Innate immune responses to sterile inflammation after acute brain injury and cardiac arrest

Clinical, methodological, and experimental considerations

PhD Thesis

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Lesya Ukrainka «Don't betray, my Dream!»

Don't betray, my Dream!
I eagered you so much,
Had these sleepless nights and days.
And now I dream at last.
Don't disappear, my dream,
The precious light of my exhausted eyes!

I haven't dreamt of anything but you.
It's no longer dreams, my life itself.
It's high time I recoiled upon my own head,
And now I have no turning back.

Translated by Tatiana Starkova, SGT-218a

Леся Українка "Мріє не зрадь"

Мріє, не зрадь!
Я так довго до тебе тужила,
Стільки безрадісних днів, стільки безсонних ночей. А тепера я в тебе остатню надію вложила.
О, не згасни ти, світло безсонних очей!

Я вже давно інших мрій відреклася для тебе. Се ж я зрікаюсь не мрій, я вже зрікаюсь життя. Вдарив час, я душею повстала сама проти себе, і тепер вже немає мені вороття.

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Viktoriia Chaban

2 Abbreviations

AP	Alternative pathway
BBB	Blood-brain barrier
C	Complement protein (followed by number)
C4BP	C4-binding protein
CD	Cluster of differentiation
CI	Confidence interval
CP	Classical pathway
CR	Complement receptor
CRP	C-reactive protein
CV	Coefficient of variation
DAMP	Damage associated molecular pattern
DC	Dendritic cell
dsDNA	Double-stranded DNA
E. coli	Escherichia coli bacteria
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FH	Factor H
FI	Factor I
FITC	Fluorescein isothiocyanate
HMGB-1	High-mobility group box-1 protein
HUVEC	Human umbilical vein endothelial cells
HUVEC	Human umbilical vein endothelial cells
IL	Interleukin
INF	Interferon
INH	Inhibitor
IQR	Interquartile range
IRI	Ischemia/Reperfusion injury
LP	Lectin pathway
MAC	Membrane attack complex
NK	Natural Killer cells

NLR	NOD-like receptor
OD	Optical density
OHCA	Out-of-hospital cardiac arrest
PAMP	Pathogen associated molecular pattern
PI	Propidium Iodide
PRM	Pattern recognition molecule
PRR	Pattern recognition receptor
PTX3	Long pentraxin 3
RAGE	Receptor for advanced glycation endproducts
ROS	Reactive oxygen species
SAP	Serum amyloid P component
SD	Standard deviation
STEMI	ST-elevation myocardial infarction
TBI	Traumatic brain injury
TCC	Terminal complement complex
TLR	Toll-like receptor
TTM	Target temperature management

3 Thesis summary

3.1 English Summary

Acute brain injury and cardiac arrest are the number one causes of death and disabilities that occur every year in the world. Over half of the patients develop long-term complications and have lower survival rates over a long period compared to healthy controls. The common hallmark for both conditions is a temporal disturbance of blood flow locally or systemically, causing ischemia that leads to cell death. The reperfusion period upon treatment leads to an aggravation of organ damage. This phenomenon is called Ischemia/Reperfusion injury (IRI). IRI is known to impact cellular metabolism and may lead to the activation of inflammatory mechanisms. In this thesis, the impact of innate immunity in general and, in particular, complement activation was investigated in the course of local IRI (mild traumatic brain injury) and systemic IRI (cardiac arrest). The aim was to describe innate immune system activation and trajectory in specific patient groups compared to healthy controls. Association with causing factors and patient outcome was investigated. In addition, we aimed for improving the diagnostic strategy of complement activation measurement in patients with acute medical conditions by comparing the predictive value of either reporting concentration of a complement activation product alone or as a ratio to its un-split parent protein. Finally, the possible treatment approach of hypothermia that is used to lower metabolic processes and which is hypothesized to reduce the innate immune inflammatory response as well was evaluated in an *in vitro* setup.

