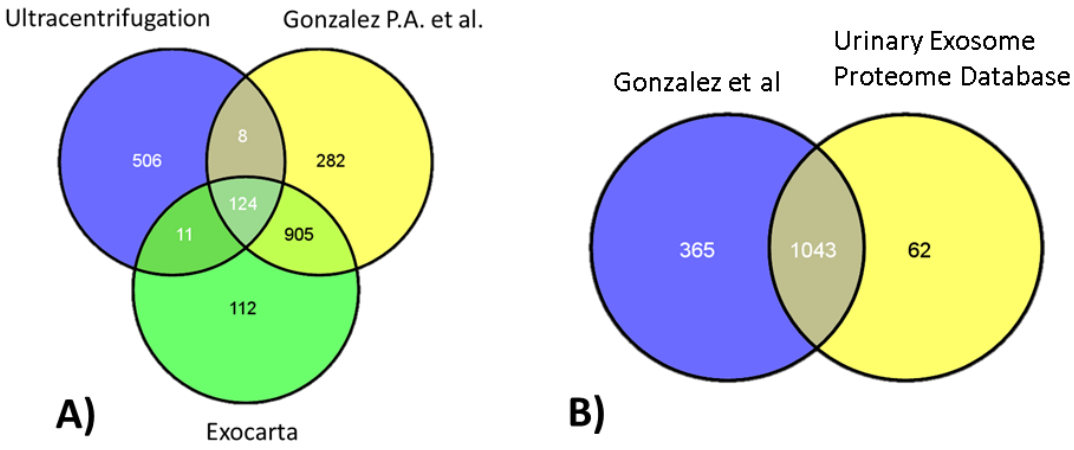

Figure 13. A) Comparison between our method, Gonzalez et al. [48] and exosome proteins deriving from uring reported in Exocarta Database. B) Comparison between Gonzalez et al. [48] and the Urinary Exosome Protein Database.

We used the Gonzalez et al. [48] publication instead of the *Urinary Exosome Protein Database* (http://dir.nhlbi.nih.gov/papers/lkem/exosome/), which is another online resource for urinary exosomes, since the letter is largely based on the former as it is apparent from Figure 13B.

3.2. Sarkosyl fractionation

The experimental scheme is reported in Figure 8. EVs isolated from 50 ml of urine were fractionated using 1% Sarkosyl, which is an anphiphilic detergent. The idea is that the uromodulin aggregates remain insoluble in sarkosyl while the exosomes will be solubilized to release the exosomal proteins into the supernatant. For this purpose, urine was centrifuge at 17,000 x g 15 minutes at 4°C. The supernatant, containing free exosomes, was ultracentrifuged at 200,000 x g 1 hour at 4°C whereas the pellet, composed by exosomes trapped by uromodulin, was resuspended in 1% Sarkosyl in Tris Buffered Saline (TBS) pH 7.6 (Biorad Laboratories) and incubated 1 hour, under costant rotation, at 4°C. Protein distribution of EV (supernatant after 200,000 ×g centrifugation, SN) were substantially different in all fractions as reported in Table 1. Mass spectrometry analysis of all the fractions identified a total of 280

proteins in the soluble fraction and 375 proteins in the insoluble fraction after separating the peptides using a linear 30 minutes gradient (1% FDR both at peptide and protein level). After the ultracentrifugation step the pellet, containing free exosomes (not trapped by uromodulin), and the supernatant (depleted of exosomes) were analyzed following the same parameters: 27 proteins were identified in the pellet containing EVs after ultracentrifugation and 84 proteins were identified in the supernatant (Table 1). To determine which fraction is enriched in/depleted of uromodulin, the main interference for the exosome isolation as it forms supramolecular structures in its non-reduced form, we calculated the ratio between uromodulin and albumin, since they are the most abundant proteins in urine (table 1). As expected, Sarkosyl insoluble fraction showed a 25 fold enrichment of uromodulin (URO/ALB of 10) compared to Sarkosyl soluble fraction (URO/ALB of 0.4).

SAMPLE	Number of proteins	Uromodulin/ Albumin
Sarkosyl soluble (ss)	280	0.4
Ultracentrifugation (UC)	27	9
ss+ UC (exosomes)	256	2
Sarkosyl insoluble (si)	375	10
Supernatant after UC	84	2.6
Ultracentrifugation + DTT (knepper protocol)	604	1.45

Table 1. Number of proteins identified after performing the method described in paragraph 2.1 of the Materials and Methods section.

In figure 14 the number of identified proteins is summarized using a Venn diagram. Comparing Sarkosyl soluble (280 proteins), Sarkosyl insoluble (375 proteins) and ultracentrifugation combined with DTT reduction following the workflow described by Gonzales (604 proteins) [48] we saw that 40% of the proteins in Sarkosyl soluble fraction overlap with Sarkosyl insoluble fraction. The overlap between proteins identified in Sarkosyl soluble fraction and

ultracentrifugation followed by DTT reduction [48] was 53%, whereas the overlap was 48% for the comparison between Sarkosyl insoluble fraction and ultracentrifugation followed by DTT reduction [48].

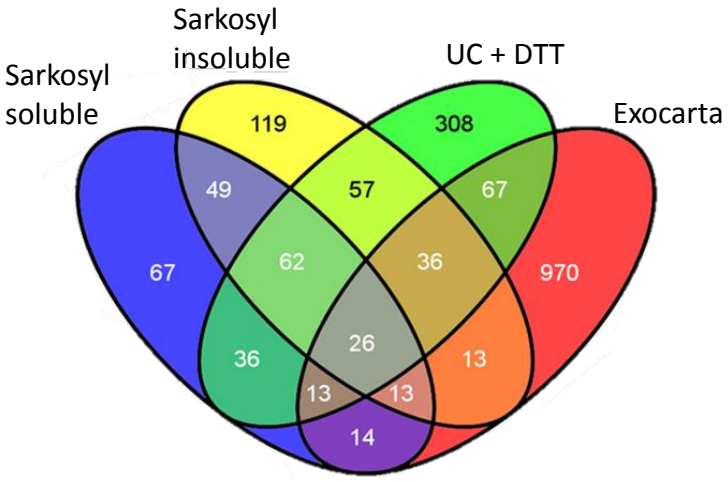

Figure 14. Diagram of proteins identified in Sarkosyl soluble, Sarkosyl insoluble, ultracentrifugation and DTT reduction and the exosomal proteins found in urine available in Exocarta database (v4.1)

To compare our EV dataset with other studies, we performed overlap analysis using Exocarta (v4.1) protein database. Results are summarized in figure 14 and show an overlap of 23% in Sarkosyl insoluble fraction and ultracentrifugation combined with DTT reduction, 26% in Sarkosyl soluble fraction compared with Exocarta database.

We found only 67 proteins unique in Sarkosyl soluble fraction, 119 in Sarkosyl insoluble fraction and 308 proteins after ultracentrifugation and DTT reduction.

3.2.1. Optimization of the Sarkosyl-based Exosome Isolation

To optimize Sarkosyl fractionation, 50 ml of urine were ultracentrifuged (200,000 x g for 1hour), avoiding the first centrifugation step at 17,000 x g for 10 min, and the pellet was resuspended in 1% Sarkosyl as represented in figure 8. After tryptic digestion the peptides were separated using 1 hour gradient by a TTOF 5600. 754 proteins were identified in Sarkosyl soluble fraction and 155 in Sarkosyl insoluble fraction.

SAMPLE	Number of proteins	Ratio uromodulin/ albumin
Sarkosyl soluble	754	1
Sarkosyl insoluble	155	5.2

Table 2. Proteins identified by Protein Pilot Software.

Comparing our protein list with previously published urine exosome protein list, 27% of proteins in Sakosyl soluble, 26% in Sarkosyl insoluble overlap with Exocarta database and 95 proteins were common between the 2 fractions, as shown in figure 15.

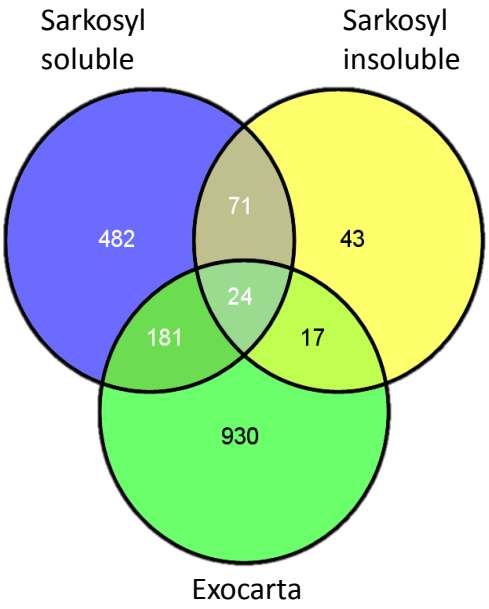

Figure 15. Diagram of proteins identified in Sarkosyl soluble, Sarkosyl insoluble and and the exosomal proteins found in urine available in Exocarta database (v4.1).

Other proteomic studies that isolate and analyze exosomes fron urine [105] show very poor overlap with both Exocarta and Gonzales et al.'s results [48], as shown in figure 16. Protein overlap analysis only 93 proteins were common to the 3 studies. This indicates that urinary exosome preparations are not as robust and/or the exosomal compositions are not as well defined as many current publications make it seem.

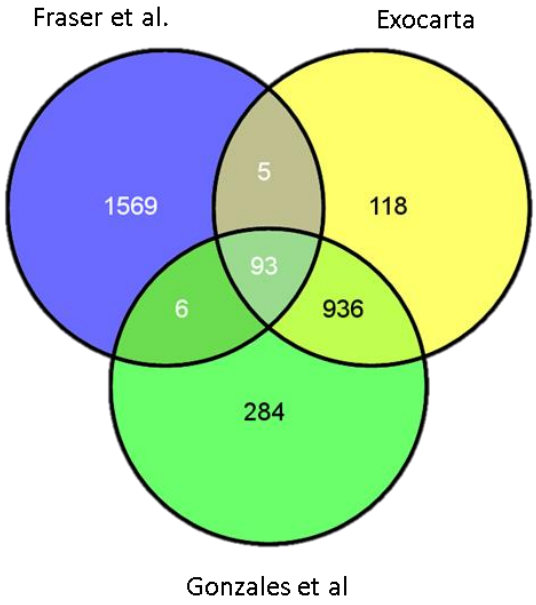

Figure 16. Comparison between different exosome proteomic studies – Exocarta, Fraser et al. [105] and Gonzales et al. [48]

To test the reproducibility of the method, 2 different biological samples were processed twice resulting in two biological repeats with two technical repeats each. Peptides were separated using a 120 minutes gradient using a QExactive mass spectrometer (Thermo Scientific); the resuting ".raw" files were converted to the .mgf format and analyzed using ProteinPilot Software (AB SCIEX). The number of proteins identified are summarized in table 3: 910 and 937 were identified in 50 ml of urine in the first biological repeat and 581 and 459 proteins were identified in 50 ml in the second biological replicate.

To identify whether lower volumes of urine can be used, 20 ml and 5 ml or urine were also processed and analyzed. A total of 930 and 667 proteins were identified in 20 ml and 5 ml of urine, respectively (table 3).

Although quite a variation was observed for the URO/ALB ratio, a successful depletion of uromodulin is apparent as none of the samples had URO/ALB ratios of 5 to 10 as reported above (table 3).

SAMPLE	Volume of urine	Sarkosyl soluble	Ratio uromodulin/ albumin
1.1	50 ml	910	0.34
1.2	50 ml	937	0.97
2.1	50 ml	581	1.3
2.2	50 ml	459	2.3
1	20 ml	930	0.7
1	5 ml	667	1.5

Table 3. Number of proteins identified in urinary exosomes.

To increase the number of proteins identified, Sarkosyl soluble and insoluble fractions were further fractionated using carbon packed TopTips: 6 fractions were analyzed with a 60 minutes gradient each. 1618 and 553 proteins were identified in Sarkosyl soluble and insoluble fractions, respectively using a 1% FDR filter. Sarkosyl soluble fraction had an overlap of 42% with Exocarta and 39% with the exosomal proteome reported by Gonzalez, P.A et al. [48]

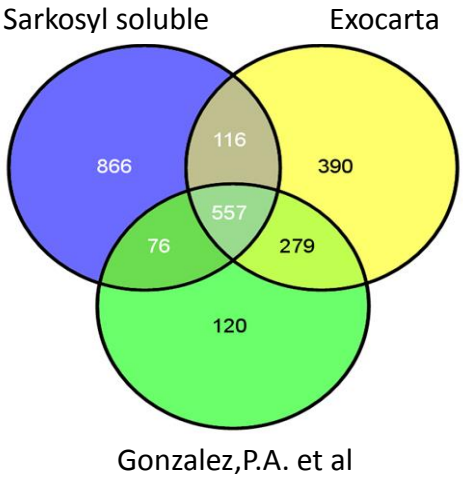

Figure 17. Overlap betweeen Sarkosyl soluble fraction, Exocarta and the exosomal proteome found by Gonzalez et al [48]

Gene ontology analysis of the EV proteins showed a 61.3% enrichment for proteins deriving from exosomes (figure 18) in Sarkosyl soluble fraction compared to (per definition) 99.9% from Exocarta. All the other cellular components show similar percentages for the Sarkosyl soluble fraction and the Exocarta database.

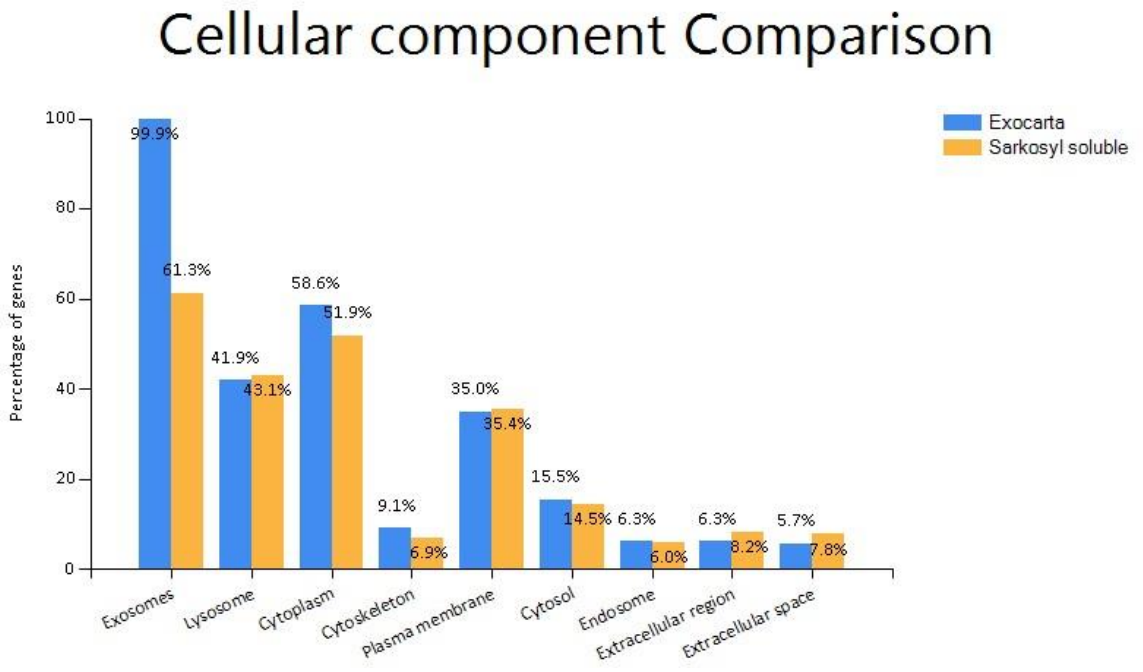

Figure 18. Gene ontology analysis of Sarkosyl soluble fraction and Exocarta database (v 4.1).

Gene ontology analysis of the Sarkosyl insoluble fraction reveal 69% enrichment for exosomal proteins (figure 19), suggesting an incomplete solubilization of the exosomes by Sarkosyl. The sarkosyl insoluble fractions contains more mitochondrial (26.6%), centrosomal (17.7%) and cytosolic (22.9%) proteins than the Exocarta database (11.7%, 9.6% and 15.5% respectively), as shown in figure 18.

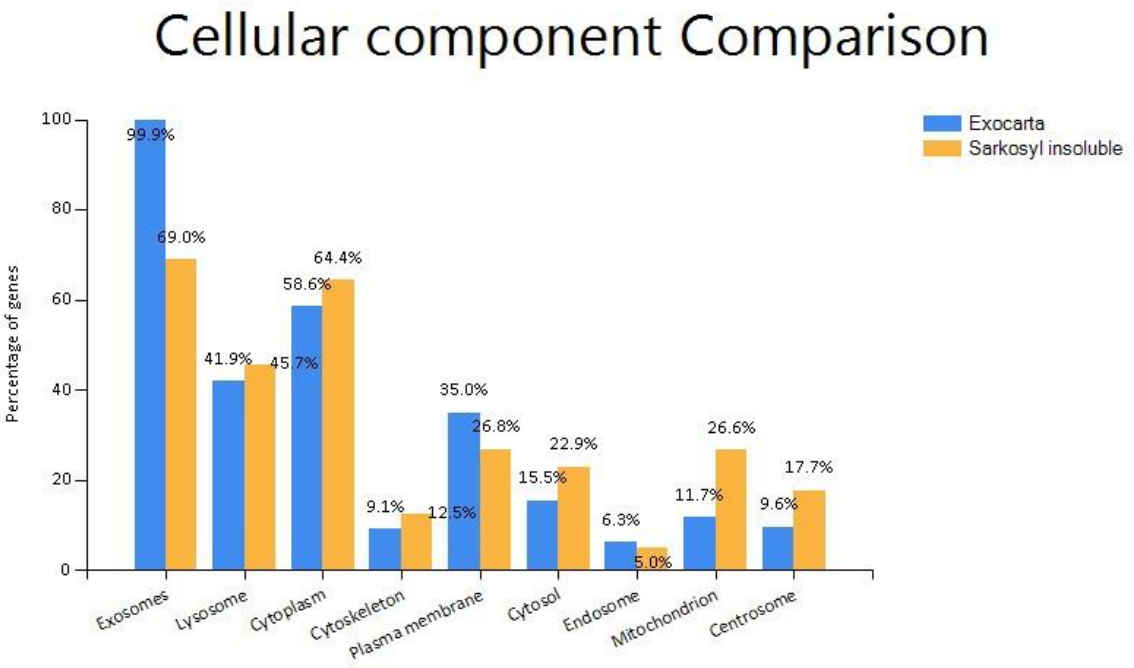

Figure 19. Gene ontology analysis of Sarkosyl insoluble fraction and Exocarta database (v 4.1).

In figure 20 the number of identified proteins is summarized using a Venn diagram. Comparing Sarkosyl soluble, Sarkosyl insoluble and Exocarta database we saw that 369 out of 1618 proteins in Sarkosyl soluble fraction overlap with Sarkosyl insoluble fraction. The overlap between proteins identified in Sarkosyl insoluble fraction and Exocarta was 44%.

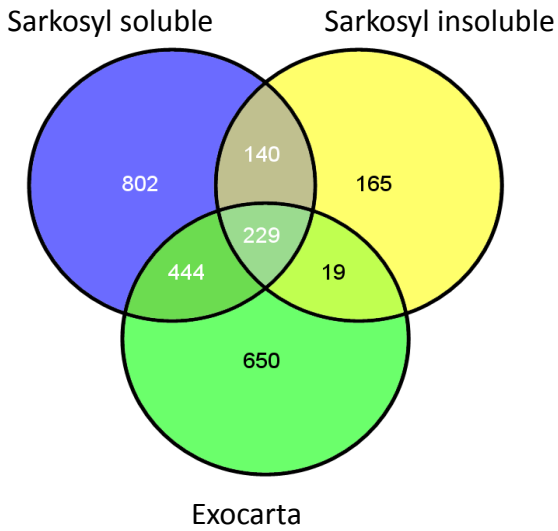

Figure 20. Venn diagram representing the overlap between Sarkosyl soluble, Sarkosyl insoluble fractions and Exocarta (v4.1)

To verify the isolation of exosomal proteins, the whole list of proteins was analyzed both for Sarkosyl soluble and insoluble fraction, looking for EVs markers. Table 4 summarized the EV markers found in the soluble fraction

Accession	Name	Spectra counts
sp|Q8WUM4|PDC6I_HUMAN	Programmed cell death 6-interacting protein (Alix)	72
sp|P11142|HSP7C_HUMAN	Heat shock cognate 71 kDa protein	51
sp|Q5VTE0|EF1A3_HUMAN	Putative elongation factor 1-alpha-like 3	36
sp|Q99816|TS101_HUMAN	Tumor susceptibility gene 101 protein	31
sp|P50395|GDIB_HUMAN	Rab GDP dissociation inhibitor beta	29
sp|P51148|RAB5C_HUMAN	Ras-related protein Rab-5C	27
sp|P07900-2|HS90A_HUMAN	Heat shock protein HSP 90-beta	24
sp|P51149|RAB7A_HUMAN	Ras-related protein Rab-7a	14
sp|P62820|RAB1A_HUMAN	Ras-related protein Rab-1A	14
sp|P08238|HS90B_HUMAN	Heat shock protein HSP 90-beta	13
sp|P21926|CD9_HUMAN	CD9 antigen	13
sp|P61026|RAB10_HUMAN	Ras-related protein Rab-10	12
sp|P61106|RAB14_HUMAN	Ras-related protein Rab-14	12
sp|P13639|EF2_HUMAN	Elongation factor 2	7
sp|P61019|RAB2A_HUMAN	Ras-related protein Rab-2A	6
sp|P08962|CD63_HUMAN	CD63 antigen	5
sp|P20339|RAB5A_HUMAN	Ras-related protein Rab-5A	5
sp|Q9HCU0|CD248_HUMAN	Endosialin	5
sp|P51153|RAB13_HUMAN	Ras-related protein Rab-13	4
sp|P14625|ENPL_HUMAN	Endoplasmin	3
sp|Q9UL26|RB22A_HUMAN	Ras-related protein Rab-22A	3
sp|P60033|CD81_HUMAN	cd81 antigen	2
sp|P05362|ICAM1_HUMAN	Intercellular adhesion molecule 1	1

Table 4. Exosomal markers in Sarkosyl soluble fraction.

Proteins deriving from endosomal sorting complexes required for transport (ESCRT) pathway, key mediator for multivesicular bodies biogenesis, are listed in table 5.

Accession	Names	Spectra counts
sp|Q9UN37|VPS4A_HUMAN	Vacuolar protein sorting-associated protein 4A	71
sp|O75351|VPS4B_HUMAN	Vacuolar protein sorting-associated protein 4B	31
sp|P53990|IST1_HUMAN	IST1 homolog	31
sp|P62805|H4_HUMAN	Histone H4	21
sp|Q9UK41|VPS28_HUMAN	Vacuolar protein sorting-associated protein 28 homolog	18
sp|Q9BRG1|VPS25_HUMAN	Vacuolar protein-sorting-associated protein 25	15
sp|P55290|CAD13_HUMAN	Cadherin-13	14
sp|Q9NP79|VTA1_HUMAN	Vacuolar protein sorting-associated protein VTA1 homolog	13
sp|Q9H9H4|VP37B_HUMAN	Vacuolar protein sorting-associated protein 37B	12
sp|Q9NZZ3|CHMP5_HUMAN	Charged multivesicular body protein 5	12
sp|P04908|H2A1B_HUMAN	Histone H2A type 1-B/E	11
sp|Q8WV92|MITD1_HUMAN	MIT domain-containing protein 1	10
sp|Q99880|H2B1L_HUMAN	Histone H2B type 1-L	7
sp|P52758|UK114_HUMAN	Ribonuclease UK114	6
sp|Q86XT2|VP37D_HUMAN	Vacuolar protein sorting-associated protein 37D	6
sp|Q9H444|CHM4B_HUMAN	Charged multivesicular body protein 4b	6
sp|Q7LBR1|CHM1B_HUMAN	Charged multivesicular body protein 1b	5
sp|Q86VN1|VPS36_HUMAN	Vacuolar protein-sorting-associated protein 36	5
sp|O43633|CHM2A_HUMAN	Charged multivesicular body protein 2a	4
sp|Q96H20|SNF8_HUMAN	Vacuolar-sorting protein SNF8	4
sp|Q96QK1|VPS35_HUMAN	Vacuolar protein sorting-associated protein 35	4
sp|Q709C8|VP13C_HUMAN	Vacuolar protein sorting-associated protein 13C	3
sp|Q13268-2|DHRS2_HUMAN	Isoform 2 of Dehydrogenase/reductase SDR family member 2	2
sp|Q96CF2|CHM4C_HUMAN	Charged multivesicular body protein 4c	2
sp|Q96FZ7|CHMP6_HUMAN	Charged multivesicular body protein 6	2
sp|Q9BY43|CHM4A_HUMAN	Charged multivesicular body protein 4a	2
sp|Q9Y3E7|CHMP3_HUMAN	Charged multivesicular body protein 3	2
sp|P16402|H13_HUMAN	Histone H13	1
sp|Q9HD42|CHM1A_HUMAN	Charged multivesicular body protein 1a	1
sp|Q9NRW7|VPS45_HUMAN	Vacuolar protein sorting-associated protein 45	1
sp|Q9UQN3|CHM2B_HUMAN	Charged multivesicular body protein 2b	1

Figure 5. ESCRT pathway proteins identified in Sarkosyl soluble fraction of urine exosomes.

The list of exosome markers for the insoluble fraction was certainly shorter, as shown in table 6.

Accession	Name	Spectra counts
sp|P11142|HSP7C_HUMAN	Heat shock cognate 71 kDa protein	45
sp|Q5VTE0|EF1A3_HUMAN	Putative elongation factor 1-alpha-like 3	33
sp|P08238|HS90B_HUMAN	Heat shock protein HSP 90-beta	11
sp|P50395|GDIB_HUMAN	Rab GDP dissociation inhibitor beta	8
sp|P07900-2|HS90A_HUMAN	Isoform 2 of Heat shock protein HSP 90-alpha	7
sp|Q8WUM4|PDC6I_HUMAN	Programmed cell death 6-interacting protein	7
sp|P13639|EF2_HUMAN	Elongation factor 2	6
sp|P62820|RAB1A_HUMAN	Ras-related protein Rab-1A	4
sp|P14625|ENPL_HUMAN	Endoplasmin	2
sp|P51149|RAB7A_HUMAN	Ras-related protein Rab-7a	2
sp|Q8WUD1|RAB2B_HUMAN	Ras-related protein Rab-2B	2
sp|P61026|RAB10_HUMAN	Ras-related protein Rab-10	1

Table 6. Exosomal markers in Sarkosyl insoluble fraction.

Some of the proteins deriving from ESCRT pathway were found also in Sarkosyl insoluble fraction, as shown in table 7. However the spectra counts are lower compare to the soluble fraction.

Accession	Name	Spectra counts
sp|P53990|IST1_HUMAN	IST1 homolog	7
sp|O43633|CHM2A_HUMAN	Charged multivesicular body protein 2a	3
sp|Q9NP79|VTA1_HUMAN	Vacuolar protein sorting-associated protein VTA1 homolog	3
sp|Q9UN37|VPS4A_HUMAN	Vacuolar protein sorting-associated protein 4A	3
sp|Q709C8|VP13C_HUMAN	Vacuolar protein sorting-associated protein 13C	1
sp|Q7LBR1|CHM1B_HUMAN	Charged multivesicular body protein 1b	1
sp|Q86XT2|VP37D_HUMAN	Vacuolar protein sorting-associated protein 37D	1
sp|Q9Y3E7|CHMP3_HUMAN	Charged multivesicular body protein 3	1

Table 7. ESCRT pathway proteins identified in Sarkosyl insoluble fraction of urine exosomes.

4. DISCUSSION AND CONCLUSION

The presence of exosomes in biological fluids has begun to be exploited as a potential source for disease-related biomarkers. The number of reports regarding mass spectrometry-based proteomic analyses of exosomes is increasing. Furthermore, it is expected that and advances in the field of mass spectrometry hardware and appropriate computational tools will further improve this trend.

One of the objectives of this study was to develop novel approaches for the isolation of urinary exosomes and to expand the existing human urinary exosome database. One of the challenges of using urine as a biological fluid is the presence of high abundant proteins as uromodulin and albumin. In order to overcome this problem, standard methods require a reduction step with 1.3 M DTT [48, 93].

Two exosome isolation strategies were used in this work: first, the standard ultracentrifugation strategy followed by a high concentration of DTT to reduce uromodulin [48, 93]; second the solubilization of exosomes in 1% Sarkosyl coupled with a carbon fractionation, which was a completely new approach. Proteins identified in the samples fractionated using the second method, such as all of the members of ESCRT pathway, enriched membrane-bound vesicle, and endosomal proteins etc., clearly indicated their multivesicular origin. The ESCRT pathway is a group of multisubunit protein complexes that play a central role in the processes of endosomal cargo sorting and MVB formation [106]. However, the function of ESCRT machinery in the formation and secretion of exosome is still unclear [80]. These samples contained also exosomal surface markers such as Alix and TG101, indicating the successfully isolation of EVs. However, some of these proteins were also present in the insoluble fraction – albeit to a much lower extent – suggesting incomplete solubilization of the exosomes by Sarkosyl.

Even though DTT reduction was used to reduce the amount of uromodulin in the 200,000 x g sediments, uromodulin was still one of the most abundant proteins in the exosome preparation probably; due to its sheer abundance, it probably associates with exosomal proteins or exosome membranes and thus is co-sedimented during the centrifugation. In an additional attempt to eliminate uromodulin, we also tried – without success – the modified urinary exosome preparation reported by Fernandez-Llama et al. [93] to recover the entrapped

exosomes in the low-speed pellets. After a low speed centrifugation (17,000 x g) uromodulin sediment, entrapping exosomes. To release exosomes from uromodulin, some papers described a method that uses a high concentration of DTT (1.3 M) [47, 48, 93]. We avoid such a high amount of DTT by using Sarkosyl, whis is amphypathic detergent. Thus, uromodulin remains in the insoluble fraction and most of the remaining proteins are in the Sarkosyl soluble fraction.

In addition to uromodulin, 3.4 - 5.4 grams per deciliter (g/dL). of serum albumin is also present in normal urine and is thus expected to be present as a common contaminant in all our samples and preparation independent of the attempts to remove the uromodulin and its aggregates. Therefore, we used the high abundance of albumin and uromodulin to estimatethe efficiency of Sarkosyl fractionation, calculating the ratio between these 2 proteins in the soluble and insoluble fraction. It is to be expected that the uromodulin and serum albumin levels in urine show biological variation; thus it was expected that the uromodulin and albumin levels will also vary in the exosome preparations from the different urine samples. However, the presence of these two proteins in some samples may explain as to why the total exosomal protein concentration is much higher in some samples than the others.