Patient blood samples were analysed from two large observational clinical studies in mild traumatic brain injury patients (n=207 patients and 82 matched healthy controls) and successfully resuscitated out-of-hospital cardiac arrest patients (n=232 patients and 12 age-matched healthy controls). For the diagnostic study of complement activation, material obtained from patients developing heart failure after myocardial infarction (n=61 patients and 44 healthy controls) was analysed in addition to the cardiac arrest patients. Blood samples from healthy volunteers were assessed in the experimental study. In this study, whole blood and cultured primary endothelial cells were exposed to different temperatures in the range of 4-41°C. Complement activation, cytokines, and surface activation, as well as apoptosis and necrosis markers, were assessed and the effect of temperature on *Escherichia coli*-induced inflammation was described. Complement activation markers, cytokines, endothelial cell activation markers, and soluble CD14 were measured by antibody-mediated assays, such as enzyme-linked

immunosorbent assay (ELISA) and 27-plex bead-based immunoassays. Statistical analyses considered the repeated measurements and exploratory nature of these studies.

Mild traumatic brain injury led to a significant systemic increase of cytokines compared to matched healthy controls, which persisted for up to one year. These findings are remarkable as previous studies had reported prolonged systemic inflammation in severe traumatic brain injury only and had been associated with worse clinical outcome. While the clinical outcome was not assessed in this study, clear associations with demographic and clinical variables were found.

Resuscitated cardiac arrest patients with poor outcome (defined as coma or death) demonstrated higher levels of the complement activation products C3bc and sC5b-9. sC5b-9 at admission was independently associated with poor outcome and sub-sequent endothelial activation.

In acute situations like cardiac arrest and circulatory shock due to heart failure, we demonstrated that the activation product sC5b-9 is the most sensitive and accurate assay for measuring the level of complement activation compared to C3bc and the sC5b-9/C5 and C3bc/C3 ratio.

Complement activation is temperature dependent with and without an inflammatory stimulus showing increasing activity at higher temperatures. Cytokine production occurs after an inflammatory stimulus only and is detectable at temperatures above 33°C, but increased to 39°C only while a decrease was observed at 41°C. Likewise, white blood cell activation and endothelial cell death were absent at lower temperatures but increased significantly at temperatures above 37°C.

This thesis has several conclusions but all highlight that the innate immune response and in particular the complement system should be understood and targeted as upstream recognition systems at the frontline of defense. The complement system was found to be associated with the induction of various inflammatory mediators that contribute to IRI, resulting in organ damage and failure, and eventually death. Interestingly, activation of the innate immune system did not seem to be limited to an acute incidence but can be induced over a long time even after a relatively mild sterile injury, and the association of prolonged systemic inflammation and injury sequelae should be investigated in the future. Targeting the complement cascade with complement activation limiting drugs seems thus promising and should also be evaluated in clinical studies in patients experiencing IRI, especially IRI affecting the whole body like cardiac arrest. To tailor therapy, patients with high complement activation probably should get identified prior to medication with a complement inhibitor. This thesis suggests that sole

measurement of the relatively stable marker sC5b-9 is sensitive in detecting complement activation in acute medical situations. Therefore, a single bedside test for sC5b-9 would be highly suggested in circumstances when complement inhibitor prescription verification is required. The temperature should also be considered as an active intervention limiting complement activation and inflammation and might be used in situations where low temperatures are possible to obtain as e.g., organ transplantation. In conclusion, this thesis highlights the prominent role of the innate immune system in sterile IRI and may be considered as a basis for future interventional clinical studies aiming at the reduction of the detrimental effects in the course of IRI described in this thesis.

3.2 Norsk sammendrag

Akutt hjerneskade og hjertestans er de viktigste årsakene til sykelighet og død i verden. Over halvparten av pasientene utvikler langtidskomplikasjoner og har lavere overlevelse over tid sammenlignet med friske kontroller. Det felles kjennetegnet for begge tilstandene er opphør av eller sterkt forstyrret blodsirkulasjon lokalt eller systemisk, som forårsaker iskemi og fører til celledød. Reperfusjonsperioden ved behandling, dvs. oppløsning av sirkulasjonsforstyrrelsen, fører til forverring av organskaden. Dette fenomenet kalles iskemi/reperfusjonsskade (engelsk: Ischemia/Reperfusion Injury, IRI). IRI påvirker cellulær metabolisme og kan føre til aktivering av inflammatoriske systemer. I dette arbeidet ble virkningen av det medfødte immunsystemet generelt, og komplementaktivering spesielt, undersøkt i forløpet av lokal IRI (mild traumatisk hjerneskade) og ved systemisk IRI (hjertestans). Målet var å beskrive medfødt immunsystemaktivering og forløp av en slik aktivering hos spesifikke pasientgrupper sammenlignet med friske kontroller. Sammenheng mellom immunsystemaktivering som årsak til IRI og pasientutfall ble undersøkt. I tillegg tok vi sikte på å forbedre den diagnostiske strategien for måling av komplementaktivering hos pasienter med akutte medisinske tilstander ved å sammenligne den prediktive verdien av enten å rapportere konsentrasjonen av et komplementaktiveringsprodukt alene eller som forholdet mellom aktiveringsproduktet og det ikke-aktiverte moderproteinet. Til slutt ble den etablerte behandlingen hypotermi som brukes til å senke metabolske prosesser studert i et *in vitro* oppsett for å evaluere om hypotermi kan også brukes til å redusere responsen fra det medfødte immunsystemet.