Besides providing information to understand exosome biogenesis and function, exosome proteomics has brought considerable research interest in finding disease-related biomarkers. Analysis of urine exosomes yielded a series of proteins involved in very important biological processes including but not limited to protein transport, membrane trafficking, metabolic process and signal transduction. Proteomics analysis of urinary exosomes also identified proteins that play important roles in kidney function, such as proteins involved in water, drug, sodium, chloride, proton and glucose transport as well as some potential disease biomarkers currently under investigation.

In summary, this study developed and utilized an innovative method to thoroughly analyze urinary exosomes obtained from normal human urine. This study provides a large set of proteins present in human urinary exosome proteomes and provides a valuable reference for future study.

CHAPTER 2

1. INTRODUCTION

1.1. Monogenoidea

Monogenoidea are a group of largely ectoparasitic members of the phylum Platyhelminthes. They are ectoparasites of fish and, less frequently, of amphibians or reptiles (turtles), that (as adults) range in size from a few micrometers to a couple of centimeters. The group is distributed worldwide and contains an estimated 25,000 species from a range of freshwater, brackish, and saltwater environments [106].

Monogeneans lack respiratory, skeletal and circulatory systems and have no or weakly developed oral suckers. Monogenea attach to hosts using hooks, clamps and a variety of other specialized structures. They are often capable of dramatically elongating and shortening as they move. Like all ectoparasites, monogeneans have well-developed attachment structures: the anterior structures (prohaptor) and the posterior (opisthaptor, or simply haptor), as shown in figure 1. The posterior opisthaptor with its hooks, anchors, clamps etc. is typically the major attachment organ.

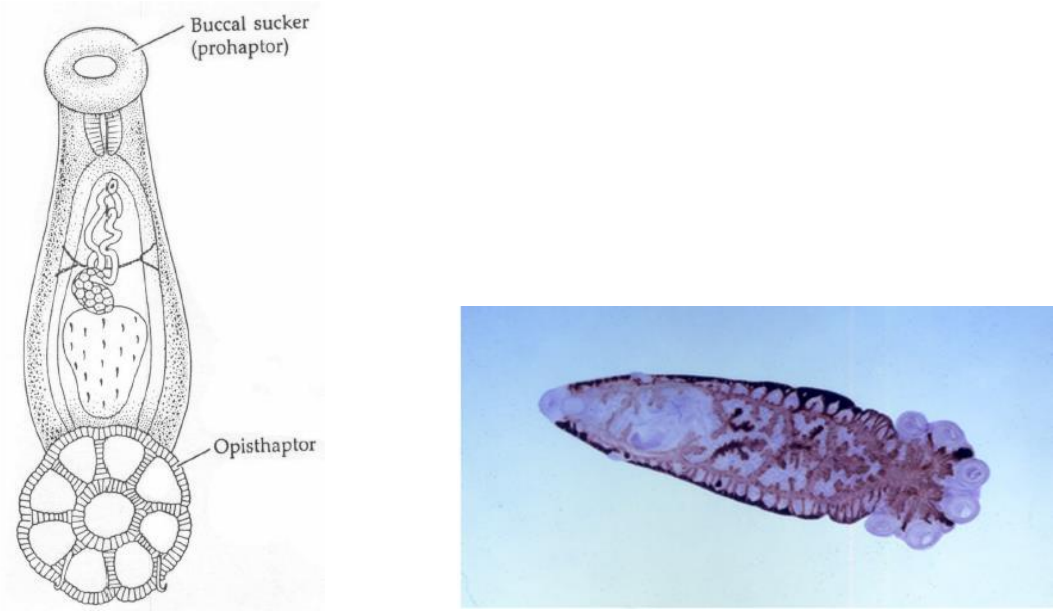

Figure 1. Example of monogenean: *Polyopisthocotylea polystoma*

Like other flatworms, Monogenea have no true body cavity (coelom). They have a simple digestive system consisting of a mouth opening with a muscular pharynx and an intestine with no terminal opening (anus). Generally, they also are hermaphroditic with functional reproductive organs of both sexes occurring in one individual. Most species are oviparous but a few are viviparous. Monogenea are Platyhelminthes and therefore are among the lowest invertebrates to possess three embryonic germ layers—endoderm, mesoderm, and ectoderm. In addition, they have a head region that contains concentrated sense organs and nervous tissue (brain).

1.1.1. Structure of adult Monogenea

The body surface of adults is formed by a non-cilated neodermis which has replaced the ciliated epidermis of the larva. The digestive system is formed by an anterior sucker or suckers, a pharynx and a blind ending caecum, usually with numerous side branches extending into most of the body. In other words, it is a gastrovascular system, combining the functions of a digestive and a vascular system. The excretory/osmoregulatory system consists of numerous flame bulbs connected to capillaries which join to form larger ducts opening through two separate excretory pores in the anterior part of the body. The flame bulbs do not openly communicate with the surrounding tissue, i.e., the system is a protonephridial system [107]. They consist of a terminal cell and a proximal canal cell, whose cytoplasmic processes (ribs) interdigitate to form the filtration apparatus. The structure of an adult monogenean is represented in Figure 2.

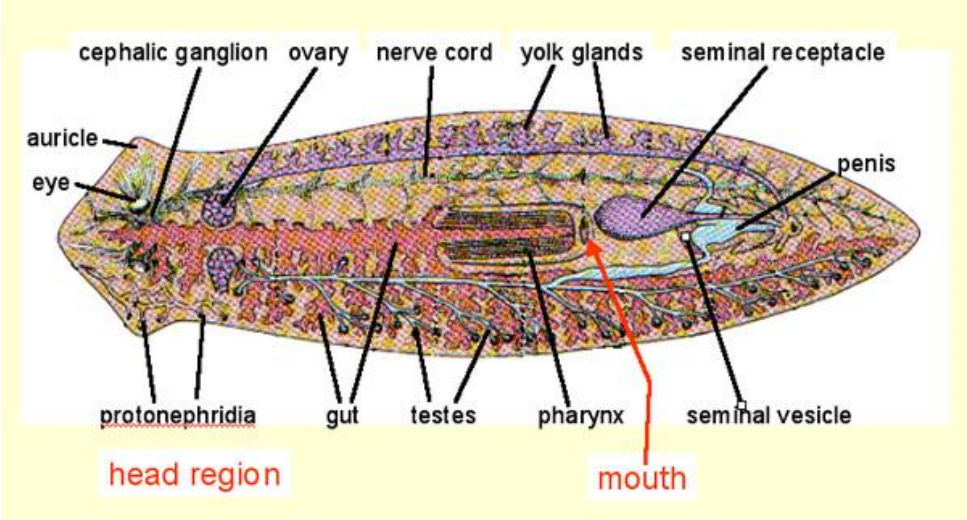

Figure 2. Structure of a monogenean.

All monogeneans are hermaphroditic, i.e., they possess male and female reproductive systems, often of extraordinary complexity. The biology and morphology of species of the genus Polystomoides from the mouth cavity and bladder of freshwater turtles have been particularly well studied [108].

1.1.2. The reproductive tract

In general, Monogenea are hermaphroditic, and they display a complexity of reproductive organs and accessory ducts that dominate the body. Typically, all parts of the male and female systems except the gonads are underlain by a muscular stratum of both circular and longitudinal fibers. The contraction and relaxation of this muscle would seem essential for the orderly process of egg formation, and regulation of this motility presumably resides in the associated nervous system [109].

Little is known about the ultrastructure of the male reproductive system in gyrodactylids. Also, there is much confusion about the anatomy of the female reproductive system. There is no distinct germarium and the most conspicuous feature is the large oocyte located in a chamber situated immediately posterior to a chamber containing the developing embryos.

The structural organisation of the reproductive apparatus in Monogenea is rather similar to flatworm's one. It consists of a male system comprising multiple testes, vas deferens, seminal vesicle and cirrus; and a female system comprising an ovary, oviduct, seminal receptacle, ootype and Mehlis' gland, and uterus, together with vitellaria, vitelline ducts and associated reservoir [110] (Figure 3).

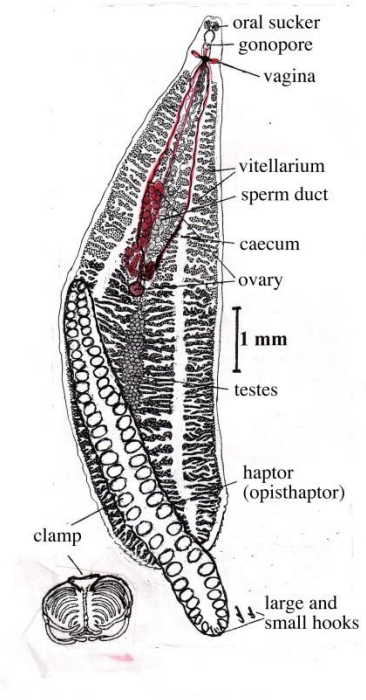

Figure 3. Monogenean reproductive tract

Gyrodactylids are unique among monogeneans in that they are viviparous and protogynous and multiply rapidly by a kind of polyembryony [111]. The uterus may contain up to four embryos one inside each other. Gyrodactylids also have a unique reproductive strategy. Mixed reproductive strategy in which asexual or parthenogenetic reproduction alternates with sex in older, crowded populations may occur [112]. Furthermore, it is likely that viviparous monogeneans have evolved from oviparous ancestors.

Some Monogenea show a genito- intestinal canal. In this respect, Kearn [113] has stated that in monogeneans that lack such an exit route, greater control is necessary in regulating the volume of vitelline cells released from the vitelline reservoir, the corollary being that the acquisition of a more sophisticated means of control may have led to the loss of the genito-intestinal canal.

1.1.3. Life cycle

Monogeneans possess the simplest life cycle among the parasitic platyhelminths. They have no intermediate hosts and are ectoparasitic on fish (seldom in the urinary bladder and rectum of cold-blooded vertebrates). Although they are hermaphrodites, the male reproductive system becomes functional before the female part.

Eggs often have filaments which entangle them in the gills or weed, or which increase their flotation ability. An operculum (egg cover) opens to allow escape of the larva (Figure 4).

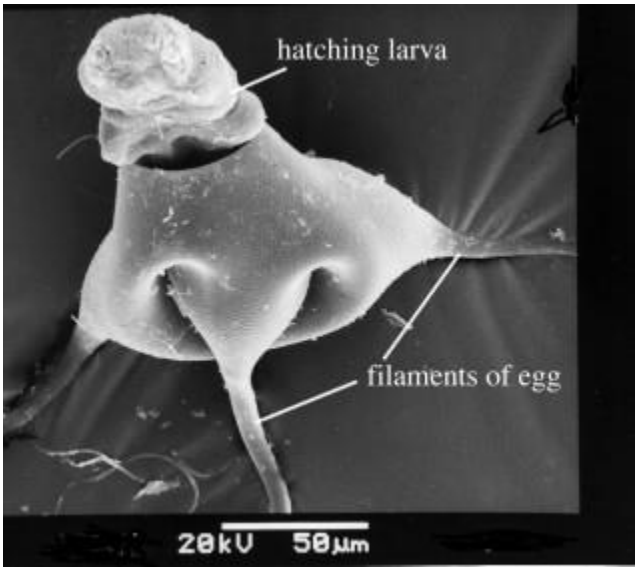

Figure 4. Original scanning electron-micrograph by Klaus Rohde.

The eggs hatch releasing a heavily ciliated larval stage known as an oncomiracidium, as shown in figure 4. The oncomiracidium has numerous posterior hooks and is generally the life stage responsible for transmission from host to host [114]. The growth from oncomiracidium to adult is marked by the haptor increasing complexity.

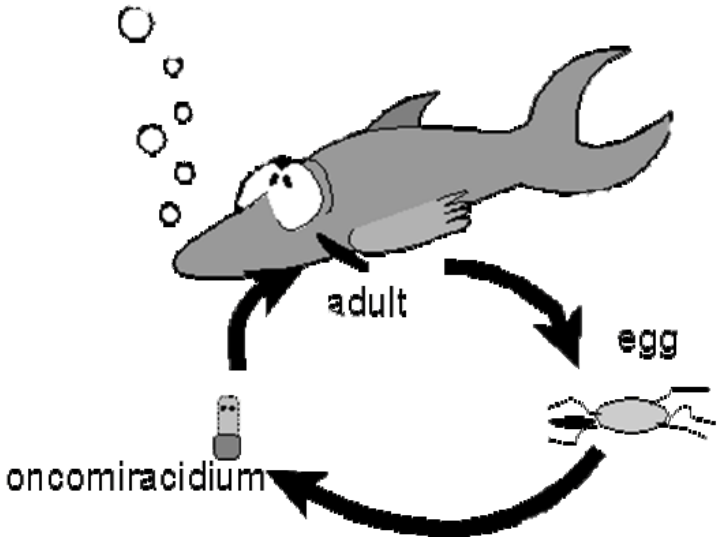

Figure 5. General monogenian life cycle.

The majority are parasites that attach to the external surface of fishes, reptiles and some invertebrates. Few species are internal, and they usually live in cavities that communicate with the external environment (i.e. the bladder). In these species, the eggs form a mass that meander in the water until a fish inhales the eggs during a respiratory action.

1.1.4. Attachment strategies

Monogeneans attach to their fish hosts by two mechanisms. Semi-permanent attachment is mediated by a posterior haptor comprising hooks, suckers, clamps and/or glue(s) [6]. Temporary attachment is controlled by secretion of an adhesive from the anterior end of the worm and is used when the parasites move around on host epidermis (most commonly skin and gills) [23]. Monopisthocotylean monogeneans loop over the host surface like a leech, extending and attaching the anterior end using temporary adhesive, then move the haptor forward and grip firmly with this organ, release the head end and stretch forward to attach again[12]. The secretions involved in this temporary anterior adhesion are strong enough to be

the only form of attachment on host surfaces subject to high shear forces from water currents and the adhesives act instantly and reversibly [10].

At least 4 types of adhesion mechanisms have been observed in multicellular animals:

1) permanent adhesion to abiotic substrate, e.g., barnacles and mussels;

2) transitory adhesion during locomotion on abiotic surfaces, e.g., limpets;

3) temporary adhesion to abiotic surfaces, e.g., starfish;

4) temporary biotic or "tissue" adhesion, which is employed by many members of the Monogenoidea.

The members of Monogenoidea are able to adhere securely and, in most cases, move on the host surface using highly specialized body organs, i.e., the posterior area of the haptor and an anterior adhesive region. While the posterior area of the haptor is specialized for attachment by physical means, due to the vast selection of hooks, anchors, clamps, and suckers, the anterior end usually lacks obvious physical attachment devices. While other flatworms can attach themselves temporarily or permanently mainly by physical means, monogenoids are able to adhere directly to biotic as well as to abiotic substrata using biochemical secretions alone. This is an important feature, considering that fish parasites must deal with physical and chemical properties of the water, as well as host mucus and immune responses. Nevertheless, monogenoids are able to achieve adhesion and move on their hosts with seemingly few chances of being disengaged [115].

In some species, the main adhesion is achieved by the posterior region of the body, and the majority of species attach their haptor to host tissue while moving the head for feeding, thus proceeding along the host surface like an inchworm. These movements are enhanced by the production of an adhesive material from specialized head glands, which create a firm, but temporary, adhesion to many substrates. This adhesive material allows these animals to adhere rapidly, even in the presence of strong water currents or even when the haptor is partially, if not completely, detached. Although the anterior end is well secured, the parasites are still able to disengage rapidly [106].

Since it has been demonstrated that these parasites are unable to swim (with some notable exceptions; [116]), it is clear that their separation from the host would presumably end in

death. The evolution of such adhesive systems has provided different solutions to several tasks, i.e., facilitating the search for a mating partner and allowing the movement to another host or, eventually, to better sites on the host surface in order to feed, grow, and reproduce [117].

1.1.5. Interaction between Monogenea and the host

The selection of a certain host species by an ectoparasitic monogenean must be governed mainly by factors in the host surface (Figure 6).

Buchmann et al., [118] suggested that chemical stimuli emitted from the host are necessary to attract the parasites and even initiate certain behavioural and physiological changes in the parasite. In addition, anatomical structures of certain host surfaces are likely to show higher compatibility with some parasite attachment mechanisms. In order to reproduce satisfactorily the monogenean must feed on host material which can be absorbed and used for production of eggs or larvae. This means that the feeding system (mouthparts and pharynx) and digestive apparatus (including enzyme array) of the parasite must be equipped to cope with the structures and molecules in the host surface [118, 119].

Investigations conducted up until now point to the fact that host specificity among monogeneans is governed by a number of dynamic interactions [118]:

The parasite is able to recognise host molecules emitted over short distances.

When contacting the host, substances present in parasite and host must be compatible.

The anatomical state of the substrate must fit the attachment structures of the monogenean.

Following attachment successful propagation of the parasite depends on appropriate host stimuli perceived by the parasite.

Nutritive host material must be recovered and utilised by the monogenean and translocated for productive purposes.

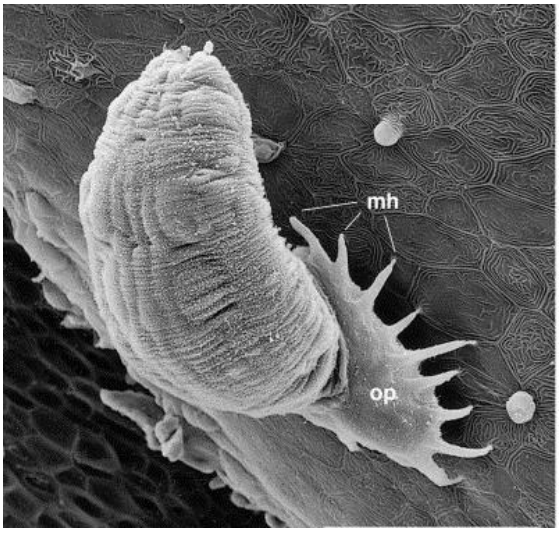

Figure 6. Example of monogenean (*Gyrodactylus salaris*) attached to the host by opishaptor, op. The image shows 16 peripheral marginal hooks, mh [120].

To comply with all these demands for performing a satisfactory life cycle, the monogenean parasite needs to avoid or exploit the various immune mechanisms used by teleosts to combat invading organisms.

Monogeneans comprise both oviparous and viviparous organisms which diversify the various types of host-seeking strategies. Thus, some oncomiracidia hatching from eggs show vivid and elegant movements when swimming through the water [118, 119]. These free-swimming larvae are able to seek the host actively over short distances. In contrast, viviparous parasites, such as most gyrodactylids, have to rely on more restricted transmission mechanisms: direct contact between fish, indirect contact mediated by the substrate or more or less random transfer of the parasite with water currents.

1.1.6. Site selection of Monogeans

Numerous studies have demonstrated that monogeneans are selective in their choice of attachment site [118]. This site selection can probably be influenced by both environmental and host-related factors and the causative mechanisms may vary between parasite types. Some monogeneans preferentially select either gills or skin. However, even a relatively simple anatomical unit such as the gill apparatus can be subdivided into numerous microhabitats.

Studies concerning the microhabitat selection of gill monogeneans have shown unequivocally that even congeneric monogeneans in many cases select different microhabitats, also in single species infections [121]. Some species attach primarily to the distal part of gill filaments, others take a proximal position. Also, the anterior versus posterior gill arches in addition to the outer and inner hemibranches seem to signal differently to the parasites. The exact explanation for this selection remains enigmatic. Environmental causes may play a role because differences in water currents through the gill apparatus could influence the settlement of oncomiracidia in various gill habitats.

1.2. Neobenedenia girellae

Many parasites are serious pathogens in intensive aquaculture in Japan [122]. One such parasite, Neobenedenia girellae (Figure 7), a capsalid monogenean, is problematic because it has broad host specificity and can cause high mortality in host fishes [122]. It is considered that N. girellae was introduced from China since imported greater amberjack fry was infected with this parasite (prevalence up to 70.0 %) [122].

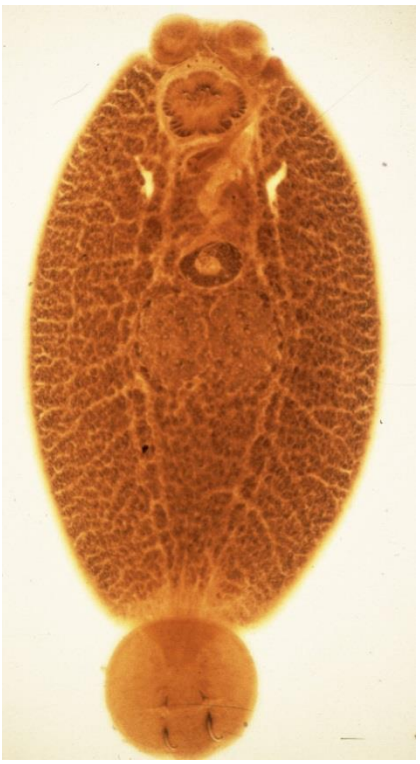

Figure 7. A stained specimen of *N. girellae.*

Neobenedenia girellae developed rapidly on Japanese flounder reaching sexual maturity in 10-11 days at 25℃ from oncomiracidia. Neobenedenia is unique in that they do not have a vagina [123].

1.2.1. Structure

Adult N. girellae measure up to 6 mm in length. They are oval parasites characterized at the anterior end by disk-shaped adhesive and feeding organs, called prophator and the posterior haptor, as all monogeneans. These structures assist in anchoring the parasite to the fish epidermis.

The mouth and pharynx are located on the mid-ventral surface. N. girellae feeds on muvus and epidermal cells and displays extracorporeal digestion; the parasites deposit digestive enzymes released from the pharynx on the fish surface and then suck up the resulting digest into the intestine.

1.2.2. Life cycle

The life cycle is direct: only the fish host is involved. Diamond-shaped eggs are produced by hermaphroditic adults on mucus strings. The eggs may detach from the adult parasite and fall to the bottom and hatch, or they may remain caught up in the excess mucus produced by the fish and hatch close to the epidermis. Likely, urea and ammonia excreted by the skin of the fish is a hatching stimulus for the enclosed larva [124]. In few day oncomiracidia are released, these resemble ciliate protozoans in size and shape.

The free swimming life of an oncomiracidium is short (4 – 36 hours), thus it quickly searches out and attaches to the host's epidermis through use of sticky cephalic glands and the developing haptor. Once attached, the oncomiracidium sheds its ciliated cells and develops into the adult.

1.2.3. Cement structure of the Capsalidea *Entobdella soleae*

The area available for bonding between parasite and host is greatly enlarged by the tegumental microvilli of the parasite's adhesive pad and by the roughly concentric arrangement of furrows in the flat surface of each host epidermal cell. Transmission electron microscopy sections through the adhesive pad of *Entobdella soleae* attached to the skin of its host reveal a layer of intervening cement, 4 or 5 µm in thickness (Fig. 8A and 8B) [117]. The cement is in intimate contact with the surfaces of the parasite and the host, infiltrating between the parasite's tegumental microvilli and penetrating into the host's epidermal furrows (Fig. 8C) [117].

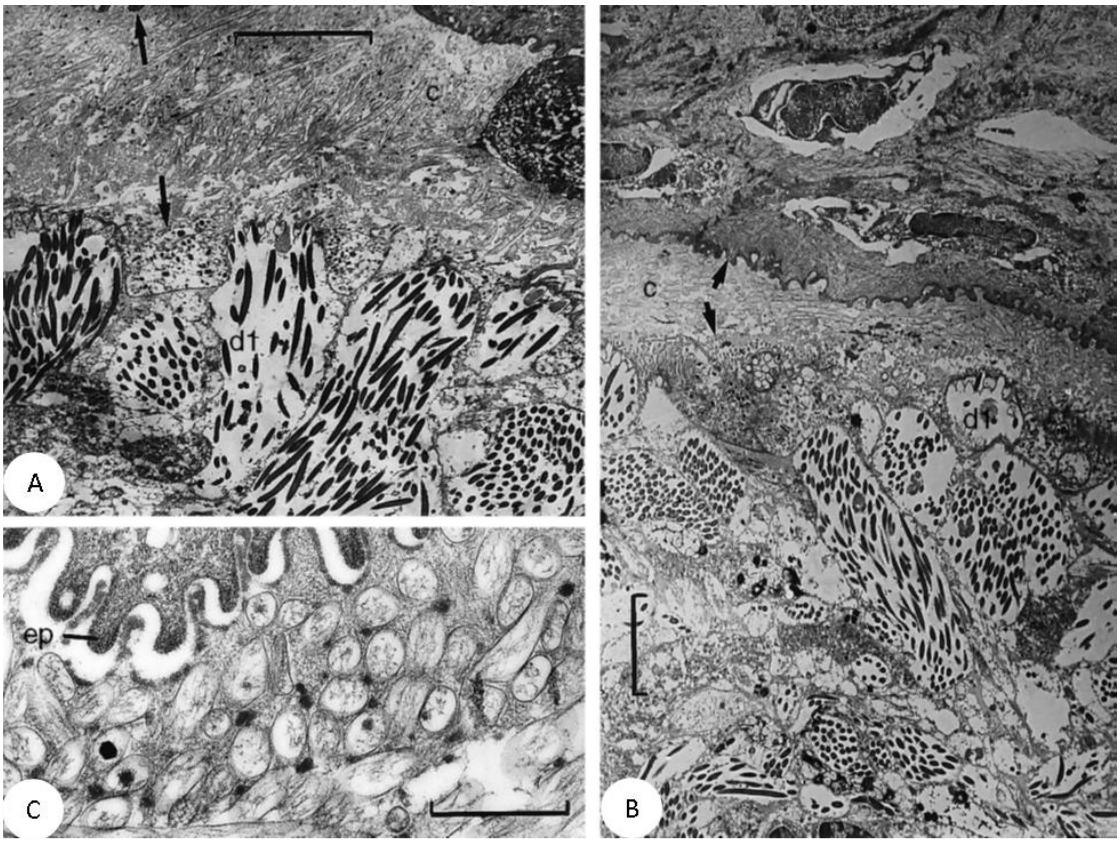

Figure 8. Transmission electron micrographs of A) and B) sections through adhesive pads of Entobdella soleae attached to the epidermal surface of the host (Solea solea). (top) and parasite (below). C) a section through the cement between parasite and host. ep, Epidermis of host; c, Cement. Arrows indicate surface of host [117].

The thin layer of cement matrix immediately in contact with the host's epidermal surface is sometimes more electron-dense than the rest of the cement (Fig. 8), indicating perhaps some kind of chemical interaction between the cement and exudate from the host's surface. No spheroidal secretory bodies were found in the cement [117].

1.2.4. Glands

Adhesive is typically produced by glands located beside the pharynx or, more specifically, in the anterolateral and/or posterolateral regions of the animal. A maximum of 3 different types of

head glands may be involved in the creation of the adhesive matrix. These glands are identified as G1, G2, and G3, and produce secretions known as S1, S2, and S3, respectively [106].

A single duct from each gland delivers the product to a single opening in the anteroventral surface of the animal. These ducts may reach the ventral surface of these animals in at least 2 different ways. First, duct endings do not open directly to the surface of the ventral tegument but instead open into a sac in which different secretions may mix in a reservoir that is not always present (Fig. 1). The adhesive matrix is expelled via a muscular (usually circular) opening. This method is typical of acanthocotylids, dactylogyrids, gyrodactylids, and some monocotylids. The epithelium of the sacs is generally microvillous, which increases the contact surface with the adhesive [125]. Second, duct openings are permanently exposed, piercing through the tegument of pads, are more or less defined from the body shape of the animal, and are usually covered by microvilli, which is typical of capsalids.

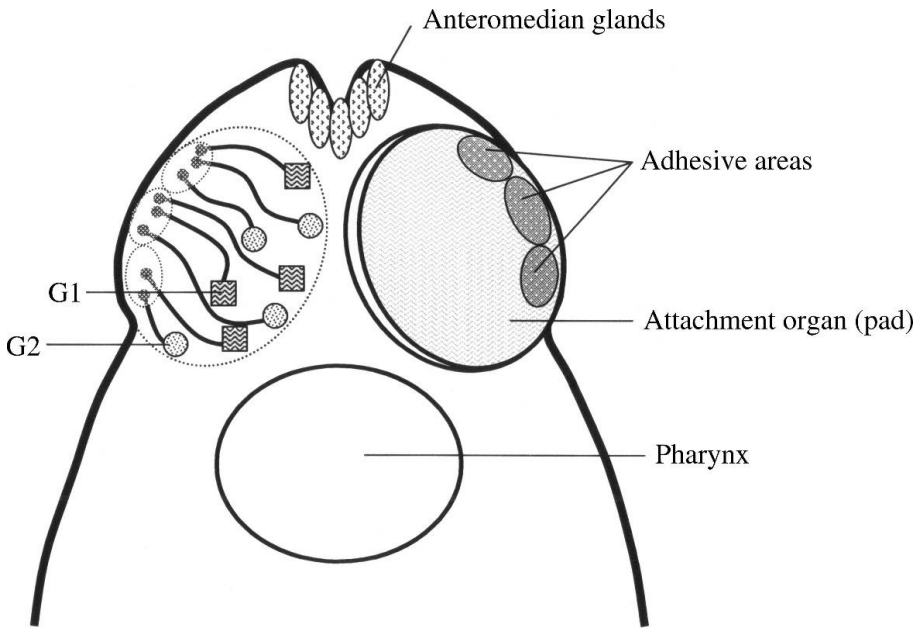

Figure 9. Schematic representation of a cephalic region of a Capsalidae.

The more common structure of capsialids, including N. girellae, is characterized by at least 2 glands, namely, G1 and G2. However, its structure has poorly been described. One of the most

intensively studied parasites with 2 secretions is the capsalid Entobdella soleae [117]. This parasite possesses 2 kinds of cells, G1 and G2, plus a third type of anteromedial gland, which are not involved in adhesion.

In some other capsalids, like Benedenia lutjani [126] and Benedenia rohdei [111, 126, 127] are somewhat different, having 2 characteristic disclike pads (Fig. 9). These pads are not simple areas of flattened tegument like those seen in E. soleae, but they are well defined [126]. In B. lutjani, adhesion is achieved at 3 areas on the top edge of these discs, where the S1 and S2 ducts terminate. The discs function like suckers and can help in securing the anterior end of the animal more firmly, thus supporting the secreted adhesive. The anterior adhesive organs of B. rohdei are microvillous, while they are free of microvilli in B. lutjani [126].This characteristic is well conserved among the Capsalidae.

There are 3 different adhesive areas and nonadhesive anteromedial glands in the Entobdellinae. Benedenia lutjani and B. rohdei achieve very firm attachment to the substratum with just 1 of the 2 pads [115].The mechanism used by most capsialids to detach from the substratum has not been described. However, since so many features appear to be common among the capsalids, Whittington and Cribb [115] suggested that detachment is achieved by tegumental secretion from the attachment discs. Apart from the capsalids, almost all of the other species possessing 2 glands have variable numbers of circular openings, most of which are also eversible.

1.3. Adhesive secretions

In the marine environment, attachment mechanisms developed by animals usually rely on highly viscous or solid adhesive secretions, which all contain specialized proteins. Functional convergences are noted among marine animals, particularly in terms of the type of adhesion used: permanent, temporary, or instantaneous. Although marine adhesive proteins from non-related organisms do not present any sequence homologies, molecular convergences have been recognized, and some adhesive motifs have been found to be shared by phylogenetically different animals. Also the adhesion of mussels has been thoroughly investigated and DOPA has

long been known as one such motif [128]. Indeed, the mussel byssus is a remarkable attachment structure that is formed by injection molding and rapid in situ hardening of concentrated solutions of proteins enriched in the catecholic amino acid 3,4-dihydroxy-L-phenylalanine (DOPA). Fe3+, found in high concentrations in the byssus, has been speculated to participate in redox reactions with DOPA that lead to protein polymerization, however direct evidence to support this hypothesis has been lacking [128].

After isolation of the first adhesive protein, Mepf-1, which contains DOPA (3,4-dihydroxy-L-phenylalanine), ten further proteins have been isolated [129]. One of them is precollagen D which possesses a central collagen domain flanked by two fibroin-like domains with sequences similar to spider silk fibroin. This protein is found in spiders' drag line [130] and also in the silkworm Bomyx mori [131]. A similar protein has been found in sea urchin [132]. Using antibodies raised against recombinant precollagen D from the mussel Mytilus galloprovincialis, we could identify a protein with adhesive properties in sea cucumber Cuvierian tubule extracts.

Now, another modified amino acid, phosphoserine (pSer), is emerging as an important motif in biological adhesives.

The diversity of adhesion mechanisms is therefore huge, although some common principles have evolved independently in different biological lineages. For instance, three types of adhesives may be distinguished depending on their mode of operation and their composition [115, 133]. Permanent adhesion involves the secretion of an adhesive that hardens with time and is characteristic of sessile organisms that remain in the same place throughout their life (such organisms have representatives among sponges, hydrozoan cnidarians, cirripede crustaceans, bivalve molluscs, tubicolous polychaetes, bryozoans, or tunicates). By contrast, nonpermanent adhesion allows simultaneous adhesion and locomotion, thus allowing adult organisms to graze, hunt, or locate a mate, and larval forms to explore immersed surfaces prior to permanent adhesion. Some organisms such as gastropod molluscs attach by a viscous film they produce between their body and the substratum, creeping on this film which is left behind them as they move (transitory adhesion). Others, such as echinoderms, attach firmly but only temporarily to the substratum, being able to attach and detach repetitively (temporary adhesion). Finally, instantaneous adhesion is characterized by an explosive release of adhesive,

allowing a very fast formation of adhesive bonds. Indeed, as opposed to all other adhesion types in which the adhesive material is secreted through exocytosis, [134, 135] the release of instantaneous adhesives destroys the adhesive organ which may be used only once. Two types of organs are known to act as instantaneous adhesive organs: the ctenophore tentilla (whose secretion functions in prey capture [136]) (figure 10A) and the Cuvierian tubules of sea cucumbers (that entangle imprudent predator [137]) (Figure 10 B).

Figure 10. Picture of A) a ctenophore; B) a sea cucumber

Also the adhesive setae of reptiles and insects have long fascinated biologists [138, 139], and interest in these structures has increased following recent demonstration of the adhesive properties of single, detached setae [139]. An understanding of the mechanism [139, 140] of adhesion of these structures is being developed. Proof of principle that artificial fibrillar arrays reminiscent of setae can, in fact, confer greater adhesive energy than unpatterned material has been published [141]. However, the simple polymer fibrillar arrays fabricated thus far fall far short of the capabilities of the biological examples. Rizzo et al., [138] have undertaken a study of the structural and material properties of gecko (Figure 11) setae to better understand the relationship between these properties and their role in reversible adhesion.

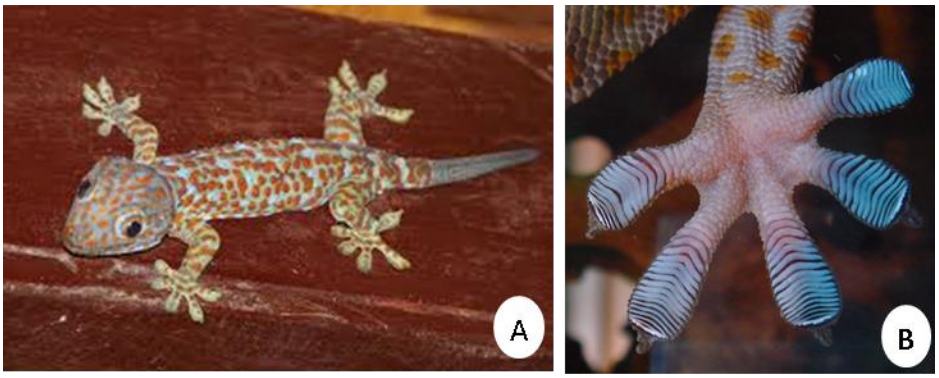

Figure 11. Picture of A) Tokay gecko Gekko gecko and B) its foot

Reptilian setae, small bristles often mistakenly referred to as hairs, exist in two types. The most studied, typified by those present on the toe lamellae of the Tokay gecko Gekko gecko (figure 12), are multibranched, hierarchical structures of 80–100 µm in length.

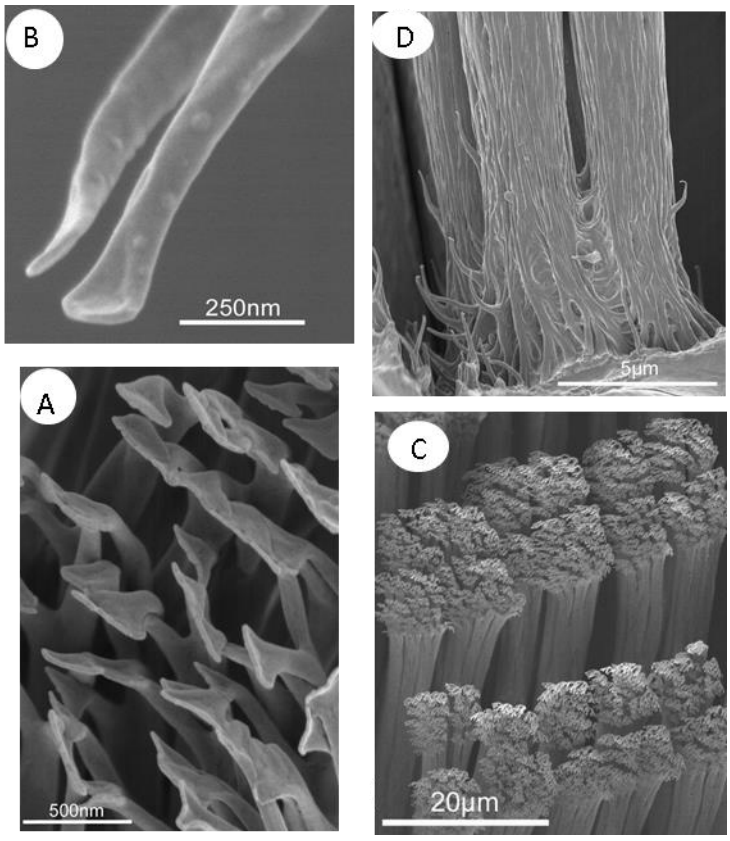

Figure 12. Scanning electron microscopy of the spatulate terminal elements of Gekko gecko setae. Detached setae were imaged to show (a) an array of spatule at the tip of a seta; (b) details of individual spatula. (c) Setae attached to a section of toepad; and () detached setae.

The second type, such as those found on the adhesive pads of anole lizards such as Anolis carolinensis, are smaller (ca 20 μm in length) and unbranched. In all cases, arrays of many millions of such setae decorate the surface of the epidermal adhesive pads of these reptiles. The hypothesis that setae are composed of β-keratin protein was assumed long ago, however only recently biochemical evidence has emerged in support of this thesis [142, 143]. β-Keratins are present only in avian and reptilian epidermis,. In un antiserum raised against an avian scale β-keratin was found to exhibit immuno cross-reactivity with gecko setae [142].

1.3.1. Biochemical features of adhesive secretions in monogeneans.

The adhesive secretion from monogenean, in particular from larvae, was first analyzed by Hamwood et al. [144], who described the presence of a substantial amount of proteins .

Amino acid analysis of secreted adhesive material (SAM) from seven species of monogeneans was conducted by Hamwood [144]. Across the seven species of monogeneans, generally there were high levels of glycine (12-19%) and alanine (6-14%) whereas tyrosine and methionine were found at low levels (1-3% and 0-2% respectively), and histidine was often absent (Table 1). However, amino acid content varied across species. Some differences were apparent when species were divided along family lines.

Only two of the 16 amino acid residue values (serine and arginine) for the capsalid, *E. soleae*, show an overlap with the range of values seen for the six monocotylid species. Also, *E. soleae* showed a noticeably lower amount of leucine and phenylalanine (Table 1) than five of the six monocotylid species. Within the monocotylids, Merizocotyle australensis differed from the other five species in having higher amounts of serine, arginine and proline, while displaying lower amounts of leucine and phenylalanine. If this species is excluded, the five remaining monocotylids show a consistent amino acid composition, except that Monocotyle helicophallus

has a lower level of serine (1%) than the other four monocotylid species. Both EAM and SAM contain significant amounts of the acidic residues Asx (asparagine or aspartate: 10%) and Glx (glutamine or glutamate: 14-18%) whereas these are absent in body tissue. EAM and SAM also contain slightly more glycine (12% vs. 8% in the body). Body tissue has a much higher content of alanine (43% vs. 6- 11%) and tyrosine (10% vs. < 3%) than either EAM or SAM. However, EAM was more similar to body composition for histidine, arginine, proline, methionine and lysine.

	Monogeneans							**Starfish**	**Barnacles**		**Limpet**
Amino acid	**Es**	**Ma**	**Mi**	**Ms**	**Mh**	**Tr**	**Nr**	**Ar**	**Be**	**Mr**	**Ll**
Asparagine or aspartate (Asx)	98	70	53	31	55	84	54	118	57	91	127
Serine	75	149	82	84	10	80	76	76	83	99	90
Glutamine or glutamate (Glx)	144	101	57	130	69	83	61	102	64	92	115
Glycine	117	193	189	187	161	152	181	97	66	79	80
Histidine	16	5	0	0	1	3	0	21	23	13	10
Arginine	43	61	39	39	43	37	41	41	75	56	33
Threonine	53	4	24	29	46	34	31	78	57	71	116
Alanine	64	103	139	104	80	97	117	62	49	75	52
Proline	45	67	54	58	53	53	56	61	86	49	79
Tyrosine	27	14	16	11	15	14	16	27	75	42	18
Valine	102	55	45	58	47	48	44	67	62	73	72
Methionine	17	0	8	11	2	11	9	17	11	16	11
Lysine	73	42	41	29	39	39	42	56	53	57	59
Isoleucine	40	39	31	29	33	33	33	45	69	53	52
Leucine	61	74	123	112	125	121	126	61	110	83	51
Phenylalanine	26	31	101	112	129	111	113	38	53	37	26
Cysteine or half-cystine	?*	?*	?*	?*	?*	?*	?*	32	6	16	20

Table 1. Amino acid composition of secreted adhesives from different marine organisms. Organisms included are the monogenean flatworms Entobdella soleae (Es), Merizocotyle australensis (Ma), M. icopae (Mi), Monocotyle spiremae (Ms), M. helicophallus (Mh), Troglocephalus rhinobatidis (Tr) and Neoheterocotyle rhinobatidis (Nr), the starfish echinoderm Asterias rubens (Ar), the barnacles Balanus eburneus (Be) and Megabalanus rosa (Mr) and the limpet Lottia limatula (Ll) [144].

SAM of Entobdella soleae does not contain polysaccharides, including acid mucins, or lipids. It is also highly insoluble. The SAM has a soft consistency. Some of these features, such as insolubility, softness and protein content, are similar to the temporary adhesive used by the starfish Asterias rubens, but carbohydrate and lipid are also present in the adhesive of this echinoderm [145].

SAM from the monogenean adhesive is a porous material comprising a network of strands; the starfish "print" has a similar, spongy appearance but this results from raised ridges of homogeneous material.

Consequently, the biochemical characterization of this temporary adhesive material is important also to develop new pharmacological strategies for the eradication of these parasites from aquaculture. Moreover, monogenoidean adhesive properties suggest the existence of new biochemical features, namely protein based molecular mechanisms leading to very rapid, strong but fully reversible protein-based adhesiveness in a water environment, which make well worth to investigate their structure.

1.3.2. Adhesive secretion of *Neobedenia girellae*

There has been only very few studies on the temporary adhesive materials from platyhelminth, mainly Capsalidae, which present very similar highly insoluble adhesive secretions [146]. The only partial characterization at the molecular level, up to now, was carried out by Hamwood et al. [144] on the monogenean capsalid parasite *Entobdella soleae*, which shares several morphological and functional features with *N. girellae*, including using hooks, ventosae and adhesive secretions to attach/detach from hosts [106]. Compared to sea star adhesive, which is the other temporary glue chemically characterized, the adhesive material presents a different amino acid composition and lacks carbohydrates and lipids. I suggested the presence of keratin and neurophysin as identified via an amino acid content search of data bases but no further attempt was undertaken so far to disclose the protein composition of the glue [144].

1.3.3. De-adhesion

In monogenean parasites, there is some evidence that the tegument of the worm may be involved in de-adhesion [117]. As starfish display a somewhat similar adhesive system, the models for de-adhesion proposed for these echinoderms may be applicable. Thomas and Hermans suggested in 1985 that de-adhesion occurs via secreted glycosaminoglycans that competitively displace the glue secreted by the tube-feet of starfish. Heparin, a glycosaminoglycan (highly acidic polysaccharide), prevents adhesion by the starfish Leptasterias hexactis [144]. Adhesion was not completely inhibited by heparin in the two monogenean species tested here, but some lessening of adhesion occurred. Perhaps lectin probes may clarify the presence or absence of glycosaminoglycans on newly detached specialized tegument of the anterior adhesive areas of monogeneans.

Flammang et al. present immunocytochemical data that support a second model for de-adhesion in starfish [145]. Here, an enzyme releases the fuzzy coat from the surface of the tube foot, leaving the fuzzy coat on the glue [145]. It is possible that the anterior adhesive area tegument of monogeneans may secrete an enzyme which is responsible for de-adhesion or perhaps the second, granular secretory bodies are involved, although Kearn et al., found evidence to dismiss the latter possibility, supporting a role for the tegument [117]. Certainly the anterior adhesive area tegument contains different inclusions from the general body tegument [115, 119]. To fully understand the de-adhesion process in monogenean flatworms, it will be necessary to pursue further electron microscopic studies of the anterior adhesive areas before and after release of the glue as has been done for E. soleae by Kearn et al., [117].

1.3.4. Biomedical relevance

Substantial impetus behind understanding these adhesive secretions are the potential technological applications that can be derived from their knowledge. These applications cover two broad fields of applied research: design of water-resistant adhesives and development of new antifouling strategies. In this context, echinoderm adhesives could offer novel features or

performance characteristics for biotechnological applications. For example, the rapidly attaching adhesive of Cuvierian tubules, the releasable adhesive of tube feet or the powerful adhesive of asteroid larvae could each be useful to address particular bioadhesion problems [133].

1.4. De novo sequencing

De novo is Latin for, "over again", or "a new". A popular definition for "de novo peptide sequencing" is, peptide sequencing performed without prior knowledge of the amino acid sequence.

The introduction of chemical protein sequencing by Edman degradation [147] in the 1950s was a milestone in the development of protein research. For identification of Edman degradation products, mainly LC was used. Intermittently, MS was introduced as alternative method to LC with optical detection. This was achieved in combination with the early soft ionization techniques chemical ionization [148], field desorption [149], and fast atom bombardment [150, 151]. Later, the gradual refinement of MS/MS techniques created the basis for peptide sequencing by MS, which finally gave fast access to multiple internal protein sequences by the analysis of proteolytic peptides. Within the last two decades, protein sequence determination by MS/MS

became more and more powerful, a development driven mainly by the improvements of LC techniques in combination with ESI [152]. The advantages of MS/MS techniques with respect to speed, sensitivity, and applicability to complex peptide mixtures gradually led to the replacement of Edman techniques by LC-MS/MS. For these reasons, tandem mass spectrometry (MS/MS) is emerging as the most reliable tool to identify proteins. There are now several configurations of mass spectrometers that provide MS/MS data with sufficient mass accuracy to deduce peptide sequences of enzymatically digested proteins from either low-energy collisionally induced (CID) or high collisional dissociation (HCD) MS/MS spectra. However, deducing peptide sequences from raw MS/MS data is slow and tedious when performed manually. Instead, the most popular approach is to search databases of known genomes with

the uninterpreted experimental MS/MS data. A number of such approaches have been described, the most popular being Mascot [153] and Sequest [154]. These methods are effective but often give false positives or incorrect identifications. Searching databases with masses and partial sequences (sequence tags) derived from MS/MS data give more reliable eesults [155]. For unknown genomes, de novo sequencing must be carried out in order to obtain sequences or partial sequences. Full sequences can then be obtained by cloning the gene of interest.

The deduction of amino acid sequences from MS/MS spectra is dependent on the quality of the data and further complicated by poor fragmentation and inaccuracies due to mass shifts caused by drifts in temperature and other instrumental parameters. To aid the assignment of sequences a number of chemical techniques have been developed to favor the formation of more stable ‘y’ or ‘b’ ions [156, 157]. A number of algorithms and software packages have been reported for the deduction of protein sequences from MS/MS data [158-164]. One software package developed independently, Lutefisk, has gained a lot of attention [161, 162]. Most of these software packages, including Lutefisk, use a graph theory approach. The spectrum is first translated into a ‘spectrum graph’ where nodes in the graph correspond to peaks in the spectrum and two nodes are connected by an edge if the mass difference between the two corresponding peaks is equal to the mass of an amino acid. The software then attempts to find a path that connects the N and C termini, and to connect all the nodes corresponding to the y ions (or b ions), as shown in Figure 13.

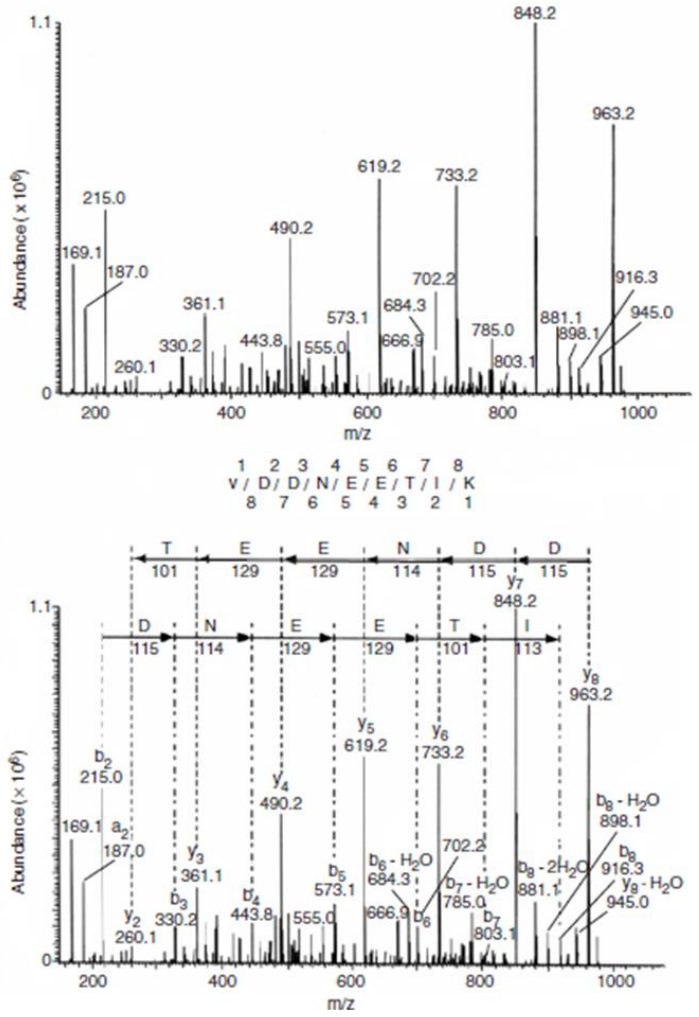

Figure 13. Example of (A) unannotated and (B) annotated MS/MS spectrum [165].

For this study, we used a software called PEAKS, for de novo sequencing of peptides from MS/MS data. PEAKS performs de novo sequencing directly from the MS/MS data and therefore does not rely on a protein database. It computes the best possible sequence among all possible amino acid combinations. Analogous approaches have been described, but were computationally inefficient and abandoned [163, 164]. Instead, PEAKS relies on a sophisticated dynamic programming algorithm and mathematical model to perform the computation efficiently. Indeed, PEAKS computes peptides whose ions correspond to as many high abundance peaks in the spectrum as possible.

The approach taken in PEAKS can be summarized into four steps, as described by Ma et al. [166]:

1) Preprocessing,

(2) Candidate computation,

(3) Refined scoring,

(4) Global and positional confidence scoring.

The first step consists of preprocessing of the raw MS/MS data. This involves a new method for noise filtering and peak centering, as well as deconvolution of the doubly and triply charged species to singly charged ions. This step is very important for the interpretation of MS/MS data by PEAKS because optimal preprocessing of data is a crucial step for de novo sequencing by MS/MS.

The second step, candidate computation, is the critical step in which the 10 000 best sequences of all possible combinations of amino acids for a given precursor ion mass are computed. For this computation, the a, b, c, x, y and b/yions are considered. The basic assumption of this model is that the greater number of high abundance peaks that are matched by those ions of a sequence, the more likely the predicted sequence is the correct sequence.

In the third step, each of the 10 000 candidates is reevaluated by a more stringent scoring scheme, and the best candidates (the number can be specified by users) under the new scoring scheme will be outputted. In this refined rescoring step, ion mass error tolerance is stricter. Finally, a recalibration of the data is performed to account for minor deviations in the MS/MS data. This recalibration method is similar to that described by Taylor et al. [162]

In the last step, PEAKS computes a confidence score for each of the top-scoring peptide sequences. The refined scores can be seen as non-normalized measures of the likelihood of correctness for each peptide, and the distribution of scores gives a measure of the overall probability of successful sequencing. Finally, the positional confidences for each residue are derived from consensus among the globally top-scoring sequences.

The purpose of this study was to identify the proteins present in the adhesive material of the capsalid N. girellae by a proteomic approach based on de novo sequencing and data base

search to overcome the lack of information concerning the genome of these parasites. Glandular secretions were obtained by a new method, set up in our laboratory, based on the electrical stimulation of the parasites.

2. MATERIALS AND METHODS

2.1. Parasites

N. girellae parasites were collected from fish belonging to the Pomacanthidae family. Parasites were removed using a buffer solution with a lower ionic strength (20 g/L NaCl) and maintained in a 35 g/L saline solution. Secreted material was collected using electrostimulation of parasites. Electrostimulation was carried out in our laboratory in an Ionoptix, by subjecting *N. girellae* parasites in a 50% PBS solution to a 40 V electric field with a 2 Hz frequency, stimulating the release of adhesive material. Secreted material was collected in test-tubes. This collection procedure, which does not involve mechanical actions to detach parasites from host and test-tube surface, allows excluding the presence, in the secretion, of contaminating material from the fish and the parasite surfaces. The idea of using electrostimulation to obtain head gland secretion was drawn from works carried out on scorpion poison [167] and the method has been adapted to be applied to Monogenoidea.

2.2. Collection of salivary extracts, solubilization and separation of samples by 2-DE

The secretion of 30 parasites was collected in a test tube and the SDS soluble (SDS-S) and insoluble (SDS-I) components were separated by centrifugation at 13,000 ×g following addition of 80 µL 2% SDS, 5 mM beta-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8 and sonication for 5 min at room temperature. The supernatant (SDS-S) was mixed with 80 µL urea buffer (7 M urea, 2 M tiourea, 4% CHAPS, 40 mM Tris). SDS-I was solubilized in 100 µL of the same urea buffer by sonication; complete solubilization was verified by the absence of visible pellet following centrifugation for 5 min at 13,000 rpm. Total protein content, determined by the Bradford method, was equal to 8 and 32 µg in SDS-S and SDS-I, respectively.

Proteins were reduced and alkylated as described in [168]. Following precipitation in anhydrous acetone/methanol (8:1, v/v) at - 20 °C, pellets (SDS-I and SDS-S) were suspended in the 2-DE sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% Resolyte 3.5–10 NL, Bromophenol

Blue), sonicated for 1 min and centrifuged. No visible pellet was detected in the samples. 2-DE was performed as follows: for the first dimension, the material was loaded by sample cups onto rehydrated IPG-strips with a non linear 3-10 pH gradient (110 mm, Amersham Biosciences, Cologno Monzese, Italy). IEF was carried out at 14 °C, 19,667 V total voltage, for 6 h and 10 min. Before the second dimension, the strips were rinsed with buffer (6 M urea in 0.375 M Tris–HCl pH 8.8, 2% SDS, 20% glycerol, Bromophenol Blue). The second dimension was performed on a homemade 11% SDS-polyacrylamide gel (8.5 × 6.5 × 0.15 cm) at 20 mA/gel constant current. After running, the gel was submitted to a silver staining protocol compatible with trypsin in-gel digestion followed by mass spectrometry analysis. Silver-stained gels were scanned using an ImageScanner (EPSON 1660 densitometer, Bruker Daltonics, Milano, Italy). Analysis of 2-D gel images was accomplished using PDQuestTM Advanced 2-D Gel Analysis Software version 8.0.1 (BioRad Laboratories, Hercules, California, USA).

2.3. Protein identification by tandem mass spectrometry and de novo sequencing

For protein identification, each 2-DE spot was excised, dried, soaked with ammonium bicarbonate 0.1 M and digested overnight with trypsin (sequence grade, Sigma-Aldrich) at 37 °C using a protease: protein ratio (1:10). Tryptic digests were extracted with 50% ACN in 0.1% TFA, desalted/concentrated on a μZipTipC18 (Millipore) using 50% ACN in 0.1% formic acid as eluent, concentrated using Savant Speed Vac (Thermo Fisher Scientific, Bremen, Germany) and submitted to mass spectrometry. LC-ESI-MS/MS analysis was performed on a Dionex UltiMate 3000 HPLC System with a Hypersil Gold column (150 mm, internal diameter of 180 μm) filled with 3 μm Reprosil-Pur C18-AQ resin. Gradient: 5-15% ACN in 0.1% formic acid for 10 min, 15-40% ACN in 0.1% formic acid for 52 min and 40-95% ACN in 0.1% formic for 68 min at a flow rate of 1.2 μl/min. The eluate was electrosprayed into an LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany) through a Proxeon nanoelectrospray ion source (Thermo Fisher Scientific). Data acquisition was controlled by Xcalibur 2.0 and Tune 2.4 software (Thermo Fisher Scientific).

The full scans (400-1800 m/z) were acquired at resolution 30,000, AGC target 500,000, maximum injection time of 500 ms; data-dependent MS/MS fragmentation was performed either with HCD or CID activation; in the case of HCD activation (0.1 ms), MS/MS scans were acquired in the Orbitrap at resolution 7500; in the case of CID activation (normalized collision energy of 35%, 10 ms activation), MS/MS scans were acquired in the linear trap. Isolation window: 3 Da, unassigned charge states: rejected, charge state 1: rejected, charge states 2 +, 3 +, and 4 +: not rejected; dynamic exclusion enabled (60 s, exclusion list size: 200).

2.4. Database searching

Protein identification in the case of the SDS insoluble material was performed by a three-step approach involving a combination of automatic and manual inspections. In the case of the SDS soluble sample only the first analysis step was performed. In detail: in the first step the MS/MS data were analyzed with Peak Studio (version 5.3, Bioinformatic Solutions Inc., Waterloo, Canada) [169] according to the filtration parameters reported in Table S1 and using release 2013_06 of UniProtKB/Swiss-Prot database (all organisms). Data processing, including peak centroiding, charge deconvolution, and deisotope, were conducted for data refinement.

Only proteins identified by at least 4 different peptides in at least one spot using both HCD and CID fragmentation were considered as positively identified.

The de novo peptides that were unassigned in the first step were manually selected and those with ALC% ≥ 80 were subjected to the second step of the analysis. Sequences were manually submitted to protein-BLAST searches (Basic Local Alignment Search Tool) (http://blast.ncbi.nlm.nih.gov.ezp-prod1.hul.harvard.edu/Blast.cgi?PAGE=Proteins) searching non-redundant protein sequence database (nr, all organisms). Peptides were assigned to a certain protein when the query sequence was identical to the database peptide sequence except for the first and the last amino acid. In the third step, the residual peptides unassigned in the second step were again manually inspected and only sequence stretches of at least 10 consecutive amino acids with local confidence ≥ 80 were analyzed by BLAST and identified based on a less strict sequence similarity (accepted conservative substitutions: D/E, K/R, T/S).

The presence of post-translational modifications (PTM) was evaluated by additional database search analyses of the mass spectrometric data; the initial analysis was conducted using Peaks Studio 5.3 in order to detect selected PTM (hydroxylation at Tyr, Arg and Lys, phosphorylation at Tyr, Ser and Thr and nitration at Tyr) which could be potentially present based on the properties of adhesive materials from other organisms. Additional investigations on the presence of unspecified PTM were performed using the “485 built-in modifications” option of the PTM module of Peaks Studio 7.0.

3. RESULTS

3.1. Sample preparation and fractionation

The novel method based on electrical stimulation of the parasites detailed above allowed collecting about 40 µg proteins from 30 parasites. According to Hamwood et al. the glue produced by capsalids is insoluble in many buffers and solvents including SDS [144]. Preliminary experiments confirmed such observation; however, to detect the potential presence of low-abundant SDS-soluble components, an SDS extraction step was performed upon collection of the secretion and the SDS soluble (SDS-S) and insoluble (SDS-I) proteins were separated by centrifugation.