Pasientblodprøver ble analysert fra to store kliniske observasjonsstudier som inkluderte pasienter med mild traumatisk hjerneskade (n=207 pasienter og 82 matchete friske kontroller) og pasienter etter vellykket gjenopplivning av hjertestans utenfor sykehus (n=232 pasienter og 12 alders-matchete friske kontroller). For den diagnostiske studien av komplementaktivering ble materiale fra pasienter som utviklet hjertesvikt etter hjerteinfarkt (n=61 pasienter og 44 friske kontroller) analysert i tillegg til hjertestanspasientene. Blodprøver fra friske frivillige ble vurdert i den eksperimentelle studien. I denne studien ble fullblod og primære endotel cellekulturer utsatt for forskjellige temperaturer i området 4-41°C. Komplementaktivering, cytokiner og overflateaktivering, samt apoptose- og nekrosemarkører, ble vurdert og effekten av temperatur på *Escherichia coli*-indusert betennelse beskrevet. Komplement-aktiveringsmarkører, cytokiner, endotelcelleaktiveringsmarkører og løselig CD14 ble målt med antistoff-baserte analyser, slik som enzymkoblet immunosorbentanalyse (ELISA) og 27-plex

kulebasert immunanalyse. Statistiske analyser la vekt på de gjentatte målingene og det eksplorative designet av disse studiene.

Mild traumatisk hjerneskade førte til betydelig systemisk økning av cytokiner sammenlignet med matchede friske kontroller. Forhøyede cytokinverdier ble observert opptil ett år etter skaden. Disse funnene er bemerkelsesverdige ettersom tidligere studier hadde rapportert langvarig systemisk betennelse kun ved alvorlig traumatisk hjerneskade assosiert med alvorlige kliniske utfall. Mens klinisk utfall ikke ble vurdert i denne studien, ble det funnet klare assosiasjoner med demografiske og kliniske variabler.

Gjenopplivede hjertestanspasienter med alvorlig utfall (definert som koma eller død) viste høyere nivåer av komplementaktiveringsproduktene C3bc og sC5b-9. sC5b-9 ved innleggelse var uavhengig assosiert med påfølgende endotelaktivering og alvorlig utfall.

I akutte situasjoner som hjertestans og sirkulasjonssjokk på grunn av hjertesvikt, var aktiveringsproduktet sC5b-9 den mest sensitive og nøyaktige analysen for å måle nivået av komplementaktivering sammenlignet med C3bc, samt med forholdene mellom aktiveringsprodukt og moderprotein; sC5b-9/C5 og C3bc/C3.

Komplementaktivering er temperaturavhengig med og uten en inflammatorisk stimulus og viser økende aktivitet ved høyere temperaturer. Cytokinproduksjonen skjer kun etter en inflammatorisk stimulus og kan påvises ved temperaturer over 33 °C med økning til 39 °C, mens en reduksjon ble observert ved 41 °C.

På samme måte var aktivering av hvite blodlegemer og endotelcelledød fraværende ved lavere temperaturer, men økte betydelig ved temperaturer over 37 °C.