Both fractions were separated using 2-DE (Fig. 14 and Fig. 15). As expected considering the properties of the glue, electrophoresis achieves only a very partial separation of the various components and SDS-I contains most of the starting material. However, the pre-fractionation step allowed a more reliable characterization of the samples and the detection of additional components besides the very abundant keratin and actin (see below).

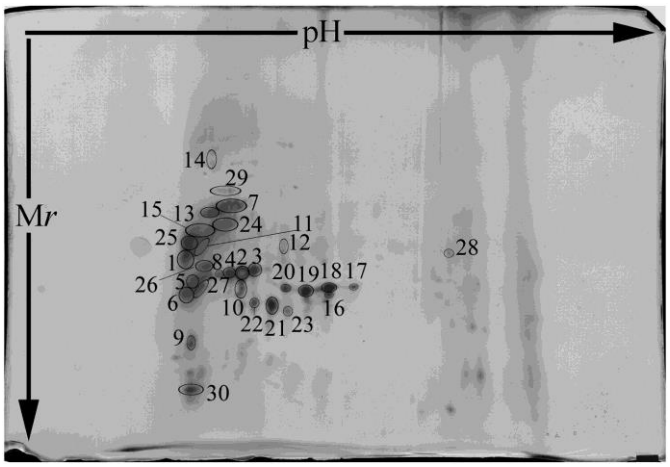

Figure 14. 2-DE of the SDS insoluble fraction of the adhesive material (SDS-I). First dimension: pH 3-10 NL 11 cm IPG; second dimension: 11% SDS PAGE. Gels were stained with mass

compatible silver. Numbers refer to spots listed in Tables 2, 3, and 4. Table 4, listing the peptides, can be found at pag 107.

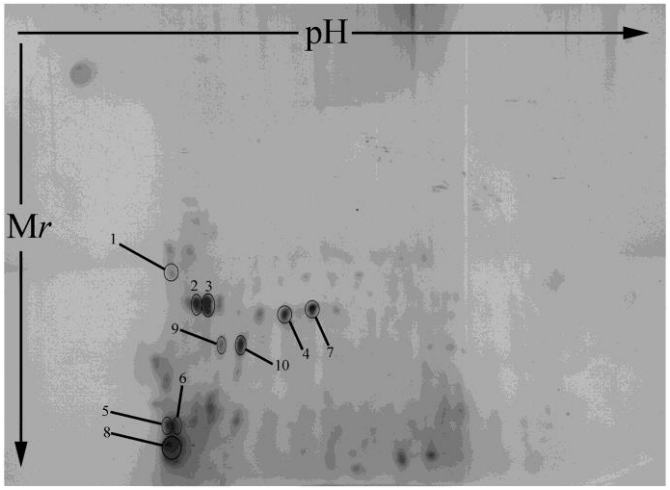

Figure. 15. 2-DE of the SDS soluble fraction of the adhesive material (SDS-S). First dimension: pH 3–10 NL 11 cm IPG; second dimension: 11% SDS PAGE. Gels were stained with mass compatible silver. Numbers refer to spots listed in Tables 2, 5 and 6. Table 6, listing the peptides, can be found at pag 145.

Proteins	SDS-I Spot n.[a]	SDS-S Spot n.[b]
Actin	2-6, 8. 10—12, 16-25, 27	4-8, 10
Albumin	3, 9, 14. 15, 18, 27	
ATP-synthase	13, 24, 25	1
78 kDa glucose regulated protein/Hsp 70/Dna/K	1, 3, 8, 14, 29	
Keratin	1-8, 11, 13-15, 18, 19, 21-27, 29, 30	1, 3, 9
αβ-Tubulin	11, 12, 13. 24. 25, 26, 28	1, 2, 9

[a] numbers refer to Fig. 14

[b] numbers refer to Fig. 15

Table 2. List of proteins identified in SDS-I and SDS-S fractions of the adhesive material.

Spot n.	**Accession**	**-10lgP**	**Cov %**	**# Peptides**	**Mass**	**Description**
1	P25691|K2M3_SHEEP	309.03	15	7	55255	Keratin, type II microfibrillar
	P11021|GRP78_HUMAN	274.37	17	7	72333	78 kDa glucose-regulated protein
	A5A6M6|K2C1_PANTR	260.03	35	20	65489	Keratin, type II cytoskeletal 1
	P08418|HSP70_SCHMA	163.92	8	4	69875	Heat shock 70 kDa protein homolog
2	P53470|ACT1_SCHMA	265.99	29	11	41731	Actin-1
	P13645|K1C10_HUMAN	342.52	54	43	58827	Keratin, type I cytoskeletal 10
	Q00214|ACTM_STYPL	140.95	18	4	42354	Actin, muscle
3	P08779|K1C16_HUMAN	311.01	28	9	51267	Keratin, type I cytoskeletal 16
	P53470|ACT1_SCHMA	238.55	18	4	41731	Actin-1
	A7ZHA4|DNAK_ECO24	218.86	13	5	69115	Chaperone protein DnaK
	P13645|K1C10_HUMAN	279.16	28	18	58827	Keratin, type I cytoskeletal 10
	Q5R1X3|ACTB_PANTR	224.74	39	9	41737	Actin, cytoplasmic 1
	P02769|ALBU_BOVIN	199.15	20	9	69294	Serum albumin
	P11142|HSP7C_HUMAN	154.15	15	5	70898	Heat shock cognate 71 kDa protein
4	P53470|ACT1_SCHMA	126.99	7	2	41731	Actin-1
	Q6IFZ9|K2C74_MOUSE	46.50	2	1	54746	Keratin, type II cytoskeletal 74
	A5A6M6|K2C1_PANTR	235.91	18	7	65489	Keratin, type II cytoskeletal 1
	Q00214|ACTM_STYPL	194.06	17	5	42354	Actin, muscle
5	P08779|K1C16_HUMAN	274.51	50	19	51267	Keratin, type I cytoskeletal 16
	Q93129|ACTC_BRABE	103.73	7	2	41704	Actin, cytoplasmic

	P04264|K2C1_HUMAN	243.79	29	17	66039	Keratin, type II cytoskeletal 1
	Q5JAK2|ACTG_RANLE	169.15	26	7	41779	Actin, cytoplasmic 2
6	P20399|ACT2_XENTR	146.60	4	27	42033	Actin, alpha cardiac muscle 2
	P04264|K2C1_HUMAN	251.49	25	10	66039	Keratin, type II cytoskeletal 1
	Q26065|ACT_PLAMG	167.81	15	3	41762	Actin, adductor muscle
7	A5A6M6|K2C1_PANTR	236.52	27	16	65489	Keratin, type II cytoskeletal 1
	P04264|K2C1_HUMAN	251,61	23	12	66039	Keratin, type II cytoskeletal 1
8	P63261|ACTG_HUMAN	223.96	15	4	41792	Actin, cytoplasmic 2
	P11021|GRP78_HUMAN	153.22	10	4	72333	78 kDa glucose-regulated protein
	P04264|K2C1_HUMAN	248.92	18	12	66039	Keratin, type II cytoskeletal 1
	P08418|HSP70_SCHMA	111.73	5	2	69875	Heat shock 70 kDa protein homolog
	P63270|ACTH_CHICK	95.02	7	2	41877	Actin, gamma-enteric smooth muscle
9	P02769|ALBU_BOVIN	334.45	30	15	69293	Serum albumin
10	P53470|ACT1_SCHMA	227.77	37	12	41731	Actin-1
11	P68365|TBA1C_CRIGR	282.81	51	14	49909	Tubulin alpha-1C chain
	P20399|ACT2_XENTR	229.25	39	6	42033	Actin, alpha cardiac muscle 2
	P13645|K1C10_HUMAN	175.51	16	7	58827	Keratin, type I cytoskeletal 10
	P69003|ACT1_HELTB	169.63	26	7	41788	Actin CyI, cytoplasmic
	P06604|TBA2_DROME	89.45	10	3	49967	Tubulin alpha-2 chain
12	P20399|ACT2_XENTR	200.92	32	6	42033	Actin, alpha 2
	P68365|TBA1C_CRIGR	146.82	15	4	49909	Tubulin alpha-1C chain
13	P13645|K1C10_HUMAN	217.94	28	13	58827	Keratin, type I cytoskeletal 10
	Q3MHM5|TBB4B_BOVIN	175.16	31	10	49831	Tubulin beta-4B chain

	P46561|ATPB_CAEEL	117.26	12	4	57527	ATP synthase subunit beta, mitochondrial
14	P13645|K1C10_HUMAN	218.20	13	4	58827	Keratin, type I cytoskeletal 10
	P02769|ALBU_BOVIN	273.21	15	7	69293	Serum albumin
	A7ZHA4|DNAK_ECO24	223.70	14	5	69115	Chaperone protein DnaK
	P04264|K2C1_HUMAN	257.28	25	15	66039	Keratin, type II cytoskeletal 1
	Q9W6Y1|HSP7C_ORYLA	185.60	9	8	76169	Heat shock cognate 71 kDa protein
15	P02769|ALBU_BOVIN	368.32	46	23	69293	Serum albumin
	P13645|K1C10_HUMAN	183.77	9	3	58827	Keratin, type I cytoskeletal 10
	P13645|K1C10_HUMAN	326.38	29	32	58827	Keratin, type I cytoskeletal 10
16	O17502|ACTM_BRALA	174.73	13	4	42187	Actin, muscle
17	P68556|ACT1_DIPDE	228.13	32	8	41744	Actin-1/4
18	Q90X97|ACTS_ATRMM	198.83	27	4	42061	Actin, alpha skeletal muscle
	P02769|ALBU_BOVIN	262.13	19	9	69294	Serum albumin
	Q91ZK5|ACTB_SIGHI	204.29	23	5	41719	Actin, cytoplasmic 1
	P02769|ALBU_BOVIN	195.08	14	6	69294	Serum albumin
	A5A6M6|K2C1_PANTR	177.21	13	6	65489	Keratin, type II cytoskeletal 1
19	P53470|ACT1_SCHMA	213.24	27	9	41731	Actin-1
	P53464|ACTM_HELTB	215.58	27	10	41762	Actin, cytoskeletal
	P04264|K2C1_HUMAN	164.22	12	6	66039	Keratin, type II cytoskeletal 1
20	Q90X97|ACTS_ATRMM	337.45	64	16	42061	Actin, alpha skeletal muscle
21	P68556|ACT1_DIPDE	233.60	31	8	41744	Actin-1/4
	A5A6M6|K2C1_PANTR	196.18	15	7	65489	Keratin, type II cytoskeletal 1
	P04264|K2C1_HUMAN	197.27	25	13	66039	Keratin, type II cytoskeletal 1

	Q5R1X3|ACTB_PANTR	122.44	21	4	41737	Actin, cytoplasmic 1
22	A5A6M6|K2C1_PANTR	252.18	16	10	65489	Keratin, type II cytoskeletal 1
	P63261|ACTG_HUMAN	212.04	15	4	41792	Actin, cytoplasmic 2
	P08779|K1C16_HUMAN	207.96	29	11	51268	Keratin, type I cytoskeletal 16
	P53480|ACTC_TAKRU	89.85	7	2	41975	Actin, alpha cardiac
23	Q9UVX4|ACT_COPC7	119.78	9	2	41612	Actin
	P04264|K2C1_HUMAN	179.64	19	8	66039	Keratin, type II cytoskeletal 1
	P84185|ACT5C_ANOGA	167.88	21	6	41721	Actin, cytoplasmic
24	P86221|TBB2C_MESAU	94.72	9	2	31916	Tubulin beta-2C chain (Fragments)
	P13645|K1C10_HUMAN	238.09	32	16	58827	Keratin, type I cytoskeletal 10
	Q9YHC3|TBB1_GADMO	181.31	21	8	49749	Tubulin beta-1 chain
	Q05825|ATPB_DROME	178,15	19	7	54108	ATP synthase subunit beta, mitochondrial
	Q5R1X3|ACTB_PANTR	125.38	16	3	41737	Actin, cytoplasmic 1
25	Q9YHC3|TBB1_GADMO	249.37	34	12	49748	Tubulin beta-1 chain
	P13645|K1C10_HUMAN	199.00	18	9	58827	Keratin, type I cytoskeletal 10
	Q05825|ATPB_DROME	188.17	15	6	54108	ATP syn-beta, mitochondrial
	P13645|K1C10_HUMAN	246.95	31	19	58827	Keratin, type I cytoskeletal 10
	P68556|ACT1_DIPDE	203.76	41	12	41773	Actin-2
	P30883|TBB4_XENLA	134.29	11	4	49816	Tubulin beta-4 chain
	P46561|ATPB_CAEEL	118.77	11	4	50537	ATP synthase subunit beta
26	P08779|K1C16_HUMAN	220.57	27	9	51267	Keratin, type I cytoskeletal 16
	Q3ZBU7|TBB4_BOVIN	133.00	8	3	49585	Tubulin beta-4 chain

	P13645|K1C10_HUMAN	296.00	23	11	58827	Keratin, type I cytoskeletal 10
	P30883|TBB4_XENLA	192.94	14	5	49816	Tubulin beta-4 chain
27	P08779|K1C16_HUMAN	158.62	17	5	51267	Keratin, type I cytoskeletal 16
	P13645|K1C10_HUMAN	239.28	15	10	58827	Keratin, type I cytoskeletal 10
	Q5R1X3|ACTB_PANTR	226.40	35	8	41373	Actin, cytoplasmic 1
	P02769|ALBU_BOVIN	223.94	26	12	69294	Serum albumin
28	Q2KJD0|TBB5_BOVIN	200.32	20	6	49670	Tubulin beta-5 chain
29	P08418|HSP70_SCHMA	171.54	10	6	69875	Heat shock 70 kDa protein homolog
	P35527|K1C9_HUMAN	141.98	9	3	62064	Keratin, type I cytoskeletal 9
	Q9W6Y1|HSP7C_ORYLA	219.09	12	7	76169	Heat shock cognate 71 kDa protein
	A5A6M6|K2C1_PANTR	211.53	15	8	65489	Keratin, type II cytoskeletal 1
30	P02538|K2C6A_HUMAN	71.72	5	2	60045	Keratin, type II cytoskeletal 6A
	P13645|K1C10_HUMAN	181.26	25	13	58827	Keratin, type I cytoskeletal 10

Table 3. Proteins identified by MS/MS analysis of SDS-I. Spot numbers refer to Fig. 14

Spot n.	**Accession**	**-10lgP**	**Number of Peptides**
1	P46561|ATPB_CAEEL	174.72	5
	Q3MHM5|TBB2C_BOVIN	138.02	3
	Q6EIY9|K2C1_CANFA	119.13	3
2	Q3ZBU7|TBB4_BOVIN	103.35	2
3	A5A6M6|K2C1_PANTR	137.79	3

4	P86700|ACT_CHIOP	129.48	3
5	P86700|ACT_CHIOP	137.88	3
6	P91754|ACT_LUMRU	181.67	6
7	A5DQP9|ACT_PICGU	113.69	2
8	P86700|ACT_CHIOP	129.93	3
9	P13645|K1C10_HUMAN	99.13	3
	P06604|TBA2_DROME	81.74	2
10	A5DQP9|ACT_PICGU	107.66	2

Table 5. Proteins identified by MS/MS analysis of SDS-S. Spot numbers refer to Fig. 15

3.2. Characterization of SDS-I

SDS-I was analyzed in duplicate using two different fragmentation techniques (HCD and CID) in order to assess overall reproducibility of the de novo mass spectrometric identification of the protein content of each spot. Since no genome is available for any monogenean parasite, protein identification was achieved by a strategy based on de novo sequencing using nano LC-ESI MS/MS with LTQ-Orbitrap Velos followed by analysis with Peaks Studio 5.3 software. Tables 1 and 2 report the proteins identified in SDS-I. Only proteins identified by at least 4 different peptides in at least one spot using both HCD and CID-based MS/MS analyses were considered as positively identified components of the adhesive material. Peptides supporting identification are reported in Table 4.

Representative MS/MS spectra of one peptide for each protein are shown in Fig. 16 A-F.

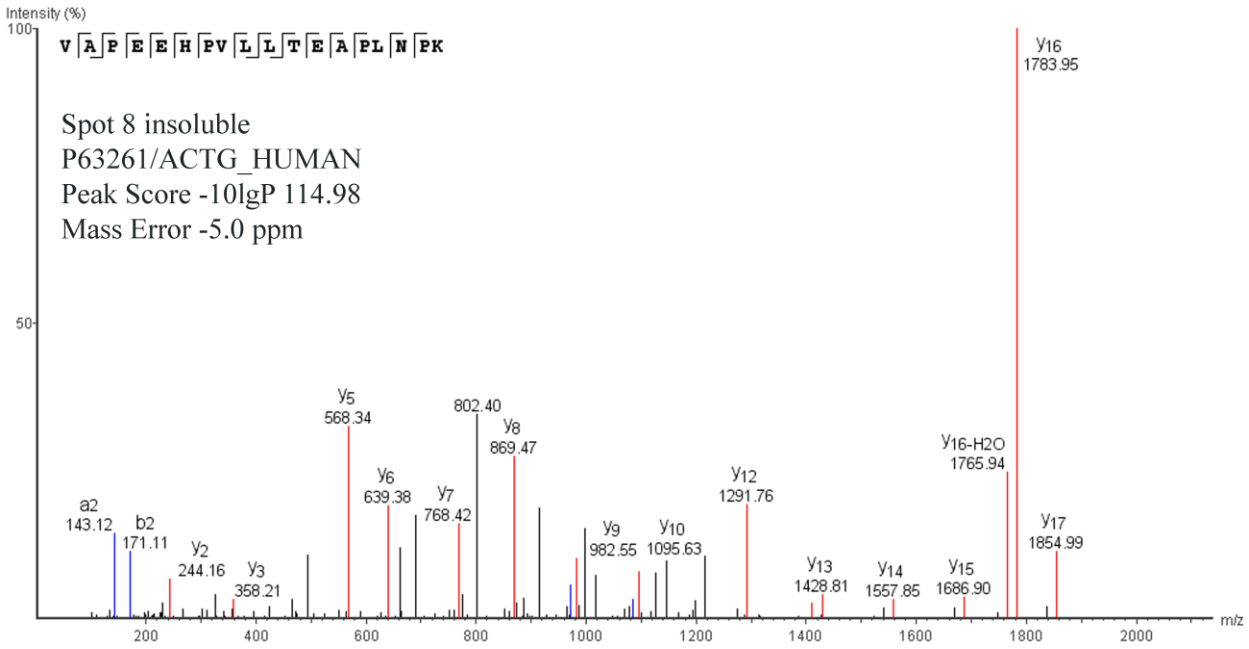

Figure 16A. Representative MS/MS spectra of human actin G.

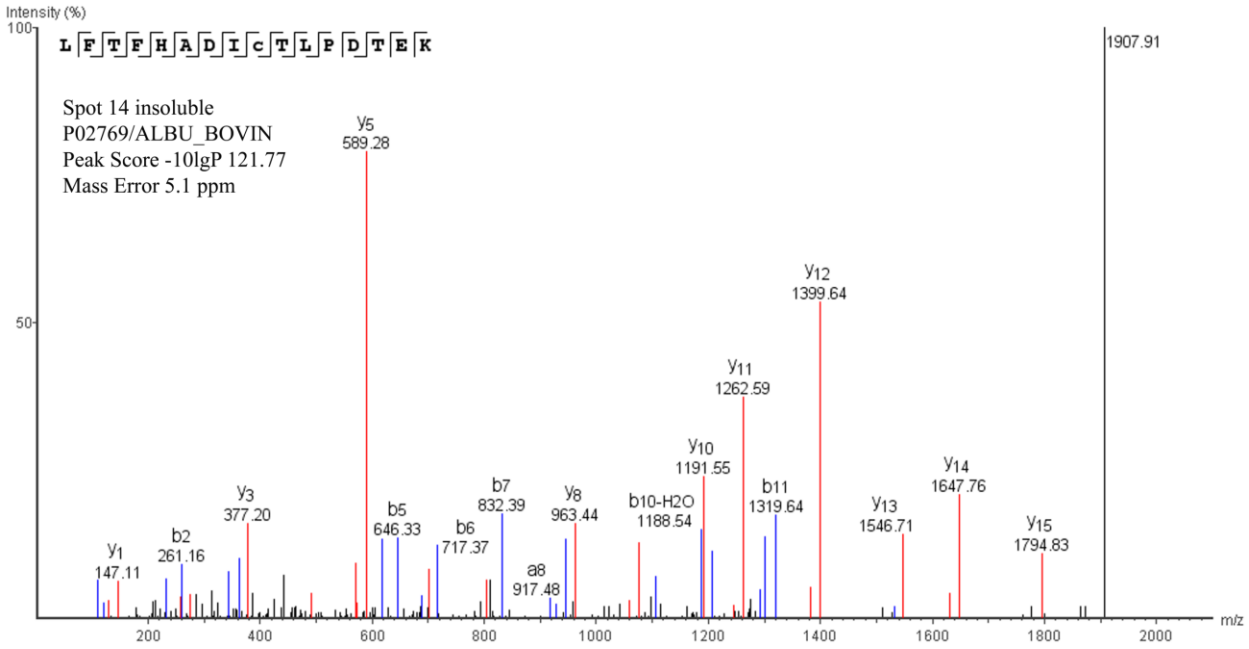

Figure 16B. Representative MS/MS spectra of bovin albumin.

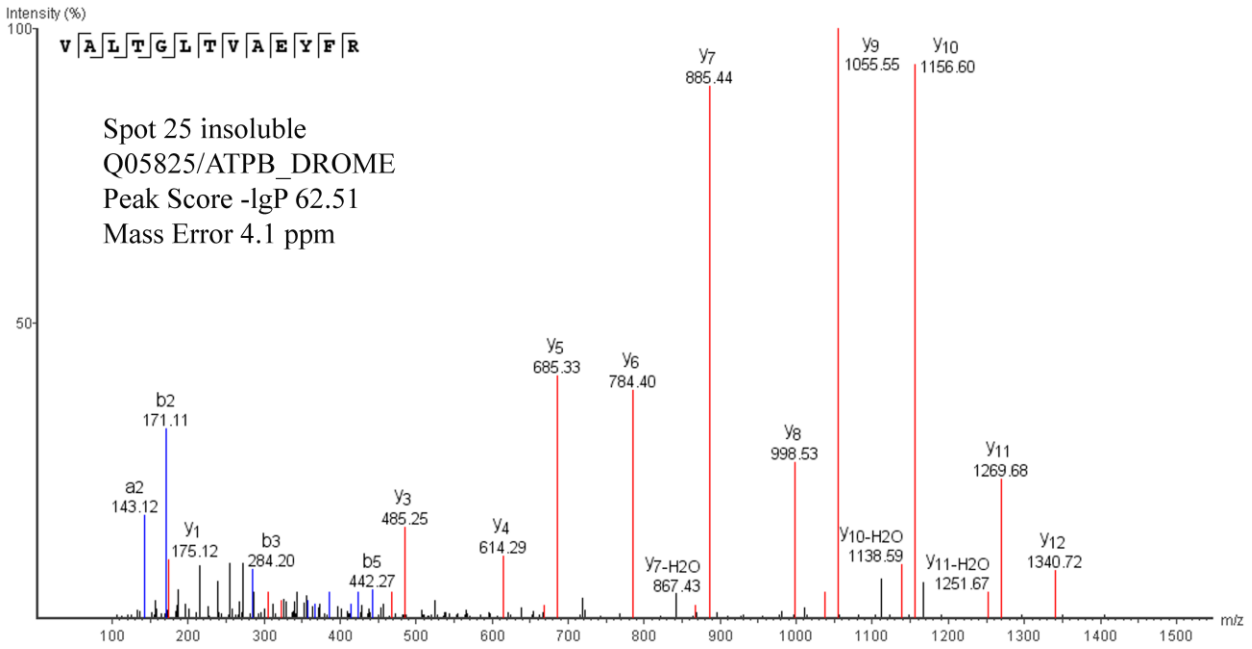

Figure 16C. Representative MS/MS spectra of *Drosophila melanogaster* ATP synthase subunit beta, mitochondrial.

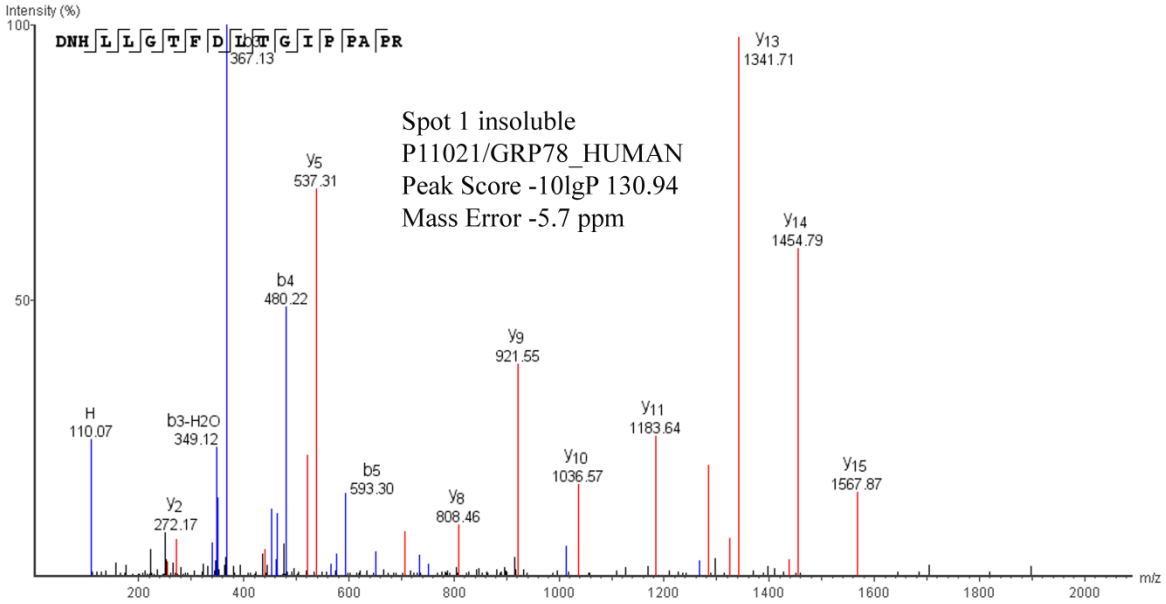

Figure 16D. Representative MS/MS spectra of human 78 kDa glucose-regulated protein.

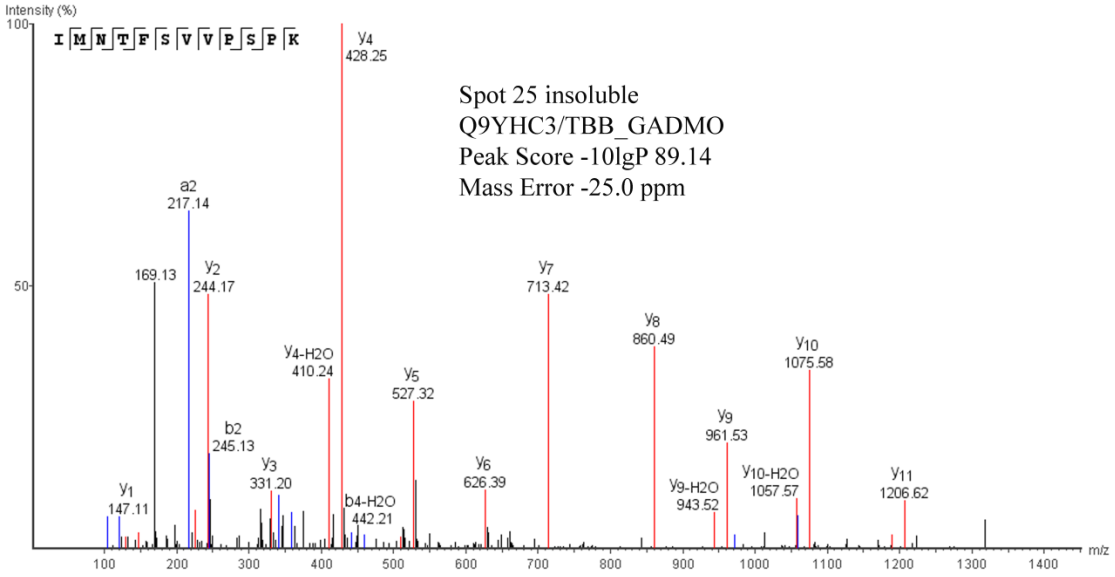

Figure 16E. Representative MS/MS spectra of atlantic cod tubulin beta-1 chain

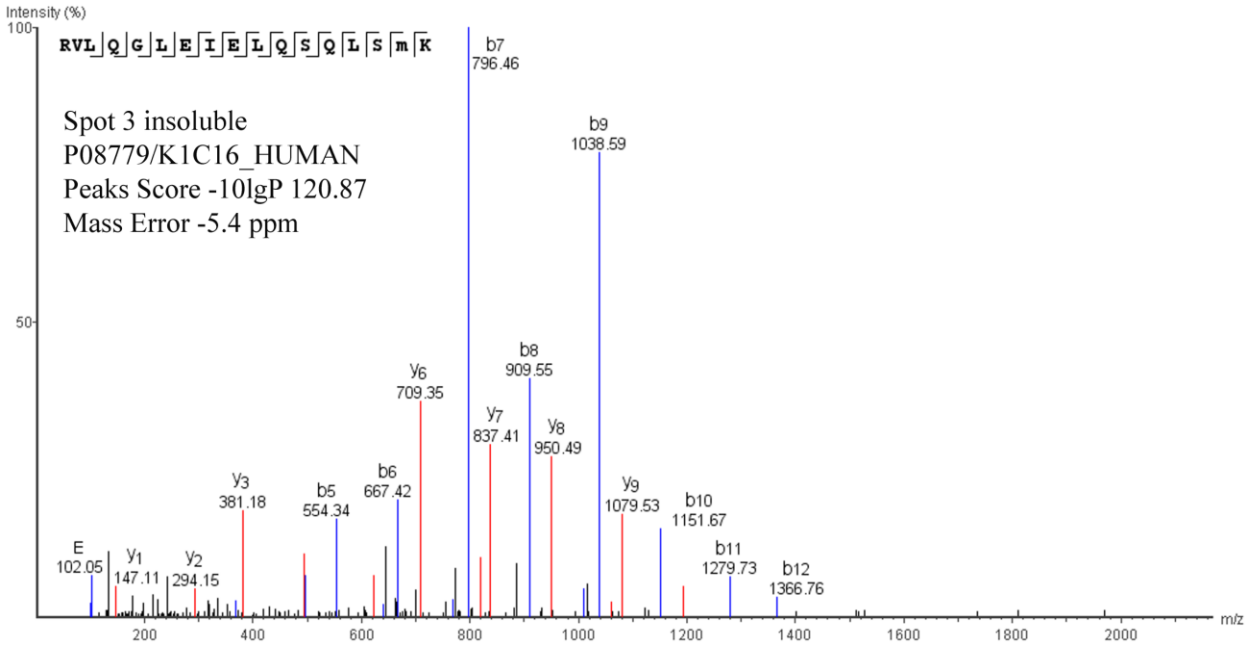

Figure 16F. Representative MS/MS spectra of human Keratin, type I cytoskeletal 16

Due to the absence of genome information for these organisms, several peptides fulfilling the required parameters for confident sequence determination by Peaks Studio could not be assigned to any protein. In order to identify further possible undetected protein components, manual BLAST searches using the unassigned peptides were performed. However, as can be

inferred from Table 4, no additional SDS-I proteins fulfilling the 4-peptide criterion detailed above were detected.

3.3. Characterization of SDS-S

Results concerning the characterization of SDS-S material are summarized in Tables 1, Table 5 and Table 6. Altogether, the data clearly confirm that the adhesive material is insoluble in SDS and that SDS-S contains low amounts of the same proteins found in SDS-I, partially solubilized by SDS; in conclusion, the adhesive material is composed only of few proteins: keratin, actin, tubulin, ATP-synthase, 78 kDa glucose regulated protein (GRP78/HSP 70/DnaK) and albumin.

3.4. Lack of some expected post-translational modifications in proteins from the bioadhesive material

As emphasized by many Authors [170] and [171], most aquatic organisms exploit modified amino acid side chains, like phosphorylated Ser and hydroxylated Tyr (3, 4 dihydroxyphenilalanine, DOPA) to gain high-surface affinity and strong adhesiveness. Barnacles are an exception and adhere with unmodified amino acids [170] and [172]. We investigated the presence of post-translational modifications (PTM) by various additional database search analyses of the mass spectrometric data, either using the “458-built-in modifications” option of the PTM module of Peaks Studio 7.0 to search for unspecified modifications or setting only specific modifications, selected on the basis of known features of adhesive materials from other organisms (i.e. hydroxylation at Tyr, Arg and Lys, phosphorylation at Tyr, Ser and Thr and nitration at Tyr as variable modifications). No significant PTM other than Cys-carbamidomethylation, Met oxidation, N-terminal acetylation and formylation, dehydration and deamidation, which are well known PTM which can be present following the usual protocols for proteomic studies, were detected. Based on these findings, and on the characterization carried out by Hamwood et al. [4] on the secretion of the monogenea E. soleae that excludes the presence of lipids, glycans and DOPA upon analyses by colorimetric assays, it

is possible to speculate that Capsalidae rely for adhesion on a mechanism different from that described in mussels.

As pointed out above, the lack of PTM in an adhesive material is quite unusual. Up to now, the only bioadhesive material from aquatic organisms which does not rely on post translationally modified residues for adhesion is the cement of barnacle, where the nanofibrillar network of proteins plays a very important role in the formation of the glue [172]. Six cement proteins have been identified so far. All of them, except for the enzymatic one, are novel, without significant homology with other proteins in databases currently available. They contain amyloid-like sequences that form a beta-sheet structure and homogenously interact with each other to form an insoluble self-assembly [172]. None of the peptides characterized in the present report shows significant similarities with barnacle cement proteins.

4. DISCUSSION AND CONCLUSION

Our data demonstrate that the composition of the glue in N. girellae is completely different from the cement of barnacle and more similar to the adhesive material described in certain reptilians, like gecko, whose ability to adhere to vertical surfaces relies on the presence of specialized adhesive setae on their feet [138]. In gecko, the characterization of these structures demonstrated that the main components of the adhesive material are α-keratins (45-60 kDa) and associated-β keratins (sKAβPs) (8-22 kDa) present in different isoforms [138]. The similarity among the gecko and the N. girellae keratins was specifically analyzed carrying out a multi alignment analysis for all the peptides ascribed to N. girellae keratin and the gecko keratins described in [138]. No significant similarity was found except for the C-terminal region of gecko keratins rich in Gly and Ser residues whose role in gecko remains, at present, still unknown. Interestingly, few studies indicate that adhesion of setae increases with increasing environmental humidity due to the modification of the contact geometry of the hydrophobic surfaces. On the other hand, a fundamental difference between the adhesive mechanisms of terrestrial animals, such as geckos, and of fish parasites, like N. girellae, is that the latter must displace water to initiate contact with a surface. It is unknown whether water displacement occurs due to the surface properties of the adhesive organ or the nature of their secretion.

Interestingly, a “keratin-like fibrous component” was discovered in ligament of the bivalve Silica radiata [173]. Characterization of the protein by MS/MS analysis revealed that it matches the sequence (27% coverage) of human K2C1/KRT1, which is one of the keratin forms matching the peptides identified in the present study (Table 2, Table 3, Table 4, Table 5 and Table 6), confirming that keratins with considerable degree of identity with a human form can be present in phylogenetically very distant organisms.

Beside keratin, another component of the N. girellae adhesive is actin, a highly conserved protein of the cytoskeleton of all eukaryotic cells. Among its different functions in a variety of cellular events such as motion, chromosome segregation, transport of macromolecules and endo- and esocytosis, this protein has been described as one of the components of the glue-like substances secreted by silkworm Bombyx mori L colleterial glands [174]. Although its role is not

understood until now, it is suggested that it might participate or regulate the secretion of these glands important for gliding motility [174]. In Monogenea the secretion is also used during locomotion for temporary adhesion to living host tissue. Therefore, actin present in the salivary extracts may play a role similar to the one described in apicomplexan parasites.

Albumin is the third protein of the N. girellae proteome already described as a glue component, being used with glutaraldehyde as a new surgical adhesive and suture support [175]. Since at the moment it is not possible to suggest any mechanism or a plausible substitute for the crosslinking agent glutaraldehyde, its role as an active component of the adhesive material cannot be established. However possible expression of an albumin-like protein in these organisms is reinforced by the observation that a protein with about 70% identity with bovine serum albumin has been sequenced from the platyhelmint Schistosoma mansoni (Uniprot accession number Q95VB7_SCHMA). As far as the other components of the secretion in N. girellae are concerned (tubulin, ATP-synthase, and 78 kDa glucose regulated protein), these proteins have never been described in secreted adhesive materials until now. However, 78 kDa glucose regulated protein/HSP70/DnaK plays a role in facilitating the assembly of multimeric protein complexes inside the ER and may have a role in the post-translational modification, assembly or packaging of proteins prior to secretion [176], while ATP-synthase has been shown to be involved in the cellular secretory mechanism [177]. Thus, both macromolecules are involved at different levels in the mechanism of eukaryotic secretions and in the modulation of the membrane plasticity, but the specific role played in the secreted adhesive material of the fish parasite is presently unknown.

In conclusion, the composition of a new bioadhesive material from the fish parasite N. girellae has been determined by a proteomic approach based on mass spectrometric de novo sequencing to overcome the absence of genomic information. The secretion of N. girellae is constituted mainly by cytoskeletal proteins. Such composition, together with the possible absence of post-translationally modified proteins, suggests that these organisms rely for adhesion on a mechanism completely different from the one described in other fish parasites or in mussels.

The results presented in this dissertation complement previous investigations concerning the role of glycoproteins produced by fish epithelium in the initial steps leading to N. girellae recognition of the host [178, 179]. Characterization at the molecular level of all processes involved control of this important pathogen in marine cultured fish.

The results reported in this PhD thesis has been published in a paper entitled “A new bioadhesive material from fish parasite Neobenedenia girellae” [180].

SUPPLEMENTARY TABLES

Table 4

Spot n.	Protein Accession	Peptide	-10lgP	m/z	z
1	P25691|K2M3_SHEEP	R.KSDLEANVEALVEESNFLKR.L	131.57	764.3967	3
		R.TKLEAAVAEAEQQGEAALNDAR.S	122.58	762.3792	3
		R.KSDLEANVEALVEESNFLK.R	111.96	1068.0411	2
		K.SDLEANVEALVEESNFLKR.L	108.62	721.6978	3
		R.LYDEEIQILNAHISDTSVIVK.M	107.53	800.7551	3
		K.SDLEANVEALVEESNFLK.R	105.77	669.6641	3
		K.LGLDIEIATYR.R	87.96	632.3477	2
	P11021|GRP78_HUMAN	K.DNHLLGTFDLTGIPPAPR.G	130.94	967.5046	2
		K.TFAPEEISAMVLTK.M	110.61	768.8978	2
		R.GVPQIEVTFEIDVNGILR.V	99.36	1000.0416	2
		R.IEIESFYEGEDFSETLTR.A	93.04	1082.9930	2
		K.TFAPEEISAM(+15.99)VLTK.M	83.56	776.8955	2
		R.IINEPTAAAIAYGLDKR.E	76.03	908.4957	2
		R.LTPEEIER.M	58.09	493.7583	2
		K.SQIFSTASDNQPTVTIK.V	47.99	918.9650	2
	A5A6M6|K2C1_PANTR	R.SGGGFSSGSAGIINYQR.R	79.59	829.3984	2
		K.WELLQQVDTSTR.T	72.93	738.3770	2
		R.FLEQQNQVLQTK.W	71.25	738.3959	2
		K.YEELQITAGR.H	70.26	590.3033	2
		K.SLNNQFASFIDK.V	70.04	692.3469	2
		K.QISNLQQSISDAEQR.G	69.96	858.9270	2

		R.TNAENEFVTIK.K	69.29	633.3219	2
		K.SKAEAESLYQSK.Y	68.57	670.8377	2
		R.DYQELMNTK.L	63.17	571.2623	2
		K.LNDLEDALQQAK.E	57.23	679.3500	2
		R.GSYGSGGSSYGSGGGSYGSGGGGGGHGSY.G	54.63	1208.9661	2
		R.TNAENEFVTIKK.D	53.87	465.2481	3
		R.GGGGGGYGSGGSSYGSGGGSYGSGGGGGGGR.G	52.73	1192.4795	2
		R.SGGGFSSGSAGII.N	51.91	548.7664	2
		R.SLDLDSIIAEVK.A	49.78	651.8616	2
		K.IEISELNR.V	49.65	487.2681	2
		K.AQYEDIAQK.S	44.50	533.2637	2
		R.SLVNLGGSK.S	43.48	437.7519	2
		W.ELLQQVDTSTR.T	42.65	645.3368	2
		R.SGGGFSSGSAGI.I	40.14	492.2247	2
	P08418|HSP70_SCHMA	R.IINEPTAAAIAYGLDK.K	66.40	830.4509	2
		K.NQVAMNPTNTVFDAK.R	57.59	825.3978	2
		K.SINPDEAVAYGAAVQA.A	52.13	788.3850	2
		K.SINPDEAVAYGAAVQAA.I	40.63	823.9027	2
2	P53470|ACT1_SCHMA	K.SYELPDGQVITIGNER.F	120.77	895.9473	2
		R.AVFPSIVGRPR.H	102.84	599.8553	2
		R.VAPEEHPVLLTEAPLNPKANR.E	95.83	765.7512	3
		R.VAPEEHPVLLTEAPLNPK.A	90.56	652.0239	3
		R.HQGVMVGMGQKDSYVGDEAQSK.R	86.56	784.3576	3
		K.AGFAGDDAPR.A	85.12	488.7275	2

		K.DSYVGDEAQSK.R	82.28	599.7626	2
		R.HQGVMVGMGQK.D	79.05	586.2862	2
		K.EISALAPSTMK.I	72.61	574.3040	2
		M.AEEDVAALVIDNGSGMC(+57.02)K.A	70.43	939.9096	2
		M.AEEDVAALVIDNGSGMC(+57.02)KAGFAGDDAPR.A	45.95	946.0854	3
	P13645|K1C10_HUMAN	K.SKELTTEIDNNIEQISSYK.S	89.02	1106.5425	2
		K.GSLGGGFSSGGFSGGSFSR.G	86.39	854.3824	2
		R.GSSGGGC(+57.02)FGGSSGGYGGLGGFGGGSFR.G	83.84	1171.9850	2
		G.SSGGGC(+57.02)FGGSSGGYGGLGGFGGGSFR.G	80.76	1143.4729	2
		R.AETEC(+57.02)QNTEYQQLLDIK.I	80.40	1041.9767	2
		R.YC(+57.02)VQLSQIQAQISALEEQLQQIR.A	78.98	1373.7164	2
		R.NVSTGDVNVEMNAAPGVDLTQLLNNMR.S	77.47	1436.6879	2
		R.ISSSKGSLGGGFSSGGFSGGSFSR.G	77.29	737.3465	3
		R.ALEESNYELEGK.I	74.84	691.3207	2
		K.QSLEASLAETEGR.Y	73.60	695.8381	2
		K.ELTTEIDNNIEQISSYK.S	73.22	998.9799	2
		F.GGSSGGYGGLGGFGGGSFR.G	68.26	817.3649	2
		R.SQYEQLAEQNR.K	67.54	683.3168	2
		R.NVQALEIELQSQLALK.Q	66.93	899.0007	2
		S.SKGSLGGGFSSGGFSGGSFSR.G	64.57	641.6312	3
		K.IRLENEIQTYR.S	63.09	717.8811	2

		S.SGGYGGLGGFGGGSFR.G	61.59	716.8284	2
		G.SLGGGFSSGGFSGGSFSR.G	61.52	825.8706	2
		R.LENEIQTYR.S	61.26	583.2907	2
		R.VLDELTLTK.A	60.66	516.2977	2
		R.LKYENEVALR.Q	59.72	617.8371	2
		R.QSVEADINGLR.R	59.35	601.3059	2
		K.VTMQNLNDR.L	58.89	545.7637	2
		R.SQYEQLAEQNRK.D	56.44	747.3631	2
		L.GGGFSSGGFSGGSFSR.G	56.25	725.8134	2
		Q.ISALEEQLQQIR.A	55.58	714.3879	2
		N.LTTDNANILLQIDNAR.L	55.33	892.9708	2
		K.NQILNLTTDNANILLQIDNAR.L	54.72	789.7501	3
		R.SLLEGEGSSGGGGR.G	54.53	631.7972	2
		I.SSSKGSLGGGFSSGGFSGGSFSR.G	54.19	699.6525	3
		S.GGYGGLGGFGGGSFR.G	53.54	673.3113	2
		K.GSLGGGFSSGGFSGGSFS.R	48.86	776.3321	2
		F.GGGGFGGGFGGGFGGDGGLLSGNEK.V	47.55	1057.9764	2
		K.TIDDLKNQILNLTTDNANILLQIDNAR.L	46.97	1018.2128	3
		R.LAADDFR.L	45.67	404.1996	2
		K.YENEVALR.Q	45.37	497.2484	2
		F.GGGFGGDGGLLSGNEK.V	45.16	711.3291	2
		L.RQSVEADINGLR.R	45.03	453.2398	3
		K.SKELTTEIDNNIEQISSY.K	44.35	1042.4968	2
		R.RVLDELTLTK.A	43.39	594.3469	2

		G.GYGGLGGFGGGSFR.G	43.27	644.8013	2
		N.TEYQQLLDIK.I	43.06	625.8302	2
		K.IRLENEIQTY.R	40.21	639.8320	2
	Q00214|ACTM_STYPL	K.SYELPDGQVITIGNER.F	73.09	895.9420	2
		K.LC(+57.02)YVALDFEQEMATAA SSSSLEK.S	55.28	1275.5864	2
		R.VAPEEHPVLLTEAPLNPK.A	53.70	977.5286	2
		R.GYSFVTTAER.E	51.60	565.7724	2
3	P08779|K1C16_HUMAN	R.RVLQGLEIELQSQLSM(+15.99)K .A	120.87	663.3627	3
		R.TDLEMQIEGLKEELAYLR.K	117.45	717.7017	3
		R.LLEGEDAHLSSQQASGQSYSSR.E	115.81	784.0320	3
		R.VLQGLEIELQSQLSMK.A	115.44	605.9995	3
		R.VLQGLEIELQSQLSM(+15.99)K. A	103.46	916.4915	2
		R.GQTGGDVNVEM(+15.99)DAAP GVDLSR.I	98.70	1052.4791	2
		R.NKIIAATIENAQPILQIDNAR.L	95.93	769.4311	3
		R.ALEEANADLEVK.I	88.03	651.3301	2
		R.TDLEM(+15.99)QIEGLKEELAYL R.K	82.06	723.0335	3
		K.EVASNSELVQSSR.S	80.06	703.3466	2
		R.LASYLDK.V	50.70	405.2224	2
	P53470|ACT1_SCHMA	R.AVFPSIVGRPR.H	107.60	400.2383	3
		K.AGFAGDDAPR.A	106.87	488.7261	2
		R.HQGVMVGMGQK.D	74.37	586.2877	2
		M.AEEDVAALVIDNGSGMC(+57.0 2)K.A	69.49	939.9116	2

	A7ZHA4|DNAK_ECO24	K.IELSSAQQTDVNLPYITADATGPK.H	112.71	844.7629	3
		K.TAIESALTALETALK.G	91.48	766.4296	2
		R.KDVNPDEAVAIGAAVQGGVLTGDVK.D	84.24	808.4277	3
		R.GM(+15.99)PQIEVTFDIDADGILHVSAK.D	80.74	791.3943	3
		K.DVNPDEAVAIGAAVQGGVLTGDVK.D	60.76	765.7276	3
	P13645|K1C10_HUMAN	K.GSLGGGFSSGGFSGGSFSR.G	95.19	854.3823	2
		K.QSLEASLAETEGR.Y	83.99	695.8384	2
		R.ALEESNYELEGK.I	83.02	691.3212	2
		R.SQYEQLAEQNR.K	75.96	683.3172	2
		K.SKELTTEIDNNIEQISSYK.S	74.29	738.0300	3
		R.SLLEGEGSSGGGGR.G	66.89	631.7972	2
		R.VLDELTLTK.A	61.98	516.2969	2
		R.QSVEADINGLR.R	60.99	601.3066	2
		R.LENEIQTYR.S	59.02	583.2905	2
		R.LKYENEVALR.Q	55.14	617.8378	2
		L.GGGFSSGGFSGGSFSR.G	52.17	725.8148	2
		R.SQYEQLAEQNRK.D	51.72	747.3648	2
		K.YENEVALR.Q	50.37	497.2484	2
		K.VTMQNLNDR.L	47.78	545.7645	2
		K.IRLENEIQTYR.S	43.22	478.9230	3
		R.AETEC(+57.02)QNTEYQQLLDIK.I	42.28	1041.9799	2
		K.DAEAWFNEK.S	41.59	555.2435	2
		K.ELTTEIDNNIEQISSYK.S	40.82	998.9791	2

	Q5R1X3|ACTB_PANTR	K.SYELPDGQVITIGNER.F	90.94	895.9414	2
		K.LC(+57.02)YVALDFEQEMATAASSSSLEK.S	77.51	1275.5778	2
		R.VAPEEHPVLLTEAPLNPK.A	75.69	977.5281	2
		F.NTPAMYVAIQAVLSLYASGR.T	65.68	709.0358	3
		K.YSVWIGGSILASLSTFQQMWISK.Q	60.60	1301.6658	2
		R.GYSFTTTAER.E	59.67	566.7614	2
		K.DLYANTVLSGGTTMYPGIADR.M	57.87	1108.0305	2
		R.DLTDYLMK.I	54.90	499.7413	2
		R.AVFPSIVGR.P	41.75	473.2750	2
	P02769|ALBU_BOVIN	K.DAFLGSFLYEYSR.R	76.00	784.3676	2
		K.LGEYGFQNALIVR.Y	69.88	740.3931	2
		K.GLVLIAFSQYLQQC(+57.02)PFDEHVK.L	59.19	1246.6290	2
		R.RHPYFYAPELLYYANK.Y	55.33	682.3407	3
		K.LVVSTQTALA	52.12	501.7898	2
		K.EYEATLEEC(+57.02)C(+57.02)AK.D	50.91	751.8031	2
		R.MPC(+57.02)TEDYLSLILNR.L	50.21	862.9115	2
		K.LVNELTEFAK.T	48.68	582.3137	2
		K.VPQVSTPTLVEVSR.S	40.49	756.4183	2
	P11142|HSP7C_HUMAN	R.GVPQIEVTFDIDANGILNVSAVDK.S	71.01	838.7689	3
		K.NQVAMNPTNTVFDAK.R	64.62	825.3937	2
		Y.GAAVQAAILSGDK.S	59.70	600.8257	2
		R.IINEPTAAAIAYGLDK.K	53.73	830.4423	2
		R.TLSSSTQASIEIDSLYEGIDFYTSIT	48.56	999.8168	3

		R.A			
4	P53470|ACT1_SCHMA	K.SYELPDGQVITIGNER.F	99.94	895.9508	2
		K.EISALAPSTMK.I	54.10	574.3060	2
	Q6IFZ9|K2C74_MOUSE	R.FLEQQNQVLQTK.W	46.50	738.3968	2
	A5A6M6|K2C1_PANTR	R.FSSC(+57.02)GGGGGSFGAGGGFGSR.S	107.03	883.3621	2
		R.SGGGFSSGSAGIINYQR.R	90.37	829.3917	2
		K.QISNLQQSISDAEQR.G	86.61	858.9211	2
		K.YEELQITAGR.H	75.81	590.2965	2
		R.FLEQQNQVLQTK.W	74.72	738.3881	2
		R.TNAENEFVTIK.K	51.56	633.3146	2
		R.GSYGSGGSSYGSGGGSYGSGGGGGGHGSY.G	40.87	1208.9606	2
	Q00214|ACTM_STYPL	K.SYELPDGQVITIGNER.F	102.30	895.9417	2
		R.GYSFVTTAER.E	74.94	565.7715	2
		R.VAPEEHPVLLTEAPLNPK.A	65.64	977.5274	2
		Y.ELPDGQVITIGNER.F	51.32	770.8942	2
		R.HQGVMVGMGQKDSYVGDEAQSK.R	45.90	588.5187	4
5	Q93129|ACTC_BRABE	K.SYELPDGQVITIGNER.F	71.77	895.9470	2
		R.AVFPSIVGRPR.H	63.92	400.2390	3
	P08779|K1C16_HUMAN	K.TEELNKEVASNSELVQSSR.S	92.61	707.3518	3
		R.VLQGLEIELQSQLSMK.A	84.74	605.9984	3
		R.ISSVLAGGSC(+57.02)RAPSTYGGGLSVSSR.F	83.16	809.4067	3
		R.LLEGEDAHLSSQQASGQSYSSR.E	81.03	784.0325	3
		K.IIAATIENAQPILQIDNAR.L	76.39	1032.5730	2
		R.NKIIAATIENAQPILQIDNAR.L	70.23	769.4300	3

		R.QTVEADVNGLR.R	67.60	601.3101	2
		K.ASLENSLEETK.G	67.43	610.8023	2
		R.QFTSSSSMKGSC(+57.02)GIGGGIGGGSSR.I	66.31	754.6765	3
		R.ALEEANADLEVKIRDWYQR.Q	63.78	773.7273	3
		R.EVFTSSSSSSSR.Q	63.52	630.7876	2
		R.QFTSSSSM(+15.99)KGSC(+57.02)GIGGGIGGGSSR.I	61.40	760.0085	3
		K.DYSPYFKTIEDLR.N	59.41	823.9019	2
		R.QRPSEIKDYSPYFK.T	59.08	586.6311	3
		K.VTMQNLNDRLASYLDKVR.A	56.69	712.7100	3
		K.EVASNSELVQSSR.S	53.57	703.3481	2
		K.VTMQNLNDRLASYLDK.V	50.20	627.6541	3
		R.QTVEADVNGLRR.V	45.92	679.3596	2
		R.DQYEQMAEK.N	40.88	571.2431	2
	P04264|K2C1_HUMAN	K.QISNLQQSISDAEQR.G	72.65	858.9209	2
		R.SGGGFSSGSAGIINYQR.R	71.50	829.3922	2
		R.FLEQQNQVLQTK.W	66.25	738.3889	2
		R.TNAENEFVTIK.K	65.07	633.3152	2
		R.SLDLDSIIAEVK.A	64.36	651.8544	2
		K.SLNNQFASFIDK.V	63.41	692.3422	2
		R.TNAENEFVTIKK.D	62.73	697.3619	2
		K.YEELQITAGR.H	62.07	590.2975	2
		K.WELLQQVDTSTR.T	61.95	738.3707	2
		K.NKLNDLEDALQQAK.E	56.59	533.9437	3
		R.DYQELMNTK.L	55.73	571.2567	2
		K.LNDLEDALQQAK.E	55.26	679.3450	2

		K.KQISNLQQSISDAEQR.G	49.20	615.6467	3
		K.SLNNQFASFIDKVR.F	48.67	819.9242	2
		K.NMQDMVEDYR.N	47.46	650.7608	2
		K.IEISELNR.V	44.37	487.2635	2
		R.GSYGSGGSSYGSGGGSYGSGGGGGGHGSYGSGSSSGGYR.G	40.47	1104.7621	3
	Q5JAK2|ACTG_RANLE	K.LC(+57.02)YVALDFEQEMATAASSSSLEK.S	64.65	850.7204	3
		K.SYELPDGQVITIGNER.F	64.00	895.9409	2
		R.VAPEEHPVLLTEAPLNPK.A	62.81	977.5270	2
		R.HQGVMVGMGQKDSYVGDEAQSK.R	51.21	588.5181	4
		K.SYELPDGQVITIGN.E	49.77	753.3698	2
		R.AVFPSIVGRPR.H	49.14	599.8499	2
		R.DLTDYLMK.I	45.61	499.7416	2
6	A7E3Q8|PLST_BOVIN	K.ISFDEFVYIFQEVK.S	71.30	882.4435	2
		K.IGLFADIELSR.N	71.24	617.3400	2
		K.LNLAFVANLFNK.Y	69.10	682.3864	2
		K.MVMTVFAC(+57.02)LMGR.G	56.99	708.3340	2
		K.TISSSLAVVDLIDAIQPGC(+57.02)INYDLVK.S	20.88	935.4946	3
	P04264|K2C1_HUMAN	R.SGGGFSSGSAGIINYQR.R	103.48	829.3904	2
		K.YEELQITAGR.H	89.61	590.2976	2
		R.FLEQQNQVLQTK.W	78.96	738.3889	2
		R.GGGGGGYGSGGSSYGSGGGSYGSGGGGGGGGR.G	73.21	1192.4720	2
		R.FSSC(+57.02)GGGGGSFGAGGGFGSR.S	70.36	883.3622	2

		K.QISNLQQSISDAEQR.G	63.70	858.9195	2
		L.LQPLNVEIDPEIQK.V	54.14	818.4437	2
		R.TNAENEFVTIK.K	49.27	633.3142	2
		R.TNAENEFVTIKK.D	43.08	697.3632	2
		R.GSYGSGGSSYGSGGGSYGSGGGGGGHGSY.G	40.11	1208.9606	2
	Q26065|ACT_PLAMG	K.SYELPDGQVITIGNER.F	84.95	895.9429	2
		R.VAPEEHPVLLTEAPLNPK.A	84.82	977.5280	2
		R.HQGVMVGMGQKDSYVGDEAQSK.R	42.88	588.5182	4
7	A5A6M6|K2C1_PANTR	R.SLVNLGGSKSISISVAR.G	79.79	844.4842	2
		R.FSSC(+57.02)GGGGGSFGAGGGFGSR.S	69.33	883.3671	2
		R.SGGGGGRFSSC(+57.02)GGGGGSFGAGGGFGSR.S	67.56	765.3257	3
		R.FLEQQNQVLQTK.W	67.20	738.3937	2
		R.SLDLDSIIAEVK.A	66.73	651.8579	2
		R.TNAENEFVTIKK.D	66.08	465.2470	3
		K.YEELQITAGR.H	65.38	590.3003	2
		R.SGYRSGGGFSSGSAGIINYQR.R	65.28	707.6719	3
		R.SGGGFSSGSAGIINYQRR.T	65.09	605.3004	3
		K.AEAESLYQSKYEELQITAGR.H	58.53	762.7106	3
		R.TNAENEFVTIK.K	57.96	633.3190	2
		R.SGGGFSSGSAGIINYQR.R	56.80	829.3943	2
		K.SLNNQFASFIDKVR.F	54.99	546.9553	3
		K.LALDLEIATYR.T	52.24	639.3559	2
		K.SLNNQFASFIDK.V	50.94	692.3447	2
		K.AEAESLYQSK.Y	46.91	563.2722	2

		K.SISISVAR.G	30.86	416.7462	2
		R.GSGGGSSGGSIGGRGSSSGGVK.S	30.44	584.6111	3
	P04264|K2C1_HUMAN	R.SGGGFSSGSAGIINYQR.R	88.64	829.3939	2
		K.WELLQQVDTSTR.T	77.47	738.3712	2
		K.YEELQITAGR.H	77.20	590.2980	2
		R.FLEQQNQVLQTK.W	75.99	738.3910	2
		R.TNAENEFVTIK.K	75.78	633.3171	2
		R.GGGGGGYGSGGSSYGSGGGSYGSGGGGGGGR.G	75.17	1192.4706	2
		K.SLNNQFASFIDK.V	64.60	692.3420	2
		K.QISNLQQSISDAEQR.G	62.91	858.9222	2
		K.IEISELNR.V	48.90	487.2648	2
		R.TNAENEFVTIKK.D	48.60	697.3663	2
		L.NVEIDPEIQK.V	41.47	592.8064	2
		K.LNDLEDALQQAK.E	40.98	679.3463	2
8	P63261|ACTG_HUMAN	R.VAPEEHPVLLTEAPLNPK.A	114.98	652.0230	3
		K.SYELPDGQVITIGNER.F	110.08	895.9458	2
		R.AVFPSIVGRPR.H	97.56	400.2378	3
		K.AGFAGDDAPR.A	85.68	488.7256	2
	P11021|GRP78_HUMAN	K.DNHLLGTFDLTGIPPAPR.G	82.06	967.5056	2
		R.AKFEELNM(+15.99)DLFR.S	73.20	764.8740	2
		R.IEIESFYEGEDFSETLTR.A	69.93	1082.9946	2
		R.GVPQIEVTFEIDVNGILR.V	44.97	1000.0419	2
	P04264|K2C1_HUMAN	R.SGGGFSSGSAGIINYQR.R	86.55	829.4000	2
		K.LNDLEDALQQAK.E	82.45	679.3514	2
		K.SLNNQFASFIDK.V	78.86	692.3474	2