Dette arbeidet har flere konklusjoner som alle fremhever at den medfødte immunresponsen og spesielt komplementsystemet bør forstås og angripes som oppstrøms gjenkjenningssystemer i forsvarets frontlinje. Komplementsystemet ble funnet å være assosiert med induksjon av ulike inflammatoriske mediatorer som bidrar til IRI, noe som resulterer i organskade og -svikt, og eventuelt til død. Interessant nok så ikke aktivering av det medfødte immunsystemet ut til å være begrenset til en akutt tilstand, men kan induseres over lang tid selv etter en relativt mild steril skade. Assosiasjon mellom langvarig systemisk inflammasjon og senskader bør undersøkes i fremtiden. Målrettet hemming av komplement kaskaden med legemidler virker lovende og bør evalueres i kliniske studier hos pasienter som gjennomgår IRI, spesielt IRI som påvirker hele kroppen slik man ser det ved hjertestans. For å skreddersy terapien, bør trolig

pasienter med forhøyet komplement aktivering identifiseres før medisinering med en komplementhemmer startes. Dette arbeidet antyder at måling av den relativt stabile markøren sC5b-9 alene er tilstrekkelig for å oppdage komplement aktivering i akutte medisinske situasjoner. Utvikling av en pasient-nær analyse for sC5b-9 ville derfor være svært ønskelig, slik at tilstander med forhøyet komplement aktivering blir tidlig identifisert og komplement hemmende terapi kun gitt ved riktig indikasjon. Temperatur bør også betraktes som en aktiv intervensjon for å begrense komplement aktivering med tilhørende betennelsesreaksjon, som kan brukes i situasjoner der lave temperaturer er mulig å oppnå, for eksempel ved organtransplantasjon. Avslutningsvis fremhever dette arbeidet den fremtredende rollen det medfødte immunsystemet har i steril IRI og kan derfor betraktes som grunnlag for fremtidige intervensjonelle kliniske studier som tar sikte på å redusere de skadelige følgene av IRI.

4 Articles in the thesis

Article I

Systemic Inflammation Persists the First Year after Mild Traumatic Brain Injury: Results from the Prospective Trondheim Mild Traumatic Brain Injury Study. V Chaban, GJB Clarke, T Skandsen, R Islam, CE Einarsen, A Vik, JK Damas, TE Mollnes, AK Haberg and SE Pischke.

Journal of Neurotrauma (2020) 37(19): 2120-2130

Article II

Complement activation is associated with poor outcome after out-of-hospital cardiac arrest. V Chaban, ER Nakstad, H Stær-Jensen, C Schjalm, I Seljeflot, J Vaage, C Lundqvist, JŠ Benth, K Sunde, TE Mollnes, GØ Andersen and SE Pischke.

Resuscitation (2021) 166: 129-136

Article III

Complement ratios C3bc/C3 and sC5b-9/C5 do not increase the sensitivity of detecting acute complement activation systemically. AM Thomas, V Chaban, SE Pischke, HL Orrem, V Bosnes, K Sunde, I Seljeflot, C Lundqvist, ER Nakstad, GØ Andersen, C Schjalm, TE Mollnes and A Barratt-Due.

Molecular Immunology (2022) 141: 273-279

Article IV

Escherichia coli-induced inflammatory responses are temperature-dependent in human whole blood *ex vivo.* V Chaban, E deBoer, KE McAdam, J Vaage, TE Mollnes, PH Nilsson, SE Pischke and R Islam.

In submission

5 Background

5.1 Inflammation and innate immune response

Inflammation is a biological response, part of our host defence system. The Latin term "*inflammo"* was used in ancient times and means blaze or burn [1]. Approximately 2000 years ago, the Roman physician Aulus Cornelius Celsus (25 BC-50 AD) described the cardinal signs of inflammation (**Figure 1**): rubor (redness), calor (heat), tumor (swelling), and dolor (pain) [2]. Later the "functio leasa" (loss of function) was added as a fifth sign by either Thomas Sydenham (1624-1689) or Rudolf Virchow (1821-1902), indicating the consequence of the four first [3].

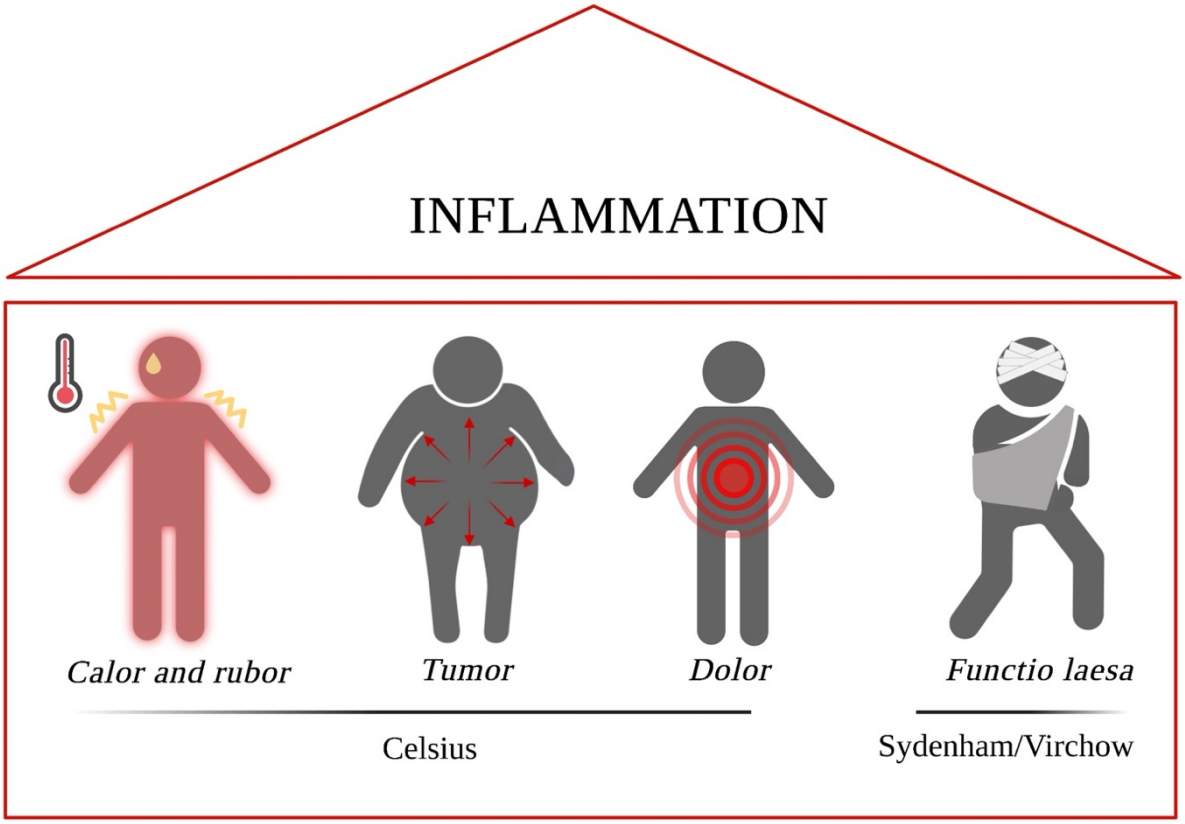


Figure1. The representation of the cardinal signs of inflammation stated by Celsus and others. *Created with BioRender.com*

The main causes of the described symptoms are immune responses to danger such as infection, tissue injury, ischemia, anaphylaxis, chemical toxins, and others. Inflammation causes vasodilation and hyperaemia, which again leads to local redness and heat. Furthermore, inflammation increases capillary permeability resulting in edema. Inflammation activates endothelial cells and induces leukocyte migration from the microcirculation into the interstitial

space at the site of the affected area [4]. The influx of immune cells leads to the formation and release of a number of vasoactive and inflammatory mediators, including eicosanoids like leukotrienes and arachidonic acid metabolites, acute-phase proteins, complement activation products, and innumerable cytokines. [5]. “Cytokine” is a general term for substances that communicate between cells and include both pro- and anti-inflammatory properties. Subgroups of cytokines are TNF, classical interleukins, interferons, chemokines, and growth factors, and are discussed below.

The innate immune system represents an early detection of “danger” and thus constitutes the first-line defence system against every injurious stimulus [6]. Major external triggers are infectious agents, such as bacteria, viruses, and fungi (infectious inflammation), whereas a typical internal trigger is ischemia/reperfusion injury (IRI) leading to sterile inflammation. The innate immune system has several branches, of which the complement system and the Toll-like receptors with the cytokine network are the focus of this thesis.