		K.QISNLQQSISDAEQR.G	78.65	858.9296	2
		K.YEELQITAGR.H	78.28	590.3024	2
		R.FLEQQNQVLQTK.W	76.88	738.3953	2
		R.TNAENEFVTIK.K	76.13	633.3217	2
		K.WELLQQVDTSTR.T	70.76	738.3780	2
		R.DYQELMNTK.L	54.76	571.2627	2
		K.IEISELNR.V	50.74	487.2685	2
		R.TNAENEFVTIKK.D	48.15	697.3691	2
		Q.FASFIDK.V	42.46	414.2179	2
	P08418|HSP70_SCHMA	K.NQVAMNPTNTVFDAK.R	87.75	825.4045	2
		R.TVSDAVITVPAYFNDSQR.Q	47.94	991.9959	2
	P63270|ACTH_CHICK	K.SYELPDGQVITIGNER.F	70.78	895.9498	2
		R.GYSFVTTAER.E	48.48	565.7770	2
9	P02769|ALBU_BOVIN	K.VPQVSTPTLVEVSR.S	127.05	756.4209	2
		K.LFTFHADIC(+57.02)TLPDTEK.Q	107.86	954.4592	2
		K.DAIPENLPPLTADFAEDK.D	98.00	978.4794	2
		K.DAFLGSFLYEYSR.R	97.60	784.3710	2
		K.HLVDEPQNLIK.Q	96.45	653.3578	2
		K.TVMENFVAFVDK.C	87.34	700.3465	2
		K.TVM(+15.99)ENFVAFVDK.C	85.36	708.3448	2
		K.DAIPENLPPLTADFAEDKDVC(+57.02)K.N	82.60	820.0610	3
		K.LGEY(+15.99)GFQNALIVR.Y	78.73	748.3943	2
		R.HPEYAVSVLLR.L	76.50	642.3566	2
		K.EYEATLEEC(+57.02)C(+57.02)AK.D	76.03	751.8063	2
		K.EAC(+57.02)FAVEGPK.L	75.03	554.2581	2

		R.M(+15.99)PC(+57.02)TEDYLSLILNR.L	67.40	870.9170	2
		K.LVNELTEFAK.T	64.60	582.3159	2
		K.YIC(+57.02)DNQDTISSK.L	54.82	722.3220	2
		K.LVVSTQTALA	52.19	501.7929	2
		K.DAFLGSFLYEY(+15.99)SR.R	15.97	792.3687	2
10	P53470|ACT1_SCHMA	K.QEYDESGPGIVHR.K	82.41	496.2355	3
		R.VAPEEHPVLLTEAPLNPK.A	77.44	977.5363	2
		K.SYELPDGQVITIGNER.F	77.12	895.9500	2
		K.AGFAGDDAPR.A	71.09	488.7278	2
		R.AVFPSIVGRPR.H	69.18	400.2403	3
		R.HQGVMVGMGQK.D	67.08	586.2893	2
		K.EISALAPSTM(+15.99)K.I	53.37	582.3024	2
		K.DSYVGDEAQSK.R	51.97	599.7653	2
		K.QEYDESGPGIVHRK.C	51.69	807.8992	2
		K.EISALAPSTMK.I	50.95	574.3049	2
		R.GYSFTTTAER.E	49.63	566.7667	2
		K.DSYVGDEAQSKR.G	46.19	677.8162	2
		R.HQGVMVGMGQKDSYVGDEAQSK.R	42.67	784.3629	3
		K.IKIVAPPER.K	39.64	511.8219	2
		M.AEEDVAALVIDNGSGMC(+57.02)K.A	35.29	939.9144	2
11	P68365|TBA1C_CRIGR	R.FDGALNVDLTEFQTNLVPYPR.I	100.83	1205.1023	2
		R.QLFHPEQLITGK.E	90.61	705.8873	2
		R.NLDIERPTYTNLNR.L	90.52	573.6296	3
		K.TIGGGDDSFNTFFSETGAGK.H	87.93	1004.4474	2

		R.IHFPLATYAPVISAEK.A	85.34	586.3235	3
		K.VGINYQPPTVVPGGDLAK.V	80.17	912.9924	2
		R.LISQIVSSITASLR.F	75.67	744.4378	2
		R.AVFVDLEPTVIDEVR.T	75.24	567.9711	3
		R.LDHKFDLMYAK.R	72.07	690.8498	2
		K.LADQC(+57.02)TGLQGFLVFHSFGGGTGSGFTSLLMER.L	69.88	1130.8711	3
		K.DVNAAIATIK.T	65.07	508.2901	2
		K.EIIDLVLDR.I	61.49	543.3116	2
		K.AYHEQLTVAEITNAC(+57.02)FEPANQMVK.C	54.59	922.1061	3
		R.TIQFVDWC(+57.02)PTGFK.V	51.03	799.8851	2
	P20399|ACT2_XENTR	K.MTQIMFETFNVPAMYVAIQAVLSLYASGR.T	98.07	1084.5416	3
		K.LC(+57.02)YVALDFENEMATAASSSSLEK.S	97.07	846.0535	3
		K.DSYVGDEAQSK.R	90.27	599.7622	2
		R.VAPEEHPTLLTEAPLNPK.A	83.13	652.6838	3
		K.SYELPDGQVITIGNER.F	74.29	895.9459	2
		K.YSVWIGGSILASLSTFQQMWITK.Q	72.82	872.7856	3
		R.C(+57.02)PETLFQPSFIGMESAGIHETTYNSIMK.C	33.48	1063.4855	3
	P13645|K1C10_HUMAN	K.GSLGGGFSSGGFSGGSFSR.G	62.85	854.3828	2
		K.ELTTEIDNNIEQISSYK.S	62.21	998.9811	2
		G.SSGGGC(+57.02)FGGSSGGYGGLGGFGGGSFR.G	54.79	1143.4698	2
		R.ALEESNYELEGK.I	49.99	691.3215	2
		K.QSLEASLAETEGR.Y	46.65	695.8367	2

		G.SLGGGFSSGGFSGGSFSR.G	41.58	825.8706	2
		K.DAEAWFNEK.S	40.61	555.2431	2
	P69003|ACT1_HELTB	R.VAPEEHPVLLTEAPLNPK.A	54.43	977.5280	2
		K.SYELPDGQVITIGNER.F	54.17	895.9420	2
		K.DLYANTVLSGGSTMFPGIADR.M	49.52	1093.0254	2
		Y.VALDFEQEMSTAASSSSLEK.S	46.66	1065.4879	2
		K.LC(+57.02)YVALDFEQEMSTAASSSSLEK.S	46.64	1283.5781	2
		K.AGFAGDDAPR.A	42.77	488.7233	2
		R.AVFPSIVGR.P	40.30	473.2743	2
	P06604|TBA2_DROME	K.VGINYQPPTVVPGGDLAK.V	50.27	912.9879	2
		R.AVFVDLEPTVVDEVR.T	48.48	844.4402	2
		K.DVNAAIATIK.T	44.83	508.2889	2
12	P20399|ACT2_XENTR	K.MTQIMFETFNVPAMYVAIQAVLSLYASGR.T	101.17	1084.5411	3
		K.SYELPDGQVITIGNER.F	81.12	895.9448	2
		K.LC(+57.02)YVALDFENEMATAASSSSLEK.S	66.39	846.0524	3
		K.YSVWIGGSILASLSTFQQMWITK.Q	64.70	872.7861	3
		R.KYSVWIGGSILASLSTFQQMWITK.Q	61.60	915.4791	3
		R.C(+57.02)PETLFQPSFIGMESAGIHETTYNSIMK.C	51.42	1063.4855	3
	P68365|TBA1C_CRIGR	R.FDGALNVDLTEFQTNLVPYPR.I	76.77	1205.1008	2
		R.LISQIVSSITASLR.F	71.07	744.4412	2
		R.AVFVDLEPTVIDEVR.T	61.35	851.4545	2
		R.IHFPLATYAPVISAEK.A	56.29	586.3224	3

13	P13645|K1C10_HUMAN	K.SKELTTEIDNNIEQISSYK.S	59.38	1106.5439	2
		K.GSLGGGFSSGGFSGGSFSR.G	53.23	854.3836	2
		K.ELTTEIDNNIEQISSYK.S	52.92	998.9813	2
		R.AETEC(+57.02)QNTEYQQLLDIK.I	52.69	1041.9785	2
		R.GSSGGGC(+57.02)FGGSSGGYGGLGGFGGGSFR.G	49.27	1171.9880	2
		R.ALEESNYELEGK.I	47.72	691.3226	2
		K.QSLEASLAETEGR.Y	46.51	695.8379	2
		K.IRLENEIQTYR.S	46.18	717.8823	2
		L.GGGFSSGGFSGGSFSR.G	45.18	725.8151	2
		R.SQYEQLAEQNR.K	44.39	683.3173	2
		R.SLLEGEGSSGGGGR.G	42.60	631.7969	2
		R.QSVEADINGLR.R	41.46	601.3071	2
		R.LKYENEVALR.Q	40.90	617.8383	2
	Q3MHM5|TBB4B_BOVIN	R.IMNTFSVVPSPK.V	47.93	660.3493	2
		R.AVLVDLEPGTMDSVR.S	47.15	801.4078	2
		K.GHYTEGAELVDSVLDVVR.K	45.69	979.9834	2
		K.MSATFIGNSTAIQELFK.R	44.66	929.4661	2
		R.ISEQFTAMFR.R	42.71	615.2978	2
		R.INVYYNEATGGK.Y	42.63	664.8225	2
		R.MSMKEVDEQMLNVQNK.N	42.16	641.9656	3
		H.SLGGGTGSGMGTLLISK.I	42.05	768.4023	2
		K.LAVNMVPFPR.L	41.97	572.3162	2
		R.FPGQLNADLR.K	40.13	565.7961	2
	P46561|ATPB_CAEEL	R.FTQAGSEVSALLGR.I	46.13	718.3746	2
		R.IPSAVGYQPTLATDMGSMQER.I	46.08	751.3554	3

		K.AHGGYSVFAGVGER.T	44.71	703.8378	2
		K.VSLVYGQMNEPPGAR.A	44.28	809.3998	2
14	P13645|K1C10_HUMAN	K.NQILNLTTDNANILLQIDNAR.L	121.63	789.7539	3
		R.NVQALEIELQSQLALK.Q	100.99	899.0049	2
		K.ADLEMQIESLTEELAYLK.K	96.09	1048.5219	2
		K.ADLEM(+15.99)QIESLTEELAYLK.K	88.28	1056.5209	2
		K.GSLGGGFSSGGFSGGSFSR.G	56.17	854.3835	2
	P02769|ALBU_BOVIN	K.LFTFHADIC(+57.02)TLPDTEK.Q	121.77	954.4591	2
		K.DAFLGSFLYEYSR.R	114.07	784.3707	2
		K.LGEY(+15.99)GFQNALIVR.Y	98.00	748.3927	2
		K.TVMENFVAFVDK.C	96.78	700.3458	2
		K.TVM(+15.99)ENFVAFVDK.C	91.59	708.3441	2
		R.HPEYAVSVLLR.L	84.17	642.3554	2
		K.DAIPENLPPLTADFAEDK.D	82.04	978.4789	2
		R.HPEY(+15.99)AVSVLLR.L	77.82	650.3536	2
		K.QTALVELLK.H	49.23	507.8102	2
	A7ZHA4|DNAK_ECO24	K.IELSSAQQTDVNLPYITADATGPK.H	110.23	844.7629	3
		K.VALQDAGLSVSDIDDVILVGGQTR.M	92.02	1221.1411	2
		K.TAIESALTALETALK.G	91.10	766.4282	2
		R.KDVNPDEAVAIGAAVQGGVLTGDVK.D	84.94	808.4265	3
		K.DVNPDEAVAIGAAVQGGVLTGDVK.D	79.29	765.7277	3
	P04264|K2C1_HUMAN	R.SGGGFSSGSAGIINYQR.R	83.62	829.3984	2
		K.WELLQQVDTSTR.T	78.96	738.3770	2

		K.YEELQITAGR.H	78.21	590.3029	2
		R.FLEQQNQVLQTK.W	73.21	738.3960	2
		R.TNAENEFVTIK.K	73.06	633.3212	2
		K.SLNNQFASFIDK.V	71.62	692.3484	2
		R.DYQELMNTK.L	70.97	571.2628	2
		K.LNDLEDALQQAK.E	70.40	679.3514	2
		K.QISNLQQSISDAEQR.G	66.97	858.9263	2
		R.SLDLDSIIAEVK.A	63.27	651.8597	2
		K.NKLNDLEDALQQAK.E	56.61	800.4199	2
		K.NMQDMVEDYR.N	49.99	650.7678	2
		K.IEISELNR.V	47.81	487.2675	2
		K.AEAESLYQSKYEELQITAGR.H	46.08	762.7122	3
		L.NVEIDPEIQK.V	43.11	592.8107	2
	Q9W6Y1|HSP7C_ORYLA	R.IINEPTAAAIAYGLDK.K	81.89	830.4507	2
		K.DAGTISGLNVLR.I	66.90	608.3370	2
		K.NQVAMNPTNTVFDAK.R	63.99	825.4003	2
		R.IINEPTAAAIAYGLDKK.V	53.57	596.6672	3
		K.SINPDEAVAYGAAVQA.A	49.40	788.3844	2
		K.SINPDEAVAYGAAVQAA.I	43.28	823.9020	2
		K.SINPDEAVAYGAAVQAAIL.S	42.12	936.9874	2
		K.SINPDEAVAYGAAVQ.A	40.24	752.8674	2
15	P13645|K1C10_HUMAN	K.TIDDLKNQILNLTTDNANILLQIDNAR.L	103.89	1018.2082	3
		R.NVSTGDVNVEMNAAPGVDLTQLLNNMR.S	90.17	958.1332	3
		R.NVQALEIELQSQLALK.Q	89.86	899.0048	2
		K.NQILNLTTDNANILLQIDNAR.L	89.47	789.7552	3

		K.ADLEMQIESLTEELAYLK.K	73.78	1048.5214	2
	P02769|ALBU_BOVIN	K.AEFVEVTKLVTDLTK.V	108.29	846.9686	2
		K.LKPDPNTLC(+57.02)DEFK.A	105.51	526.2585	3
		K.C(+57.02)C(+57.02)AADDKEAC(+57.02)FAVEGPK.L	104.16	643.2679	3
		K.DAFLGSFLYEYSR.R	101.47	523.2494	3
		R.M(+15.99)PC(+57.02)TEDYLSLILNR.L	99.73	870.9145	2
		K.LFTFHADIC(+57.02)TLPDTEK.Q	97.27	954.4578	2
		K.EYEATLEEC(+57.02)C(+57.02)AK.D	96.58	751.8069	2
		K.DAIPENLPPLTADFAEDKDVC(+57.02)K.N	93.44	820.0610	3
		K.DAIPENLPPLTADFAEDK.D	92.80	978.4775	2
		R.KVPQVSTPTLVEVSR.S	92.63	820.4683	2
		K.VPQVSTPTLVEVSR.S	91.09	756.4215	2
		K.DAFLGSFLYEY(+15.99)SR.R	89.93	792.3692	2
		K.EAC(+57.02)FAVEGPK.L	88.54	554.2581	2
		K.HLVDEPQNLIK.Q	88.10	653.3582	2
		K.TVMENFVAFVDK.C	86.91	700.3466	2
		K.TVM(+15.99)ENFVAFVDK.C	86.23	708.3431	2
		R.HPEYAVSVLLR.L	86.22	428.5729	3
		K.EC(+57.02)C(+57.02)HGDLLEC(+57.02)ADDR.A	86.08	583.8899	3
		K.GLVLIAFSQYLQQC(+57.02)PFDEHVK.L	85.13	831.4206	3
		R.HPEY(+15.99)AVSVLLR.L	82.03	433.9044	3
		K.DDPHAC(+57.02)YSTVFDK.L	80.48	777.8263	2

		K.YNGVFQEC(+57.02)C(+57.02)QAEDK.G	75.27	874.3519	2
		R.RPC(+57.02)FSALTPDETYVPK.A	73.79	940.9597	2
		K.YIC(+57.02)DNQDTISSK.L	72.73	722.3215	2
		K.LVNELTEFAK.T	65.75	582.3162	2
	P13645|K1C10_HUMAN	K.GSLGGGFSSGGFSGGSFSR.G	103.53	854.3891	2
		K.ELTTEIDNNIEQISSYK.S	91.49	998.9890	2
		R.SQYEQLAEQNR.K	82.78	683.3214	2
		R.ALEESNYELEGK.I	82.75	691.3257	2
		R.ISSSKGSLGGGFSSGGFSGGSFSR.G	75.98	737.3527	3
		L.GGGFSSGGFSGGSFSR.G	69.35	725.8202	2
		R.VLDELTLTK.A	66.04	516.3018	2
		K.SKELTTEIDNNIEQISSYK.S	63.72	1106.5593	2
		F.GGSSGGYGGLGGFGGGSFR.G	61.01	817.3705	2
		K.SKELTTEIDNNIEQISS.Y	60.63	960.9712	2
		K.GSLGGGFSSGGFSGGSF.S	59.46	732.8218	2
		G.SLGGGFSSGGFSGGSFSR.G	57.27	825.8782	2
		R.QSVEADINGLR.R	55.67	601.3098	2
		I.SSSKGSLGGGFSSGGFSGGSFSR.G	55.58	699.6583	3
		K.QSLEASLAETEGR.Y	53.04	695.8424	2
		L.TTEIDNNIEQISSYK.S	51.91	877.9235	2
		R.LENEIQTYR.S	51.20	583.2949	2
		K.GSLGGGFSSGGF.S	50.95	515.2343	2
		K.GSLGGGFSSGGFSGGS.F	50.08	659.2883	2
		K.DAEAWFNEK.S	49.88	555.2476	2

		R.KDAEAWFNEK.S	48.78	619.2962	2
		E.IDNNIEQISSYK.S	47.82	712.3557	2
		S.SKGSLGGGFSSGGFSGGSFSR.G	47.70	641.6356	3
		G.FSSGGFSGGSFSR.G	44.34	640.2863	2
		K.GSLGGGFSSGGFSGGSFS.R	43.16	776.3391	2
		K.VTMQNLNDR.L	42.84	545.7679	2
		K.DAEAWFNEKSK.E	42.60	662.8128	2
		L.EIELQSQLALK.Q	42.59	636.3633	2
		K.SKELTTEIDNNIEQ.I	42.47	817.4003	2
		G.GFSSGGFSGGSFSR.G	41.69	668.7985	2
		D.NNIEQISSYK.S	41.65	598.3003	2
		R.SLLEGEGSSGGGGR.G	40.70	631.8019	2
16	O17502|ACTM_BRALA	K.SYELPDGQVITIGNER.F	97.21	895.9510	2
		K.AGFAGDDAPR.A	78.49	488.7282	2
		R.GYSFVTTAER.E	70.94	565.7782	2
		K.DAYVGDEAQSKR.G	58.53	669.8179	2
		R.FLEQQNQVLQTK.W	64.05	738.3962	2
17	P68556|ACT1_DIPDE	K.SYELPDGQVITIGNER.F	96.62	895.9556	2
		R.VAPEEHPVLLTEAPLNPK.A	75.96	652.0303	3
		K.LC(+57.02)YVALDFEQEMATAA SSSSLEK.S	75.70	850.7382	3
		K.QEYDESGPGIVHR.K	74.22	496.2382	3
		R.AVFPSIVGRPR.H	73.17	599.8603	2
		R.HQGVMVGMGQK.D	71.43	586.2906	2
		K.DSYVGDEAQSK.R	70.30	599.7672	2
		K.AGFAGDDAPR.A	68.93	488.7289	2

		K.IKIVAPPER.K	40.82	511.8244	2
18	Q90X97|ACTS_ATRMM	K.SYELPDGQVITIGNER.F	97.72	895.9466	2
		R.VAPEEHPTLLTEAPLNPK.A	96.48	652.6829	3
		K.YSVWIGGSILASLSTFQQMWITK.Q	95.34	872.7842	3
		K.LC(+57.02)YVALDFENEMATAASSSSLEK.S	84.36	1268.5775	2
		K.LC(+57.02)YVALDFENEM(+15.99)ATAASSSSLEK.S	54.89	1276.5742	2
		K.YSVWIGGSILASLSTFQQM(+15.99)WITK.Q	16.68	1316.6689	2
		K.DLYANNVMSGGTTMYPGIADR.M	11.84	1123.5105	2
	P02769|ALBU_BOVIN	K.LGEYGFQNALIVR.Y	103.16	740.3992	2
		K.DAFLGSFLYEYSR.R	102.92	784.3708	2
		R.M(+15.99)PC(+57.02)TEDYLSLILNR.L	95.58	870.9139	2
		K.TVMENFVAFVDK.C	87.61	700.3467	2
		K.SLHTLFGDELC(+57.02)K.V	86.45	710.3480	2
		R.HPEYAVSVLLR.L	78.60	642.3566	2
		R.MPC(+57.02)TEDYLSLILNR.L	77.67	862.9158	2
		K.LVNELTEFAK.T	67.62	582.3171	2
		K.EYEATLEEC(+57.02)C(+57.02)AK.D	58.56	751.8066	2
		K.LFTFHADIC(+57.02)TLPDTEK.Q	57.36	954.4592	2
	Q91ZK5|ACTB_SIGHI	K.LC(+57.02)YVALDFEQEMATAASSSSLEK.S	106.31	1275.5778	2
		R.VAPEEHPVLLTEAPLNPK.A	80.36	977.5281	2
		K.SYELPDGQVITIGNER.F	79.65	895.9401	2

		R.TTGIVMDSGDGVTHTVPIYEGYALPHAILR.L	77.34	1061.8661	3
		R.VAPEEHPVLLTEAPLN.P	40.06	864.9536	2
	P02769|ALBU_BOVIN	K.LGEYGFQNALIVR.Y	89.71	740.3955	2
		K.LVNELTEFAK.T	74.73	582.3140	2
		R.RHPEYAVSVLLR.L	65.63	480.6046	3
		K.HLVDEPQNLIK.Q	64.53	653.3553	2
		K.LVVSTQTALA	47.88	501.7900	2
		K.SHC(+57.02)IAEVEKDAIPENLPPLTADFAEDKDVC(+57.02)K.N	40.46	878.6646	4
	A5A6M6|K2C1_PANTR	K.YEELQITAGR.H	79.14	590.2976	2
		R.SGGGFSSGSAGIINYQR.R	76.86	829.3912	2
		K.SLNNQFASFIDK.V	65.02	692.3413	2
		R.SGYRSGGGFSSGSAGIINY.Q	59.96	918.9222	2
		R.TNAENEFVTIK.K	54.23	633.3152	2
		R.GGGGGGYGSGGSSYGSGGGSYGSGGGGGGGR.G	40.20	1192.4698	2
19	P53470|ACT1_SCHMA	K.SYELPDGQVITIGNER.F	82.44	895.9576	2
		R.AVFPSIVGRPR.H	80.60	400.2426	3
		K.QEYDESGPGIVHR.K	79.21	496.2365	3
		R.VAPEEHPVLLTEAPLNPK.A	76.14	977.5430	2
		K.AGFAGDDAPR.A	71.47	488.7286	2
		R.HQGVMVGMGQK.D	67.72	586.2924	2
		K.EISALAPSTMK.I	53.47	574.3082	2
		K.QEYDESGPGIVHRK.C	51.61	807.9025	2
		K.DSYVGDEAQSK.R	48.52	599.7650	2
	P53464|ACTM_HELTB	K.DLYANTVLSGGTSMYPGIADR.M	81.48	1101.0237	2

		K.SYELPDGQVITIGNER.F	79.01	895.9410	2
		R.VAPEEHPVLLTEAPLNPK.A	66.68	977.5290	2
		H.PVLLTEAPLNPK.A	61.95	646.3804	2
		R.GYSFTTTAER.E	56.39	566.7623	2
		R.AVFPSIVGRPR.H	53.40	599.8505	2
		R.VAPEEHPVLLTEAPLNPKANR.E	51.67	765.7463	3
		Y.ELPDGQVITIGNER.F	50.67	770.8947	2
		R.VAPEEHPVLLTEAPLN.P	42.65	864.9548	2
		R.HQGVMVGMGQKDSYVGDEAQSK.R	40.27	588.5208	4
	P04264|K2C1_HUMAN	R.FSSC(+57.02)GGGGGSFGAGGGFGSR.S	71.79	883.3640	2
		K.YEELQITAGR.H	67.08	590.2986	2
		R.TNAENEFVTIK.K	64.43	633.3169	2
		K.QISNLQQSISDAEQR.G	62.58	858.9219	2
		R.SGGGFSSGSAGIINYQR.R	51.40	829.3940	2
		R.SGYRSGGGFSSGSAGIIN.Y	48.03	837.3890	2
20	Q90X97|ACTS_ATRMM	K.DLYANNVM(+15.99)SGGTTM(+15.99)YPGIADR.M	124.10	1139.5037	2
		R.KDLYANNVMSGGTTMYPGIADR.M	98.08	1187.5520	2
		R.TTGIVLDSGDGVTHNVPIYEGYALPHAIMR.L	95.68	1066.2029	3
		K.SYELPDGQVITIGNER.F	94.32	895.9491	2
		R.VAPEEHPTLLTEAPLNPK.A	94.25	978.5168	2
		K.DLYANNVMSGGTTMYPGIADR.M	86.98	1123.5054	2
		K.AGFAGDDAPR.A	86.64	488.7253	2

		R.AVFPSIVGRPR.H	85.33	400.2383	3
		K.QEYDEAGPSIVHR.K	82.62	750.8542	2
		K.EITALAPSTMK.I	80.73	581.3096	2
		R.HQGVMVGMGQK.D	80.44	586.2862	2
		K.DLYANNVM(+15.99)SGGTTMYPGIADR.M	77.38	1131.5059	2
		R.DLTDYLMKILTER.G	75.90	805.9221	2
		K.DLYANNVMSGGTTM(+15.99)YPGIADR.M	74.73	1131.5059	2
		K.YSVWIGGSILASLSTFQQM(+15.99)WITK.Q	74.70	1316.6703	2
		R.GYSFVTTAER.E	73.62	565.7748	2
		K.EITALAPSTM(+15.99)K.I	72.22	589.3070	2
		R.HQGVM(+15.99)VGM(+15.99)GQK.D	68.93	401.8564	3
		K.LC(+57.02)YVALDFENEMATAASSSSLEK.S	68.35	1268.5806	2
		K.LC(+57.02)YVALDFENEM(+15.99)ATAASSSSLEK.S	66.76	1276.5736	2
		R.DLTDYLM(+15.99)KILTER.G	65.28	813.9210	2
		K.IWHHTFYNELR.V	63.22	758.3728	2
		K.LC(+57.02)Y(+15.99)VALDFENEMATAASSSSLEK.S	59.77	1276.5741	2
		R.DLTDYLMK.I	55.93	499.7433	2
		R.DLTDYLM(+15.99)K.I	53.86	507.7414	2
		K.DSYVGDEAQSK.R	52.67	599.7617	2
21	P68556|ACT1_DIPDE	R.VAPEEHPVLLTEAPLNPK.A	106.56	652.0280	3
		K.SYELPDGQVITIGNER.F	86.99	895.9539	2
		K.QEYDESGPGIVHR.K	83.16	496.2359	3

		K.QEYDESGPGIVHRK.C	76.68	807.8989	2
		K.DSYVGDEAQSK.R	65.35	599.7668	2
		K.LC(+57.02)YVALDFEQEMATAASSSSLEK.S	57.51	850.7329	3
		K.DSYVGDEAQSKR.G	48.02	677.8156	2
		R.TTGIVLDSGDGVTHSVPIYEGYALPHAILR.L	40.29	788.6683	4
	A5A6M6|K2C1_PANTR	K.WELLQQVDTSTR.T	82.73	738.3777	2
		R.FSSC(+57.02)GGGGGSFGAGGGFGSR.S	79.73	883.3718	2
		R.FLEQQNQVLQTK.W	77.26	738.3964	2
		R.SLDLDSIIAEVK.A	66.83	651.8622	2
		R.TNAENEFVTIKK.D	64.77	697.3642	2
		K.SLNNQFASFIDK.V	63.52	692.3506	2
		R.SGGGFSSGSAGIINYQR.R	53.05	829.4178	2
	P04264|K2C1_HUMAN	K.QISNLQQSISDAEQR.G	57.83	858.9217	2
		K.WELLQQVDTSTR.T	55.33	738.3708	2
		R.FLEQQNQVLQTK.W	53.31	738.3895	2
		K.YEELQITAGR.H	52.11	590.2986	2
		R.TNAENEFVTIK.K	51.11	633.3156	2
		K.SLNNQFASFIDK.V	50.15	692.3417	2
		R.DYQELMNTK.L	47.34	571.2575	2
		R.SGGGFSSGSAGIINYQR.R	46.27	829.3926	2
		K.AEAESLYQSKYEELQITAGR.H	44.21	1143.5537	2
		K.NMQDMVEDYR.N	42.39	650.7622	2
		K.LNDLEDALQQAK.E	42.18	679.3447	2
		K.IEISELNR.V	40.18	487.2643	2

		R.FSSC(+57.02)GGGGGSFGAGGGFGSR.S	40.05	883.3635	2
	Q5R1X3|ACTB_PANTR	K.LC(+57.02)YVALDFEQEMATAASSSSLEK.S	54.18	1275.5759	2
		K.SYELPDGQVITIGNER.F	53.91	895.9414	2
		R.VAPEEHPVLLTEAPLNPK.A	44.62	652.0198	3
		K.DLYANTVLSGGTTMYPGIADR.M	42.22	1108.0287	2
22	A5A6M6|K2C1_PANTR	K.WELLQQVDTSTR.T	97.25	738.3749	2
		K.YEELQITAGR.H	86.38	590.3011	2
		R.FLEQQNQVLQTK.W	85.77	738.3931	2
		R.THNLEPYFESFINNLR.R	82.50	665.3277	3
		R.SLDLDSIIAEVK.A	79.30	651.8582	2
		K.LALDLEIATYR.T	78.47	639.3550	2
		K.SLNNQFASFIDKVR.F	74.35	546.9559	3
		R.TNAENEFVTIKK.D	73.55	697.3665	2
		K.SLNNQFASFIDK.V	68.92	692.3448	2
		R.TNAENEFVTIK.K	61.14	633.3180	2
	P63261|ACTG_HUMAN	K.SYELPDGQVITIGNER.F	112.85	895.9460	2
		R.VAPEEHPVLLTEAPLNPK.A	98.51	652.0233	3
		R.AVFPSIVGRPR.H	93.26	400.2378	3
		K.AGFAGDDAPR.A	75.38	488.7257	2
	P08779|K1C16_HUMAN	R.LLEGEDAHLSSQQASGQS.Y	66.34	928.9247	2
		K.IIAATIENAQPILQIDNAR.L	59.22	1032.5648	2
		R.ALEEANADLEVK.I	59.01	651.3271	2
		R.APSTYGGGLSVSSR.F	58.24	669.8305	2
		K.TEELNKEVASNSELVQSSR.S	54.51	707.3472	3
		R.VLDELTLAR.T	50.22	515.2963	2