Inflammation is a general term including both infectious and sterile conditions. They may frequently occur simultaneously, like when a trauma that initially is sterile, is complicated by invasion of microbes, either directly through an open trauma, or indirectly when the patient is immobilized and the risk of infection increases [7]. The aim of the inflammatory reaction is to discard and eliminate the injurious agent or stimuli, to stop the harmful effect and restore homeostasis [4]. Depending on the extent of the infection and amount and pathogenicity of the microbes, the inflammatory response can be classified as local, limited to the particular area affected by the pathogen, or systemic, causing sepsis and a whole-body inflammatory reaction. Systemic inflammation is characterized by the criteria defined as a systemic inflammatory response, which is a “life-threatening organ dysfunction caused by a dysregulated host response to infection” [8]. Hallmark is an uncontrolled inflammatory reaction, which is principally the same whether it is induced by a pathogenic or sterile stimulus. Importantly, crosstalk of the inflammatory reaction with other plasma cascade systems such as the coagulation system exacerbates the homeostatic imbalance. In this case, the immune response has turned into being a threat to the host.

The immune system is traditionally divided into two entities: innate immunity, which is the focus of this thesis, and adaptive immunity. Although highly specialized for their tasks, both are closely cross-talking, and they secure a substantial degree of redundancy. Furthermore, the

acute or chronic inflammatory responses evoked by a sterile stimulus are in many ways similar to activation of the innate immune system by infection [9]. The main differences between innate and adaptive immunity are described in **Table 1**. and illustrated **Figure 2**.

Characteristics	Innate immunity	Adaptive immunity
Response	First line defense has rapid response to the trigger	Second line of the defence, longer time of response (1-2 weeks)
Presence	Innate immunity is present from birth	Adaptive immunity is created in response to exposure to a foreign substance.
Specificity	Non-Specific	Specific
Potency	Limited and lower potency	High potency
Presence	Present at birth	Develops during a person's lifetime and can be short-lived.
Components	The innate immune system is composed of physical and chemical barriers, phagocytic leukocytes, dendritic cells, natural killer cells, and plasma proteins.	Adaptive immune system is composed of B- and T cells
Effectors	Cytokines	Antibodies, cytokines