		R.ISSVLAGGSC(+57.02)R.A	46.50	553.7800	2
		R.QTVEADVNGLR.R	45.88	601.3064	2
		R.LLEGEDAHLSSQQASGQSYSSR.E	45.55	1175.5400	2
		R.LLEGEDAHLSSQQASGQSY.S	41.62	1010.4570	2
		R.GQTGGDVNVEMDAAPGVDLSR.I	41.06	1044.4794	2
	P53480|ACTC_TAKRU	R.GYSFVTTAER.E	51.88	565.7720	2
		K.SYELPDGQVITIGNER.F	48.89	895.9406	2
23	Q9UVX4|ACT_COPC7	K.SYELPDGQVITIGNER.F	87.91	895.9506	2
		R.VAPEEHPVLLTEAPLNPK.A	63.74	977.5371	2
	A5A6M6|K2C1_PANTR	R.SLDLDSIIAEVK.A	63.67	651.8625	2
		K.SLNNQFASFIDK.V	59.70	692.3502	2
	P04264|K2C1_HUMAN	R.FLEQQNQVLQTK.W	59.43	738.3892	2
		K.YEELQITAGR.H	57.20	590.2975	2
		K.WELLQQVDTSTR.T	55.95	738.3710	2
		K.QISNLQQSISDAEQR.G	53.62	858.9202	2
		R.TNAENEFVTIK.K	53.49	633.3154	2
		R.SGGGFSSGSAGIINYQR.R	51.79	829.3881	2
		K.SLNNQFASFIDK.V	48.34	692.3406	2
		R.GGGGGGYGSGGSSYGSGGGSYGSGGGGGGGR.G	41.90	1192.4718	2
	P84185|ACT5C_ANOGA	R.VAPEEHPVLLTEAPLNPK.A	61.21	977.5255	2
		K.LC(+57.02)YVALDFEQEMATAASSSSLEK.S	60.66	1275.5774	2
		K.SYELPDGQVITIGNER.F	56.06	895.9387	2
		K.AGFAGDDAPR.A	47.29	488.7224	2
		R.GYSFTTTAER.E	46.98	566.7609	2

		K.DLYANTVLSGGTTMYPGIADR.M	44.73	1108.0267	2
		K.SYELPDGQVITIGN.E	41.42	753.3693	2
24	P86221|TBB2C_MESAU	K.LAVNMVPFPR.L	65.50	572.3218	2
		R.AVLVDLEPGTMDSVR.S	58.44	801.4161	2
		K.LAVNM(+15.99)VPFPR.L	49.66	580.3181	2
	P13645|K1C10_HUMAN	K.GSLGGGFSSGGFSGGSFSR.G	76.68	854.3823	2
		K.QSLEASLAETEGR.Y	71.43	695.8372	2
		R.ALEESNYELEGK.I	69.72	691.3212	2
		R.GSSGGGC(+57.02)FGGSSGGYG GLGGFGGGSFR.G	63.82	1171.9835	2
		K.ELTTEIDNNIEQISSYK.S	62.64	998.9788	2
		R.SLLEGEGSSGGGGR.G	59.56	631.7955	2
		R.VLDELTLTK.A	59.23	516.2977	2
		R.QSVEADINGLR.R	58.18	601.3059	2
		R.LENEIQTYR.S	56.14	583.2897	2
		R.AETEC(+57.02)QNTEYQQLLDIK. I	54.73	1041.9766	2
		K.VTMQNLNDR.L	52.85	545.7639	2
		K.DAEAWFNEK.S	52.65	555.2431	2
		R.LKYENEVALR.Q	50.96	617.8370	2
		K.SKELTTEIDNNIEQISSYK.S	44.96	738.0306	3
		L.GGGFSSGGFSGGSFSR.G	43.11	725.8136	2
		R.LAADDFR.L	40.05	404.1987	2
	Q9YHC3|TBB1_GADMO	R.IMNTFSVVPSPK.V	71.85	660.3483	2
		R.AVLVDLEPGTMDSVR.S	64.17	801.4061	2
		R.INVYYNEASGGK.Y	59.12	657.8135	2
		K.LAVNMVPFPR.L	59.07	572.3145	2

		R.FPGQLNADLR.K	57.78	565.7958	2
		K.NSSYFVEWIPNNVK.T	56.09	848.9114	2
		K.TAVC(+57.02)DIPPR.G	49.14	514.7585	2
		K.EVDEQMLNVQNK.N	47.34	723.8420	2
	Q05825|ATPB_DROME	R.FTQAGSEVSALLGR.I	72.01	718.3731	2
		R.IPSAVGYQPTLATDMGSMQER.I	68.51	751.3527	3
		R.TIAMDGTEGLVR.G	66.66	631.8178	2
		K.AHGGYSVFAGVGER.T	61.50	703.8376	2
		K.VVDLLAPYAK.G	58.28	544.8149	2
		K.IGLFGGAGVGK.T	55.03	488.2797	2
		R.VALTGLTVAEYFR.D	45.11	720.3911	2
	Q5R1X3|ACTB_PANTR	K.LC(+57.02)YVALDFEQEMATAA SSSSLEK.S	62.58	1275.5751	2
		K.SYELPDGQVITIGNER.F	59.92	895.9416	2
		K.DLYANTVLSGGTTMYPGIADR.M	50.34	1108.0262	2
25	Q9YHC3|TBB1_GADMO	K.NSSYFVEWIPNNVK.T	89.33	848.9203	2
		R.IMNTFSVVPSPK.V	89.14	660.3566	2
		R.ISEQFTAMFR.R	80.41	615.3033	2
		R.AVLVDLEPGTM(+15.99)DSVR.S	78.72	809.4127	2
		K.LAVNMVPFPR.L	74.75	572.3217	2
		K.GHYTEGAELVDSVLDVVR.K	73.60	653.6667	3
		R.INVYYNEASGGK.Y	68.48	657.8196	2
		R.AVLVDLEPGTMDSVR.S	67.72	801.4149	2
		R.LHFFM(+15.99)PGFAPLTSR.G	66.85	546.2808	3
		R.YLTVAAIFR.G	65.66	527.3104	2
		K.EVDEQMLNVQNK.N	63.45	723.8488	2

		R.LHFFMPGFAPLTSR.G	63.35	810.9218	2
		K.TAVC(+57.02)DIPPR.G	57.79	514.7632	2
		R.EIVHLQAGQC(+57.02)GNQIGAK.F	56.86	911.9662	2
	P13645|K1C10_HUMAN	R.NVQALEIELQSQLALK.Q	82.14	899.0120	2
		R.AETEC(+57.02)QNTEYQQLLDIK.I	77.10	1041.9874	2
		K.IRLENEIQTYR.S	66.83	717.8892	2
		R.QSVEADINGLRR.V	66.55	679.3636	2
		K.VTM(+15.99)QNLNDR.L	57.47	553.7661	2
		R.LENEIQTYR.S	54.56	583.2952	2
		K.VRALEESNYELEGK.I	52.58	818.9135	2
		K.ADLEMQIESLTEELAYLK.K	50.58	1048.5312	2
		R.LASYLDK.V	44.72	405.2234	2
	Q05825|ATPB_DROME	R.FTQAGSEVSALLGR.I	86.14	718.3812	2
		R.DQEGQDVLLFIDNIFR.F	80.08	961.4869	2
		K.VVDLLAPYAK.G	75.91	544.8224	2
		K.IGLFGGAGVGK.T	62.80	488.2861	2
		R.VALTGLTVAEYFR.D	62.51	720.4012	2
		R.TIAM(+15.99)DGTEGLVR.G	50.96	639.8212	2
	P13645|K1C10_HUMAN	K.GSLGGGFSSGGFSGGSFSR.G	76.35	854.3817	2
		K.SKELTTEIDNNIEQISSYK.S	70.62	738.0312	3
		K.ELTTEIDNNIEQISSYK.S	69.72	998.9785	2
		K.QSLEASLAETEGR.Y	64.67	695.8366	2
		R.SQYEQLAEQNR.K	64.49	683.3167	2
		R.ALEESNYELEGK.I	63.90	691.3192	2
		R.QSVEADINGLR.R	61.76	601.3065	2

		R.VLDELTLTK.A	61.51	516.2972	2
		K.IRLENEIQTYR.S	59.85	717.8814	2
		R.LKYENEVALR.Q	59.16	617.8367	2
		R.AETEC(+57.02)QNTEYQQLLDIK.I	58.56	1041.9772	2
		L.GGGFSSGGFSGGSFSR.G	52.85	725.8140	2
		K.DAEAWFNEK.S	48.55	555.2428	2
		R.LENEIQTYR.S	47.31	583.2896	2
		N.AAPGVDLTQLLNNMR.S	46.57	806.9175	2
		R.NVSTGDVNVEMN.A	41.86	639.7795	2
		R.LAADDFR.L	41.09	404.1990	2
		I.SSSKGSLGGGFSSGGFSGGSFSR.G	40.38	699.6532	3
		R.RVLDELTLTK.A	40.10	396.5666	3
	P68556|ACT1_DIPDE	K.LC(+57.02)YVALDFEQEMATAASSSSLEK.S	71.62	1275.5778	2
		K.SYELPDGQVITIGNER.F	69.82	895.9412	2
		R.VAPEEHPVLLTEAPLNPK.A	68.62	977.5269	2
		R.HQGVMVGMGQKDSYVGDEAQSK.R	56.18	784.3561	3
		K.YSVWIGGSILASLSTFQQMWISK.Q	54.62	1301.6680	2
		R.AVFPSIVGRPR.H	54.50	599.8505	2
		R.GYSFTTTAER.E	54.25	566.7605	2
		R.DLTDYLMK.I	53.85	499.7412	2
		K.SYELPDGQVITIGN.E	52.24	753.3689	2
		R.AVFPSIVGR.P	47.10	473.2750	2
		R.HQGVMVGMGQKDSYVGDEAQ	43.26	627.5428	4

		SKR.G			
		K.DLYANTVLSGGTTMYPGIADR.M	42.59	1108.0271	2
	P30883|TBB4_XENLA	R.AVLVDLEPGTMDSVR.S	68.74	801.4056	2
		R.IMNTFSVVPSPK.V	61.74	660.3475	2
		R.FPGQLNADLR.K	46.04	565.7949	2
		K.LAVNMVPFPR.L	45.64	572.3135	2
	P46561|ATPB_CAEEL	R.FTQAGSEVSALLGR.I	70.98	718.3729	2
		K.VSLVYGQMNEPPGAR.A	62.88	809.3976	2
		K.AHGGYSVFAGVGER.T	43.84	469.5605	3
		K.VVDLLAPYAK.G	43.20	544.8156	2
26	P08779|K1C16_HUMAN	K.IIAATIENAQPILQIDNAR.L	88.95	1032.5770	2
		R.VLQGLEIELQSQLSM(+15.99)K.A	76.77	916.4974	2
		R.NKIIAATIENAQPILQIDNAR.L	72.70	769.4329	3
		R.ALEEANADLEVK.I	69.90	651.3327	2
		R.GQTGGDVNVEM(+15.99)DAAPGVDLSR.I	68.06	1052.4857	2
		R.TDLEMQIEGLK.E	65.41	638.8270	2
		R.QTVEADVNGLRR.V	61.64	679.3629	2
		K.VTM(+15.99)QNLNDR.L	56.70	553.7667	2
		R.DQYEQMAEK.N	41.51	571.2452	2
	Q3ZBU7|TBB4_BOVIN	R.IMNTFSVVPSPK.V	75.69	660.3555	2
		K.LAVNMVPFPR.L	69.06	572.3221	2
		R.AVLVDLEPGTMDSVR.S	68.35	801.4139	2
	P13645|K1C10_HUMAN	K.GSLGGGFSSGGFSGGSFSR.G	108.84	854.3830	2
		R.AETEC(+57.02)QNTEYQQLLDIK.I	107.89	1041.9778	2

		R.ALEESNYELEGK.I	94.20	691.3224	2
		K.QSLEASLAETEGR.Y	92.61	695.8384	2
		R.GSSGGGC(+57.02)FGGSSGGYGGLGGFGGGSFR.G	79.56	1171.9846	2
		K.SKELTTEIDNNIEQISSYK.S	78.38	738.0303	3
		K.ELTTEIDNNIEQISSYK.S	67.74	998.9795	2
		R.VLDELTLTK.A	65.75	516.2979	2
		L.GGGFSSGGFSGGSFSR.G	59.53	725.8135	2
		K.DAEAWFNEK.S	51.31	555.2441	2
		R.LKYENEVALR.Q	46.19	617.8383	2
	P30883|TBB4_XENLA	R.AVLVDLEPGTMDSVR.S	100.01	801.4074	2
		R.IMNTFSVVPSPK.V	84.88	660.3494	2
		K.NSSYFVEWIPNNVK.T	52.67	848.9125	2
		K.LAVNMVPFPR.L	51.58	572.3154	2
		R.ISEQFTAMFR.R	44.53	615.2976	2
27	P08779|K1C16_HUMAN	K.EVASNSELVQSSR.S	73.27	703.3500	2
		R.NKIIAATIENAQPILQIDNAR.L	65.68	769.4349	3
		R.ISSVLAGGSC(+57.02)R.A	62.96	553.7853	2
		R.QTVEADVNGLRR.V	54.06	679.3635	2
		R.LASYLDK.V	42.64	405.2244	2
	P13645|K1C10_HUMAN	K.GSLGGGFSSGGFSGGSFSR.G	108.64	854.3823	2
		R.ALEESNYELEGK.I	78.30	691.3212	2
		R.NVSTGDVNVEMN.A	66.01	639.7795	2
		R.VLDELTLTK.A	53.97	516.2970	2
		K.ELTTEIDNNIEQISSYK.S	51.89	998.9786	2
		R.NVSTGDVNVEMNA.A	49.86	675.2971	2

		K.DAEAWFNEK.S	45.81	555.2438	2
		L.GGGFSSGGFSGGSFSR.G	45.71	725.8158	2
		G.SLGGGFSSGGFSGGSFSR.G	44.52	825.8727	2
		R.QSVEADINGLR.R	41.71	601.3061	2
	Q5R1X3|ACTB_PANTR	K.SYELPDGQVITIGNER.F	98.73	895.9419	2
		K.LC(+57.02)YVALDFEQEMATAASSSSLEK.S	82.26	850.7227	3
		R.VAPEEHPVLLTEAPLNPK.A	65.24	652.0214	3
		F.NTPAMYVAIQAVLSLYASGR.T	64.06	709.0360	3
		R.KYSVWIGGSILASLSTFQQMWISK.Q	62.03	910.8092	3
		K.DLYANTVLSGGTTMYPGIADR.M	59.42	1108.0305	2
		R.GYSFTTTAER.E	52.31	566.7630	2
		K.YSVWIGGSILASLSTFQQMWISK.Q	51.36	868.1229	3
	P02769|ALBU_BOVIN	K.DAFLGSFLYEYSR.R	83.26	784.3680	2
		R.MPC(+57.02)TEDYLSLILNR.L	73.91	862.9183	2
		K.LGEYGFQNALIVR.Y	70.01	740.3944	2
		K.LVVSTQTALA	65.12	501.7909	2
		K.GLVLIAFSQYLQQC(+57.02)PFDEHVK.L	58.47	831.4199	3
		K.LVNELTEFAK.T	57.21	582.3134	2
		R.RHPEYAVSVLLR.L	56.36	480.6046	3
		K.SLHTLFGDELC(+57.02)K.V	54.07	473.8986	3
		K.TVMENFVAFVDK.C	49.88	700.3425	2
		K.HLVDEPQNLIK.Q	47.44	653.3564	2
		Y.FYAPELLYYANK.Y	45.11	746.3723	2
		K.LFTFHADIC(+57.02)TLPDTEK.Q	41.70	954.4558	2

28	Q2KJD0|TBB5_BOVIN	K.GHYTEGAELVDSVLDVVR.K	92.21	979.9882	2
		R.ALTVPELTQQVFDAK.N	77.42	830.4472	2
		R.LHFFM(+15.99)PGFAPLTSR.G	77.42	546.2792	3
		K.MAVTFIGNSTAIQELFK.R	76.48	935.4867	2
		R.AILVDLEPGTM(+15.99)DSVR.S	70.19	816.4146	2
		K.M(+15.99)AVTFIGNSTAIQELFK.R	70.12	943.4847	2
		K.LAVNM(+15.99)VPFPR.L	62.61	580.3156	2
29	P08418|HSP70_SCHMA	K.NQVAMNPTNTVFDAKR.L	79.60	602.6379	3
		R.IINEPTAAAIAYGLDKK.V	71.62	596.6688	3
		R.IINEPTAAAIAYGLDK.K	65.49	830.4517	2
		K.NQVAM(+15.99)NPTNTVFDAK.R	60.78	833.3989	2
		K.VEIIANDQGNR.T	57.49	614.8181	2
		R.TVSDAVITVPAYFNDSQR.Q	45.59	991.9963	2
	P35527|K1C9_HUMAN	R.HGVQELEIELQSQLSKK.A	87.57	656.0261	3
		R.HGVQELEIELQSQLSK.K	56.31	919.4888	2
		K.VQALEEANNDLENK.I	41.81	793.8872	2
		R.LASYLDK.V	40.88	405.2239	2
	Q9W6Y1|HSP7C_ORYLA	K.NQVAMNPTNTVFDAK.R	97.87	825.3956	2
		R.IINEPTAAAIAYGLDK.K	85.67	830.4452	2
		K.SINPDEAVAYGAAVQAAILSGDK.S	66.79	1130.5618	2
		K.DAGTISGLNVLR.I	66.48	608.3344	2
		K.NQVAMNPTNTVFDAKR.L	46.99	602.6325	3
		R.IINEPTAAAIAYGLDKK.V	46.76	894.4915	2
		K.VEIIANDQGNR.T	45.50	614.8131	2

	A5A6M6|K2C1_PANTR	R.FLEQQNQVLQTK.W	81.10	738.3911	2
		R.TNAENEFVTIK.K	75.94	633.3168	2
		K.YEELQITAGR.H	70.11	590.2988	2
		K.SLNNQFASFIDK.V	64.16	692.3423	2
		K.WELLQQVDTSTR.T	56.62	738.3724	2
		K.QISNLQQSISDAEQR.G	53.50	858.9225	2
		K.IEISELNR.V	47.00	487.2633	2
		R.SGGGFSSGSAGIINYQR.R	45.30	829.3955	2
30	P02538|K2C6A_HUMAN	R.FLEQQNKVLETK.W	51.20	492.9408	3
		R.GSGGLGGAC(+57.02)GGAGFGSR.S	41.04	712.8224	2
	P13645|K1C10_HUMAN	K.GSLGGGFSSGGFSGGSFSR.G	54.59	854.3817	2
		K.QSLEASLAETEGR.Y	53.09	695.8366	2
		R.ALEESNYELEGK.I	52.90	691.3212	2
		R.SLLEGEGSSGGGGR.G	45.99	631.7961	2
		K.SKELTTEIDNNIEQISSYK.S	45.71	1106.5424	2
		R.LENEIQTYR.S	45.09	583.2901	2
		R.VLDELTLTK.A	44.60	516.2971	2
		L.GGGFSSGGFSGGSFSR.G	43.44	725.8125	2
		K.ELTTEIDNNIEQISSYK.S	43.21	998.9813	2
		R.QSVEADINGLR.R	41.35	601.3061	2
		K.IRLENEIQTYR.S	40.75	717.8810	2
		R.GSSGGGC(+57.02)FGGSSGGYGGLGGFGGGSFR.G	40.57	1171.9844	2
		K.DAEAWFNEK.S	40.23	555.2428	2

Table 4. Supporting peptides for the proteins identified by MS/MS analysis of SDS-I listed in Table 3. Spot numbers refer to Fig. 14

Table 6

Spot n.	Protein Accession	Peptide	-10lgP	m/z	z
1	P46561|ATPB_CAEEL	AHGGYSVFAGVGER	86.76	703.8434	2
		DQEGQDVLLFIDNIFR	74.15	961.4874	2
		VSLVYGQMNEPPGAR	70.77	809.4055	2
		VVDLLAPYAK	69.59	544.8220	2
		VSLVYGQM(+15.99)NEPPGAR	64.41	817.4019	2
		IGLFGGAGVGK	49.51	488.2841	2
	Q3MHM5|TBB2C_BOVIN	IMNTFSVVPSPK	74.82	660.3542	2
		LAVNM(+15.99)VPFPR	66.40	580.3181	2
		LAVNMVPFPR	66.11	572.3214	2
		AVLVDLEPGTM(+15.99)DSVR	60.88	809.4100	2
		AVLVDLEPGTMDSVR	59.35	801.4162	2
	Q6EIY9|K2C1_CANFA	TNAENEFVTIKK	69.83	465.2471	3
		FLEQQNQVLQTK	64.22	738.3936	2
		SLNNQFASFIDK	51.56	692.3497	2
		ALEESNYELEGK	62.98	691.3265	2
2	Q3ZBU7|TBB4_BOVIN	R.AVLVDLEPGTM(+15.99)DSVR.S	73.03	809.4104	2
		R.AVLVDLEPGTMDSVR.S	71.75	801.4130	2
		R.IMNTFSVVPSPK.V	60.65	660.3341	2
3	A5A6M6|K2C1_PANTR	R.TNAENEFVTIKK.D	78.17	465.2481	3

		R.FLEQQNQVLQTK.W	76.77	738.3968	2
		K.AQYEDIAQK.S	63.68	533.2637	2
4	P86700|ACT_CHIOP	R.SYELPDGQVITIGNER.C	73.70	895.9501	2
		R.AVFPSIVGRPR.D	69.15	400.2397	3
		AGFAGDDAPR.A	63.59	488.7275	2
5	P86700|ACT_CHIOP	R.SYELPDGQVITIGNER.C	82.21	895.9490	2
		R.AVFPSIVGRPR.D	69.67	400.2397	3
		AGFAGDDAPR.A	62.49	488.7268	2
6	P91754|ACT_LUMRU	R.VAPEEHPVLLTEAPLNPK.A	80.22	977.5341	2
		K.SYELPDGQVITIGNER.F	80.18	895.9476	2
		R.AVFPSIVGRPR.H	72.87	400.2391	3
		K.AGFAGDDAPR.A	70.78	488.7277	2
		R.HQGVMVGMGQK.D	59.18	586.2876	2
		R.GYSFTTTAER.E	45.26	566.7665	2
7	A5DQP9|ACT_PICGU	R.AVFPSIVGRPR.H	76.51	400.2400	3
		K.SYELPDGQVITIGNER.F	74.36	895.9512	2
8	P86700|ACT_CHIOP	R.AVFPSIVGRPR.D	79.35	400.2404	3
		AGFAGDDAPR.A	73.23	488.7280	2
		K.IIAPPER.K	41.91	398.2402	2
9	P13645|K1C10_HUMAN	R.SLLEGEGSSGGGGR.G	59.13	631.8001	2
		R.LAADDFR.L	40.71	404.2024	2
		R.LASYLDK.V	40.30	405.2233	2

	P06604|TBA2_DROME	K.VGINYQPPTVVPGGDLAK.V	51.83	912.9938	2
		R.AVFVDLEPTVVDEVR.T	40.98	844.4468	2
10	A5DQP9|ACT_PICGU	K.SYELPDGQVITIGNER.F	72.44	895.9477	2
		R.AVFPSIVGRPR.H	70.44	599.8553	2

Table 6. Supporting peptides for the proteins identified by MS/MS analysis of SDS-I listed in Table 5. Spot numbers refer to Fig. 15

REFERENCES

1. Anderson, H.C., *Matrix vesicles and calcification.* Curr Rheumatol Rep, 2003. **5**(3): p. 222-6.
2. Devaux, P.F., *Static and dynamic lipid asymmetry in cell membranes.* Biochemistry, 1991. **30**(5): p. 1163-73.
3. Zwaal, R.F. and A.J. Schroit, *Pathophysiologic implications of membrane phospholipid asymmetry in blood cells.* Blood, 1997. **89**(4): p. 1121-32.
4. Sims, P.J. and T. Wiedmer, *Unraveling the mysteries of phospholipid scrambling.* Thromb Haemost, 2001. **86**(1): p. 266-75.
5. Freyssinet, J.M., *Cellular microparticles: what are they bad or good for?* J Thromb Haemost, 2003. **1**(7): p. 1655-62.
6. Thery, C., M. Ostrowski, and E. Segura, *Membrane vesicles as conveyors of immune responses.* Nat Rev Immunol, 2009. **9**(8): p. 581-93.
7. Pilzer, D., et al., *Emission of membrane vesicles: roles in complement resistance, immunity and cancer.* Springer Semin Immunopathol, 2005. **27**(3): p. 375-87.
8. Johnstone, R.M., *Exosomes biological significance: A concise review.* Blood Cells Mol Dis, 2006. **36**(2): p. 315-21.
9. Stein, J.M. and J.P. Luzio, *Ectocytosis caused by sublytic autologous complement attack on human neutrophils. The sorting of endogenous plasma-membrane proteins and lipids into shed vesicles.* Biochem J, 1991. **274 (Pt 2)**: p. 381-6.
10. Cocucci, E., G. Racchetti, and J. Meldolesi, *Shedding microvesicles: artefacts no more.* Trends Cell Biol, 2009. **19**(2): p. 43-51.
11. Lee, T.L., et al., *Stress-relaxation of fibroblasts in collagen matrices triggers ectocytosis of plasma membrane vesicles containing actin, annexins II and VI, and beta 1 integrin receptors.* J Cell Sci, 1993. **105 (Pt 1)**: p. 167-77.
12. Dolo, V., et al., *Selective localization of matrix metalloproteinase 9, beta1 integrins, and human lymphocyte antigen class I molecules on membrane vesicles shed by 8701-BC breast carcinoma cells.* Cancer Res, 1998. **58**(19): p. 4468-74.
13. Al-Nedawi, K., et al., *Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells.* Nat Cell Biol, 2008. **10**(5): p. 619-24.
14. Pascual, M., et al., *Identification of membrane-bound CR1 (CD35) in human urine: evidence for its release by glomerular podocytes.* J Exp Med, 1994. **179**(3): p. 889-99.

15. Lescuyer, P., et al., *Proteomic analysis of a podocyte vesicle-enriched fraction from human normal and pathological urine samples.* Proteomics Clin Appl, 2008. **2**(7-8): p. 1008-18.
16. Kerr, J.F., A.H. Wyllie, and A.R. Currie, *Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics.* Br J Cancer, 1972. **26**(4): p. 239-57.
17. Hristov, M., et al., *Apoptotic bodies from endothelial cells enhance the number and initiate the differentiation of human endothelial progenitor cells in vitro.* Blood, 2004. **104**(9): p. 2761-6.
18. Beyer, C. and D.S. Pisetsky, *The role of microparticles in the pathogenesis of rheumatic diseases.* Nat Rev Rheumatol, 2010. **6**(1): p. 21-9.
19. Ihara, T., et al., *The process of ultrastructural changes from nuclei to apoptotic body.* Virchows Arch, 1998. **433**(5): p. 443-7.
20. Elmore, S., *Apoptosis: a review of programmed cell death.* Toxicol Pathol, 2007. **35**(4): p. 495-516.
21. Takizawa, F., S. Tsuji, and S. Nagasawa, *Enhancement of macrophage phagocytosis upon iC3b deposition on apoptotic cells.* FEBS Lett, 1996. **397**(2-3): p. 269-72.
22. Martinez, M.C. and J.M. Freyssinet, *Deciphering the plasma membrane hallmarks of apoptotic cells: phosphatidylserine transverse redistribution and calcium entry.* BMC Cell Biol, 2001. **2**: p. 20.
23. Friedl, P., P. Vischer, and M.A. Freyberg, *The role of thrombospondin-1 in apoptosis.* Cell Mol Life Sci, 2002. **59**(8): p. 1347-57.
24. Mevorach, D., et al., *Complement-dependent clearance of apoptotic cells by human macrophages.* J Exp Med, 1998. **188**(12): p. 2313-20.
25. Savill, J., et al., *Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis.* J Clin Invest, 1992. **90**(4): p. 1513-22.
26. van Engeland, M., et al., *Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure.* Cytometry, 1998. **31**(1): p. 1-9.
27. Samos, J., et al., *Circulating nucleic acids in plasma/serum and tumor progression: are apoptotic bodies involved? An experimental study in a rat cancer model.* Ann N Y Acad Sci, 2006. **1075**: p. 165-73.
28. Bergsmedh, A., et al., *Horizontal transfer of oncogenes by uptake of apoptotic bodies.* Proc Natl Acad Sci U S A, 2001. **98**(11): p. 6407-11.

29. Thery, C., L. Zitvogel, and S. Amigorena, *Exosomes: composition, biogenesis and function.* Nat Rev Immunol, 2002. **2**(8): p. 569-79.
30. Gruenberg, J. and H. Stenmark, *The biogenesis of multivesicular endosomes.* Nat Rev Mol Cell Biol, 2004. **5**(4): p. 317-23.
31. Keller, S., et al., *Exosomes: from biogenesis and secretion to biological function.* Immunol Lett, 2006. **107**(2): p. 102-8.
32. Williams, R.L. and S. Urbe, *The emerging shape of the ESCRT machinery.* Nat Rev Mol Cell Biol, 2007. **8**(5): p. 355-68.
33. Shields, S.B., et al., *ESCRT ubiquitin-binding domains function cooperatively during MVB cargo sorting.* J Cell Biol, 2009. **185**(2): p. 213-24.
34. Woodman, P.G. and C.E. Futter, *Multivesicular bodies: co-ordinated progression to maturity.* Curr Opin Cell Biol, 2008. **20**(4): p. 408-14.
35. Razi, M. and C.E. Futter, *Distinct roles for Tsg101 and Hrs in multivesicular body formation and inward vesiculation.* Mol Biol Cell, 2006. **17**(8): p. 3469-83.
36. Slagsvold, T., et al., *Endosomal and non-endosomal functions of ESCRT proteins.* Trends Cell Biol, 2006. **16**(6): p. 317-26.
37. van Niel, G., et al., *Exosomes: a common pathway for a specialized function.* J Biochem, 2006. **140**(1): p. 13-21.
38. Kobayashi, T., et al., *A lipid associated with the antiphospholipid syndrome regulates endosome structure and function.* Nature, 1998. **392**(6672): p. 193-7.
39. Wurmser, A.E. and S.D. Emr, *Phosphoinositide signaling and turnover: PtdIns(3)P, a regulator of membrane traffic, is transported to the vacuole and degraded by a process that requires lumenal vacuolar hydrolase activities.* EMBO J, 1998. **17**(17): p. 4930-42.
40. Xiao, Z., et al., *Proteomic analysis of extracellular matrix and vesicles.* J Proteomics, 2009. **72**(1): p. 34-45.
41. Marsh, M. and G. van Meer, *Cell biology. No ESCRTs for exosomes.* Science, 2008. **319**(5867): p. 1191-2.
42. Trajkovic, K., et al., *Ceramide triggers budding of exosome vesicles into multivesicular endosomes.* Science, 2008. **319**(5867): p. 1244-7.
43. Raposo, G., et al., *Distinct protein sorting and localization to premelanosomes, melanosomes, and lysosomes in pigmented melanocytic cells.* J Cell Biol, 2001. **152**(4): p. 809-24.