Table 1. Some main characteristics of the innate and adaptive immune system

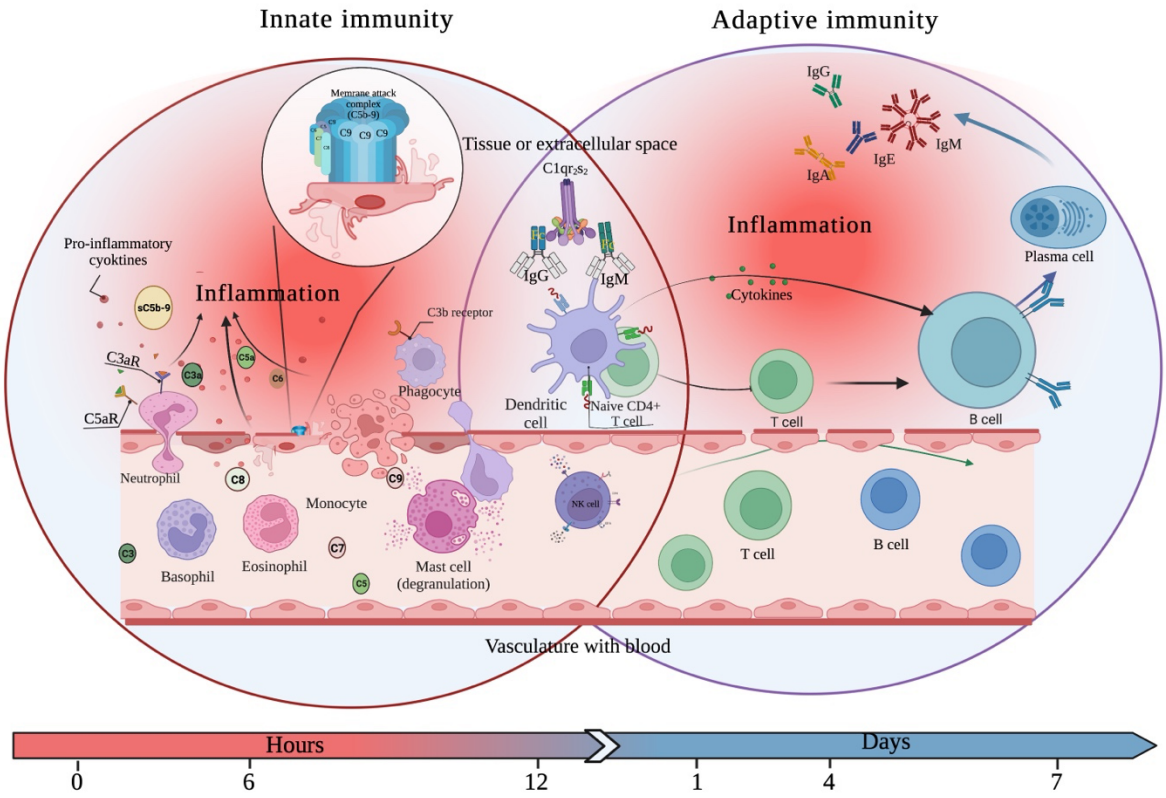


Figure 2. **Components of innate and adaptive immunity.** Innate immunity mechanisms represent the first barrier against infection; the development of adaptive responses requires cross-talking events between these two types of immunity. *Created with BioRender.com*

5.2 Pattern recognition mechanisms

The host is always threatened by danger. The statement "danger model" in immunology describes the theory of how the immune system is activated by substances that are normally not seen by the immune system. The principal idea of this theory is that the immune system does not differentiate between self and non-self, but rather between structures that might cause damage and structures that will not [10]. The danger theory was first introduced by Matzinger [11]. According to her, an immune response differentiates between dangerous and safe by recognition of exogenous pathogens or endogenous alarm signals from injured tissue. The host recognizes so-called danger signals with induction of an initial innate and a late adaptive immune response (**Figure 2)**. The recognition occurs by pattern recognition receptors (PRR), synonymously termed pattern recognition molecules (PRM). PRRs are germline-encoded sensors located on cells of the host or soluble in the plasma like e.g., complement molecules. There are at least five main families of cellular PRRs that initiate pro-inflammatory signalling

pathways: Toll-like receptors (TLR), NOD-like receptors, RIG-I-Like receptors, C-type lectin receptors, and 13 cytosolic dsDNA sensors.

PRRs can recognize the molecular patterns, termed pathogen associated molecular patterns (PAMPs) when expressed on microbes. In contrast to PAMPs, damage associated molecular patterns (DAMPs) are host cell-derived molecules, which get released as endogenous, sterile molecules upon cell damage caused by trauma, and ischemia, and they are like the PAMPs recognized by the PRRs [12].

5.3 Toll-like receptors and CD14

5.3.1 Toll-like receptors

TLRs recognize the major PAMPs including microbial nucleic acids, double- and single-stranded RNA, lipoproteins, surface glycoproteins, and membrane components (peptidoglycans, lipoteichoic acid, lipopolysaccharide). TLRs are expressed on dendritic cells (DCs), macrophages and non-immune cells such as fibroblasts and epithelial cells. TLRs are classified into two groups: cell surface TLRs and intracellular TLRs. Cell surface TLRs include TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10, while intracellular TLRs are localized in the endosome and include TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13 [13].

5.3.2 CD14

CD14, a glycophosphatidylinositol-anchored protein, is an important co-receptor for several TLRs, in particular as the LPS receptor for TLR4/MD-2, but also for TLR2 and several other TLRs [14]. This places CD14 as a key targeting molecule for inhibition of the TLR system as presented in **Figure 3.** This observation has led to the development of a hypothesis in our group that combined inhibition of CD14 of the TLR system, and key molecules of the complement system (C3 and C5) may inhibit innate immune dys- or over-activation very efficiently, as reviewed in [15].