44. Mathivanan, S. and R.J. Simpson, *ExoCarta: A compendium of exosomal proteins and RNA.* Proteomics, 2009. **9**(21): p. 4997-5000.

45. Thery, C., et al., *Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles.* J Immunol, 2001. **166**(12): p. 7309-18.

46. Mears, R., et al., *Proteomic analysis of melanoma-derived exosomes by two-dimensional polyacrylamide gel electrophoresis and mass spectrometry.* Proteomics, 2004. **4**(12): p. 4019-31.

47. Pisitkun, T., R.F. Shen, and M.A. Knepper, *Identification and proteomic profiling of exosomes in human urine.* Proc Natl Acad Sci U S A, 2004. **101**(36): p. 13368-73.

48. Gonzales, P.A., et al., *Large-scale proteomics and phosphoproteomics of urinary exosomes.* J Am Soc Nephrol, 2009. **20**(2): p. 363-79.

49. Potolicchio, I., et al., *Proteomic analysis of microglia-derived exosomes: metabolic role of the aminopeptidase CD13 in neuropeptide catabolism.* J Immunol, 2005. **175**(4): p. 2237-43.

50. Valadi, H., et al., *Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells.* Nat Cell Biol, 2007. **9**(6): p. 654-9.

51. Choi, D.S., et al., *Proteomic analysis of microvesicles derived from human colorectal cancer cells.* J Proteome Res, 2007. **6**(12): p. 4646-55.

52. Mathivanan, S., et al., *Proteomics analysis of A33 immunoaffinity-purified exosomes released from the human colon tumor cell line LIM1215 reveals a tissue-specific protein signature.* Mol Cell Proteomics, 2010. **9**(2): p. 197-208.

53. Hegmans, J.P., et al., *Proteomic analysis of exosomes secreted by human mesothelioma cells.* Am J Pathol, 2004. **164**(5): p. 1807-15.

54. Graner, M.W., et al., *Proteomic and immunologic analyses of brain tumor exosomes.* FASEB J, 2009. **23**(5): p. 1541-57.

55. Kramer-Albers, E.M., et al., *Oligodendrocytes secrete exosomes containing major myelin and stress-protective proteins: Trophic support for axons?* Proteomics Clin Appl, 2007. **1**(11): p. 1446-61.

56. Kesimer, M., et al., *Characterization of exosome-like vesicles released from human tracheobronchial ciliated epithelium: a possible role in innate defense.* FASEB J, 2009. **23**(6): p. 1858-68.

57. Conde-Vancells, J., et al., *Characterization and comprehensive proteome profiling of exosomes secreted by hepatocytes.* J Proteome Res, 2008. **7**(12): p. 5157-66.

58. Fevrier, B., et al., *Cells release prions in association with exosomes.* Proc Natl Acad Sci U S A, 2004. **101**(26): p. 9683-8.

59. Looze, C., et al., *Proteomic profiling of human plasma exosomes identifies PPARgamma as an exosome-associated protein.* Biochem Biophys Res Commun, 2009. **378**(3): p. 433-8.

60. Admyre, C., et al., *Exosomes with immune modulatory features are present in human breast milk.* J Immunol, 2007. **179**(3): p. 1969-78.

61. Staubach, S., H. Razawi, and F.G. Hanisch, *Proteomics of MUC1-containing lipid rafts from plasma membranes and exosomes of human breast carcinoma cells MCF-7.* Proteomics, 2009. **9**(10): p. 2820-35.

62. Gonzalez-Begne, M., et al., *Proteomic analysis of human parotid gland exosomes by multidimensional protein identification technology (MudPIT).* J Proteome Res, 2009. **8**(3): p. 1304-14.

63. Ji, H., et al., *Difference gel electrophoresis analysis of Ras-transformed fibroblast cell-derived exosomes.* Electrophoresis, 2008. **29**(12): p. 2660-71.

64. Mathivanan, S., H. Ji, and R.J. Simpson, *Exosomes: extracellular organelles important in intercellular communication.* J Proteomics, 2010. **73**(10): p. 1907-20.

65. Corbeel, L. and K. Freson, *Rab proteins and Rab-associated proteins: major actors in the mechanism of protein-trafficking disorders.* Eur J Pediatr, 2008. **167**(7): p. 723-9.

66. Stenmark, H., *Rab GTPases as coordinators of vesicle traffic.* Nat Rev Mol Cell Biol, 2009. **10**(8): p. 513-25.

67. Futter, C.E. and I.J. White, *Annexins and endocytosis.* Traffic, 2007. **8**(8): p. 951-8.

68. Denzer, K., et al., *Follicular dendritic cells carry MHC class II-expressing microvesicles at their surface.* J Immunol, 2000. **165**(3): p. 1259-65.

69. Raposo, G., et al., *Accumulation of major histocompatibility complex class II molecules in mast cell secretory granules and their release upon degranulation.* Mol Biol Cell, 1997. **8**(12): p. 2631-45.

70. Raposo, G., et al., *B lymphocytes secrete antigen-presenting vesicles.* J Exp Med, 1996. **183**(3): p. 1161-72.

71. Segura, E., et al., *ICAM-1 on exosomes from mature dendritic cells is critical for efficient naive T-cell priming.* Blood, 2005. **106**(1): p. 216-23.

72. Veron, P., et al., *Accumulation of MFG-E8/lactadherin on exosomes from immature dendritic cells.* Blood Cells Mol Dis, 2005. **35**(2): p. 81-8.

73. Raiborg, C., et al., *FYVE and coiled-coil domains determine the specific localisation of Hrs to early endosomes.* J Cell Sci, 2001. **114**(Pt 12): p. 2255-63.
74. Nair, J., et al., *Sec2 protein contains a coiled-coil domain essential for vesicular transport and a dispensable carboxy terminal domain.* J Cell Biol, 1990. **110**(6): p. 1897-909.
75. Li, X.B., et al., *Role of exosomes in immune regulation.* J Cell Mol Med, 2006. **10**(2): p. 364-75.
76. Camussi, G., et al., *Exosome/microvesicle-mediated epigenetic reprogramming of cells.* Am J Cancer Res, 2011. **1**(1): p. 98-110.
77. Denzer, K., et al., *Exosome: from internal vesicle of the multivesicular body to intercellular signaling device.* J Cell Sci, 2000. **113 Pt 19**: p. 3365-74.
78. Cheruvanky, A., et al., *Rapid isolation of urinary exosomal biomarkers using a nanomembrane ultrafiltration concentrator.* Am J Physiol Renal Physiol, 2007. **292**(5): p. F1657-61.
79. Schorey, J.S. and S. Bhatnagar, *Exosome function: from tumor immunology to pathogen biology.* Traffic, 2008. **9**(6): p. 871-81.
80. Simons, M. and G. Raposo, *Exosomes--vesicular carriers for intercellular communication.* Curr Opin Cell Biol, 2009. **21**(4): p. 575-81.
81. Hu, C., et al., *Analytical strategies in lipidomics and applications in disease biomarker discovery.* J Chromatogr B Analyt Technol Biomed Life Sci, 2009. **877**(26): p. 2836-46.
82. Subra, C., et al., *Exosome lipidomics unravels lipid sorting at the level of multivesicular bodies.* Biochimie, 2007. **89**(2): p. 205-12.
83. Wubbolts, R., et al., *Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation.* J Biol Chem, 2003. **278**(13): p. 10963-72.
84. de Gassart, A., et al., *Lipid raft-associated protein sorting in exosomes.* Blood, 2003. **102**(13): p. 4336-44.
85. Zhou, H., et al., *Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery.* Kidney Int, 2006. **69**(8): p. 1471-6.
86. Adachi, J., et al., *The human urinary proteome contains more than 1500 proteins, including a large proportion of membrane proteins.* Genome Biol, 2006. **7**(9): p. R80.
87. Kourembanas, S., *Exosomes: Vehicles of Intercellular Signaling, Biomarkers, and Vectors of Cell Therapy.* Annu Rev Physiol, 2014.
88. Arslan, F., et al., *Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent*

adverse remodeling after myocardial ischemia/reperfusion injury. Stem Cell Res, 2013. **10**(3): p. 301-12.

89. Bryniarski, K., et al., *Antigen-specific, antibody-coated, exosome-like nanovesicles deliver suppressor T-cell microRNA-150 to effector T cells to inhibit contact sensitivity.* J Allergy Clin Immunol, 2013. **132**(1): p. 170-81.
90. Yeo, R.W., et al., *Mesenchymal stem cell: an efficient mass producer of exosomes for drug delivery.* Adv Drug Deliv Rev, 2013. **65**(3): p. 336-41.
91. Tian, Y., et al., *A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy.* Biomaterials, 2014. **35**(7): p. 2383-90.
92. Momen-Heravi, F., et al., *Impact of biofluid viscosity on size and sedimentation efficiency of the isolated microvesicles.* Front Physiol, 2012. **3**: p. 162.
93. Fernandez-Llama, P., et al., *Tamm-Horsfall protein and urinary exosome isolation.* Kidney Int, 2010. **77**(8): p. 736-42.
94. Cantin, R., et al., *Discrimination between exosomes and HIV-1: purification of both vesicles from cell-free supernatants.* J Immunol Methods, 2008. **338**(1-2): p. 21-30.
95. Thery, C., et al., *Isolation and characterization of exosomes from cell culture supernatants and biological fluids.* Curr Protoc Cell Biol, 2006. **Chapter 3**: p. Unit 3 22.
96. Chen, C.Y., M.C. Hogan, and C.J. Ward, *Purification of exosome-like vesicles from urine.* Methods Enzymol, 2013. **524**: p. 225-41.
97. Alvarez, M.L., et al., *Comparison of protein, microRNA, and mRNA yields using different methods of urinary exosome isolation for the discovery of kidney disease biomarkers.* Kidney Int, 2012. **82**(9): p. 1024-32.
98. Rood, I.M., et al., *Comparison of three methods for isolation of urinary microvesicles to identify biomarkers of nephrotic syndrome.* Kidney Int, 2010. **78**(8): p. 810-6.
99. Merchant, M.L., et al., *Microfiltration isolation of human urinary exosomes for characterization by MS.* Proteomics Clin Appl, 2010. **4**(1): p. 84-96.
100. Striefel, S., *Ethical research issues: going beyond the Declaration of Helsinki.* Appl Psychophysiol Biofeedback, 2001. **26**(1): p. 39-59; discussion 67-71.
101. Karande, P., et al., *Synergistic effects of chemical enhancers on skin permeability: a case study of sodium lauroylsarcosinate and sorbitan monolaurate.* Eur J Pharm Sci, 2007. **31**(1): p. 1-7.

102. Brown, R.N., et al., *Mapping the subcellular proteome of Shewanella oneidensis MR-1 using sarkosyl-based fractionation and LC-MS/MS protein identification.* J Proteome Res, 2010. **9**(9): p. 4454-63.

103. Frankel, S., R. Sohn, and L. Leinwand, *The use of sarkosyl in generating soluble protein after bacterial expression.* Proc Natl Acad Sci U S A, 1991. **88**(4): p. 1192-6.

104. Huang da, W., B.T. Sherman, and R.A. Lempicki, *Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources.* Nat Protoc, 2009. **4**(1): p. 44-57.

105. Fraser, K.B., et al., *LRRK2 secretion in exosomes is regulated by 14-3-3.* Hum Mol Genet, 2013. **22**(24): p. 4988-5000.

106. Galli, P., et al., *Head glands of Monogenoidea: morphology, functionality, and potentialities in industrial production of surgery bioadhesives.* J Parasitol, 2009. **95**(6): p. 1330-41.

107. Sinnappah, N.D., et al., *A Paedomorphic parasite associated with a neotenic amphibian host: phylogenetic evidence suggests a revised systematic position for Sphyranuridae within anuran and turtle Polystomatoineans.* Mol Phylogenet Evol, 2001. **18**(2): p. 189-201.

108. Rohde, K., *Studies on the cytology and histology of helminths.* Med J Malaya, 1965. **20**(1): p. 55-6.

109. Maule, A.G., et al., *A cytochemical study of the serotoninergic, cholinergic and peptidergic components of the reproductive system in the monogenean parasite, Diclidophora merlangi.* Parasitol Res, 1990. **76**(5): p. 409-19.

110. Frankland, H.M., *The life history and bionomics of Diclidophora denticulata (Trematoda: Monogenea).* Parasitology, 1955. **45**(3-4): p. 313-51.

111. Kearn, G.C., *Evolutionary expansion of the Monogenea.* Int J Parasitol, 1994. **24**(8): p. 1227-71.

112. Arafa, S.Z., M.M. El-Naggar, and G.C. Kearn, *On some ultrastructural features of the reproductive system of the monogenean parasite Macrogyrodactylus congolensis from Clarias gariepinus inhabiting the River Nile in Egypt.* Acta Parasitol, 2014. **59**(2): p. 238-46.

113. Kearn, G.C., *The eggs of monogeneans.* Adv Parasitol, 1986. **25**: p. 175-273.

114. Whittington, I.D. and G.C. Kearn, *Two new species of Neoentobdella (Monogenea: Capsalidae: Entobdellinae) from the skin of Australian stingrays (Dasyatidae).* Folia Parasitol (Praha), 2009. **56**(1): p. 29-35.

115. Whittington, I.D. and B.W. Cribb, *Adhesive secretions in the Platyhelminthes.* Adv Parasitol, 2001. **48**: p. 101-224.

116. El-Naggar, M.M., et al., *Chaetotaxy of the monogeneans Macrogyrodactylus clarii and m. congolensis from the gills and skin of the catfish clarias gariepinus in Egypt, with a note on argentophilic elements in the nervous system.* Folia Parasitol (Praha), 2001. **48**(3): p. 201-8.

117. Kearn, G.C. and R. Evans-Gowing, *Attachment and detachment of the anterior adhesive pads of the monogenean (platyhelminth) parasite Entobdella soleae from the skin of the common sole (Solea solea).* Int J Parasitol, 1998. **28**(10): p. 1583-93.

118. Buchmann, K. and T. Lindenstrom, *Interactions between monogenean parasites and their fish hosts.* Int J Parasitol, 2002. **32**(3): p. 309-19.

119. Whittington, I.D., et al., *Host-specificity of monogenean (platyhelminth) parasites: a role for anterior adhesive areas?* Int J Parasitol, 2000. **30**(3): p. 305-20.

120. Kay, J.W., A.P. Shinn, and C. Sommerville, *Towards an automated system for the identification of notifiable pathogens: using as an example.* Parasitol Today, 1999. **15**(5): p. 201-6.

121. Buchmann, K., *Binding and lethal effect of complement from Oncorhynchus mykiss on Gyrodactylus derjavini (Platyhelminthes: Monogenea).* Dis Aquat Organ, 1998. **32**(3): p. 195-200.

122. Ogawa, K., et al., *Neobenedenia girellae (Hargis, 1955) Yamaguti, 1963 (Monogenea: Capsalidae) from cultured marine fishes of Japan.* J Parasitol, 1995. **81**(2): p. 223-7.

123. Yamaguti, S., *Preparation of stained whole mounts of flatworms.* Trans Am Microsc Soc, 1965. **84**(4): p. 602-3.

124. Kearn, G.C. and S. MacDonald, *The chemical nature of host hatching factors in the monogenean skin parasites Entobdella soleae and Acanthocotyle lobianchi.* Int J Parasitol, 1976. **6**(6): p. 457-6.

125. Whittington, I.D. and B.W. Cribb, *Glands associated with the anterior adhesive areas of the monogeneans, Entobdella sp. and Entobdella australis (Capsalidae) from the skin of Himantura fai and Taeniura lymma (Dasyatididae).* Int J Parasitol, 1998. **28**(4): p. 653-65.

126. Whittington, I.D. and B.W. Cribb, *Morphology and ultrastructure of the anterior adhesive areas of the capsalid monogenean parasites Benedenia rohdei from the gills and B. lutjani from the pelvic fins of Lutjanus carponotatus (Pisces: Lutjanidae).* Parasitol Res, 1999. **85**(5): p. 399-408.

127. Beverley-Burton, M. and I.D. Whittington, *Clemacotyle australis (Monogenea: Monocotylidae) from the white-spotted eagle ray Aetobatus narinari (Rajiformes: Myliobatididae) on the Great Barrier Reef: redescription, emended generic diagnosis, and oncomiracidium.* J Parasitol, 1995. **81**(4): p. 616-25.

128. Fullenkamp, D.E., et al., *pH-dependent cross-linking of catechols through oxidation via Fe and potential implications for mussel adhesion.* RSC Adv, 2014. **4**(48): p. 25127-25134.

129. Silverman, H.G. and F.F. Roberto, *Understanding marine mussel adhesion.* Mar Biotechnol (NY), 2007. **9**(6): p. 661-81.

130. Gosline, J.M., et al., *The mechanical design of spider silks: from fibroin sequence to mechanical function.* J Exp Biol, 1999. **202**(Pt 23): p. 3295-303.

131. Chirila, T., et al., *Bombyx mori silk fibroin membranes as potential substrata for epithelial constructs used in the management of ocular surface disorders.* Tissue Eng Part A, 2008. **14**(7): p. 1203-11.

132. Schutze, J., et al., *Molecular evolution of the metazoan extracellular matrix: cloning and expression of structural proteins from the demosponges Suberites domuncula and Geodia cydonium.* J Mol Evol, 2001. **53**(4-5): p. 402-15.

133. Flammang, P., R. Santos, and D. Haesaerts, *Echinoderm adhesive secretions: from experimental characterization to biotechnological applications.* Prog Mol Subcell Biol, 2005. **39**: p. 201-20.

134. Hennebert, E., et al., *Characterization of the protein fraction of the temporary adhesive secreted by the tube feet of the sea star Asterias rubens.* Biofouling, 2012. **28**(3): p. 289-303.

135. Jonker, J.L., et al., *Unusual adhesive production system in the barnacle Lepas anatifera: an ultrastructural and histochemical investigation.* J Morphol, 2012. **273**(12): p. 1377-91.

136. Demeuldre, M., et al., *Instantaneous adhesion of Cuvierian tubules in the sea cucumber Holothuria forskali.* Biointerphases, 2014. **9**(2): p. 029016.

137. Van Dyck, S., P. Gerbaux, and P. Flammang, *Qualitative and quantitative saponin contents in five sea cucumbers from the Indian ocean.* Mar Drugs, 2010. **8**(1): p. 173-89.

138. Rizzo, N.W., et al., *Characterization of the structure and composition of gecko adhesive setae.* J R Soc Interface, 2006. **3**(8): p. 441-51.

139. Autumn, K., et al., *Adhesive force of a single gecko foot-hair.* Nature, 2000. **405**(6787): p. 681-5.

140. Autumn, K. and A.M. Peattie, *Mechanisms of adhesion in geckos.* Integr Comp Biol, 2002. **42**(6): p. 1081-90.

141. Geim, A.K., et al., *Microfabricated adhesive mimicking gecko foot-hair.* Nat Mater, 2003. **2**(7): p. 461-3.

142. Alibardi, L., *Ultrastructural autoradiographic and immunocytochemical analysis of setae formation and keratinization in the digital pads of the gecko Hemidactylus turcicus (Gekkonidae, Reptilia).* Tissue Cell, 2003. **35**(4): p. 288-96.

143. Alibardi, L. and R.H. Sawyer, *Immunocytochemical analysis of beta keratins in the epidermis of chelonians, lepidosaurians, and archosaurians.* J Exp Zool, 2002. **293**(1): p. 27-38.

144. Hamwood, T.E., et al., *Preliminary characterisation and extraction of anterior adhesive secretion in monogenean (platyhelminth) parasites.* Folia Parasitol (Praha), 2002. **49**(1): p. 39-49.

145. Flammang, P., et al., *A study of the temporary adhesion of the podia in the sea star asterias rubens (Echinodermata, asteroidea) through their footprints.* J Exp Biol, 1998. **201 (Pt 16)**: p. 2383-95.

146. Dodou, D., et al., *Mechanisms of temporary adhesion in benthic animals.* Biol Rev Camb Philos Soc, 2011. **86**(1): p. 15-32.

147. Edman, P., *A method for the determination of amino acid sequence in peptides.* Arch Biochem, 1949. **22**(3): p. 475.

148. Fales, H.M., et al., *Use of chemical ionization mass spectrometry in analysis of amino acid phenylthiohydantoin derivatives formed during Edman degradation of proteins.* Anal Biochem, 1971. **43**(1): p. 288-99.

149. Schulten, H.R. and B. Wittman-Liebold, *High resolution field desorption mass spectrometry. V. Mixtures of amino acid phenylthiohydantoins and Edman degradation products.* Anal Biochem, 1976. **76**(l): p. 300-10.

150. Bradley, C.V., D.H. Williams, and M.R. Hanley, *Peptide sequencing using the combination of edman degradation, carboxypeptidase digestion and fast atom bombardment mass spectrometry.* Biochem Biophys Res Commun, 1982. **104**(4): p. 1223-30.

151. Williams, D.H., et al., *Fast-atom-bombardment mass spectrometry. A new technique for the determination of molecular weights and amino acid sequences of peptides.* Biochem J, 1982. **201**(1): p. 105-17.

152. Fenn, J.B., et al., *Electrospray ionization for mass spectrometry of large biomolecules.* Science, 1989. **246**(4926): p. 64-71.

153. Perkins, D.N., et al., *Probability-based protein identification by searching sequence databases using mass spectrometry data.* Electrophoresis, 1999. **20**(18): p. 3551-67.

154. Eng, J.K., A.L. McCormack, and J.R. Yates, *An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database.* J Am Soc Mass Spectrom, 1994. **5**(11): p. 976-89.

155. Mann, M. and M. Wilm, *Error-tolerant identification of peptides in sequence databases by peptide sequence tags.* Anal Chem, 1994. **66**(24): p. 4390-9.

156. Keough, T., M.P. Lacey, and R.S. Youngquist, *Derivatization procedures to facilitate de novo sequencing of lysine-terminated tryptic peptides using postsource decay matrix-assisted laser*

desorption/ionization mass spectrometry. Rapid Commun Mass Spectrom, 2000. **14**(24): p. 2348-56.

157. Munchbach, M., et al., *Quantitation and facilitated de novo sequencing of proteins by isotopic N-terminal labeling of peptides with a fragmentation-directing moiety.* Anal Chem, 2000. **72**(17): p. 4047-57.

158. Bartels, C., *Fast algorithm for peptide sequencing by mass spectroscopy.* Biomed Environ Mass Spectrom, 1990. **19**(6): p. 363-8.

159. Chen, T., et al., *A dynamic programming approach to de novo peptide sequencing via tandem mass spectrometry.* J Comput Biol, 2001. **8**(3): p. 325-37.

160. Dancik, V., et al., *De novo peptide sequencing via tandem mass spectrometry.* J Comput Biol, 1999. **6**(3-4): p. 327-42.

161. Taylor, J.A. and R.S. Johnson, *Sequence database searches via de novo peptide sequencing by tandem mass spectrometry.* Rapid Commun Mass Spectrom, 1997. **11**(9): p. 1067-75.

162. Taylor, J.A. and R.S. Johnson, *Implementation and uses of automated de novo peptide sequencing by tandem mass spectrometry.* Anal Chem, 2001. **73**(11): p. 2594-604.

163. Hamm, C.W., W.E. Wilson, and D.J. Harvan, *Peptide sequencing program.* Comput Appl Biosci, 1986. **2**(2): p. 115-8.

164. Hines, W.M., et al., *Pattern-based algorithm for peptide sequencing from tandem high energy collision-induced dissociation mass spectra.* J Am Soc Mass Spectrom, 1992. **3**(4): p. 326-36.

165. Addona, T. and K. Clauser, *De novo peptide sequencing via manual interpretation of MS/MS spectra.* Curr Protoc Protein Sci, 2002. **Chapter 16**: p. Unit 16 11.

166. Ma, B., et al., *PEAKS: powerful software for peptide de novo sequencing by tandem mass spectrometry.* Rapid Commun Mass Spectrom, 2003. **17**(20): p. 2337-42.

167. Batista, C.V., et al., *Proteomics of the venom from the Amazonian scorpion Tityus cambridgei and the role of prolines on mass spectrometry analysis of toxins.* J Chromatogr B Analyt Technol Biomed Life Sci, 2004. **803**(1): p. 55-66.

168. Tedeschi, G., et al., *Characterization of nitroproteome in neuron-like PC12 cells differentiated with nerve growth factor: identification of two nitration sites in alpha-tubulin.* Proteomics, 2005. **5**(9): p. 2422-32.

169. Zhang, J., et al., *PEAKS DB: de novo sequencing assisted database search for sensitive and accurate peptide identification.* Mol Cell Proteomics, 2012. **11**(4): p. M111 010587.

170. Stewart, R.J., T.C. Ransom, and V. Hlady, *Natural Underwater Adhesives.* J Polym Sci B Polym Phys, 2011. **49**(11): p. 757-771.

171. Gantayet, A., D.J. Rees, and E.D. Sone, *Novel proteins identified in the insoluble byssal matrix of the freshwater zebra mussel.* Mar Biotechnol (NY), 2014. **16**(2): p. 144-55.

172. Kamino, K., *Underwater adhesive of marine organisms as the vital link between biological science and material science.* Mar Biotechnol (NY), 2008. **10**(2): p. 111-21.

173. Huang, Z. and G. Zhang, *Identification and secondary structure analysis of a keratin-like fibrous protein discovered in ligament of the bivalve Siliqua radiata.* Biochemistry (Mosc), 2011. **76**(11): p. 1227-32.

174. Skillman, K.M., et al., *Evolutionarily divergent, unstable filamentous actin is essential for gliding motility in apicomplexan parasites.* PLoS Pathog, 2011. **7**(10): p. e1002280.

175. Traver, M.A. and D.G. Assimos, *New generation tissue sealants and hemostatic agents: innovative urologic applications.* Rev Urol, 2006. **8**(3): p. 104-11.

176. Luna, M.S., et al., *Activation of Bothrops jararaca snake venom gland and venom production: a proteomic approach.* J Proteomics, 2013. **94**: p. 460-72.

177. Buttner, D., *Protein export according to schedule: architecture, assembly, and regulation of type III secretion systems from plant- and animal-pathogenic bacteria.* Microbiol Mol Biol Rev, 2012. **76**(2): p. 262-310.

178. Yoshinaga, T., et al., *Attachment-inducing capacities of fish tissue extracts on oncomiracidia of Neobenedenia girellae (Monogenea, Capsalidae).* J Parasitol, 2000. **86**(2): p. 214-9.

179. Ohashi, H., et al., *Purification and identification of a glycoprotein that induces the attachment of oncomiracidia of Neobenedenia girellae (Monogenea, Capsalidae).* Int J Parasitol, 2007. **37**(13): p. 1483-90.

180. Maffioli, E., et al., *A new bioadhesive material from fish parasite Neobenedenia girellae.* J Proteomics, 2014. **110C**: p. 1-6.

ACKNOWLEDGEMENTS

It would not have been possible to write this doctoral thesis without the help and support of the kind people around me, to only some of whom it is possible to give particular mention here.
First of all I would like to thank my advisors: Prof. Gabriella Tedeschi and Prof. Armando Negri for giving me the opportunity to start my PhD at the University of Milan and introducing me to the world of proteomics; Prof. Hanno Steen, for giving me the opportunity to spend 18 months in his laboratory at Boston Children's Hospital, for guiding my work and providing nice discussions and Prof. Judith Steen for the interesting inputs and ideas.
The exosome part of this work could not have been done without the help of Kevin Broadbelt. Therefore I would like to express all my gratitude for his time and dedication to my work, for showing me how to perform experiments, for all the commitment with my personal growth in science and continuous support, for being a great co-worker and a perfect friend.
I also would like to thank the present Steen lab members for all the everyday help and support. Especially I would like to thank Saima Ahmed for being the best lab manager; Jan Muntel for all his patience and for sharing his knowledge about the Q Exactive and TTOF 5600, Ruchi Chauhan for helping me with a couple of projects, Watraud Mair for all her precious advices and suggestions, especially regarding the Sarkosyl fractionation; Sebastian Berger for sharing his knowledge about the TTOF 5600, John Sauld for the great moments we spent together in the lab. Last, but not least, I would like to thank Ceren Uncu for introducing me to the cell culture and Torsten Mueller for helping me with the FASP protocol.
I would like to thank also all my Italian colleagues; in particular, Simona Nonnis for being present despite the long distance and Francesco Corniola.
For the non-scientific side of my thesis, I particularly want to thank my husband Gabriele for understanding the need to be far away for this step of my life, all the support with his love during this hard time of distance.
The most special thank goes to my mother for giving me the strength to reach for the stars and chase my dreams, for her love and support throughout my life and because, without her, I would not be the same person I am today.

I would like to express my heart-felt gratitude to my extended family that has aided and encouraged me throughout this endeavor; in particular, to my grandmother for respecting the decision of leaving.

I'll always be grateful to Isabella for being one of the greatest people I've ever met. She was fundamental in supporting me in those difficult and stressful moments. Moreover, I would never forget all the chats and great moments I shared with some of my Italian friends Eleonora, Stella, Ludovica, Claudia, Alice, Marco and Andrea.

The last thank goes to all the people that made the stay in Boston unique and unforgettable: first of all thanks to my roommates Stefano e Rafael for being like a family, for the good and bad moments spent together. Federica for being a great friend, for laughing and crying together, but also Cate, Teresa, Ali, Carla, Kevin, Sam, Leo, Frida, Tobias, Fiona, Tais, Kate, Omar and Clair for sharing tea time, winter house parties and summer BBQs, Halloween in Salem and the great time spent together.

.