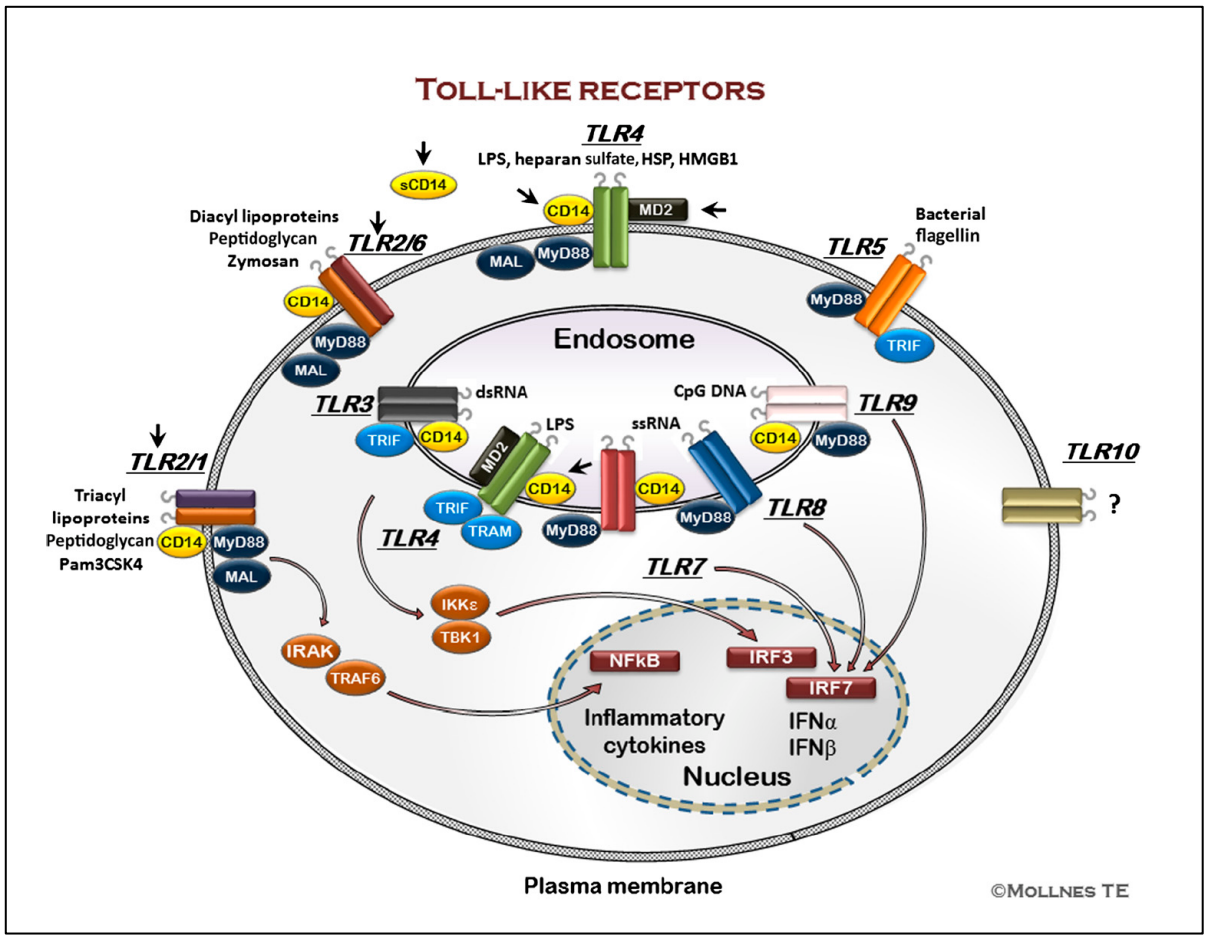


Figure 3. Overview of the TLRs. TLRs are transmembrane proteins that recognize common microbial structural patterns and molecules that are exposed by damaged cells i.e., cell debris. Ten TLRs have been described in humans. TLR1, -2, -4, -5, and -6 are found on the outer cellular membrane, whereas TLR-3, -7, -8, and -9 are endosomal receptors. MyD88 functions as an adapter protein for all TLRs except TLR3. TLR4 is the LPS receptor, with MD2 and CD14 serving as co-receptors, and is translocated from the plasma membrane to the endosomal membrane upon activation. CD14 is a co-receptor for almost all TLRs (apart from TLR5 and TLR10), making it a viable target molecule for broad TLR regulation. Black arrows indicate probable TLR target compounds. HSP, heat shock protein; HMGB1, high mobility group box 1; MAL, MyD88 adapter-like; Pam3CSK4, palmitoyl-3-cysteine-serine-lysine-4; TBK1, TANK-binding kinase 1; IKKε, IkB kinase ε [15]. *Published with permission of T.E. Mollnes*

5.4 The complement system

The complement system is part of the innate immune surveillance system. It has a crucial role in host defence against pathogens and is an important inducer of inflammation. It was first described in the 1890s by Jules Bordet as a heat-sensitive factor circulating in the human serum that aided or "complemented", the reason for the name complement, the antibodies in the killing of bacteria [16].

The complement system consists of more than 40 proteins that are either soluble proteins in blood plasma or present as membrane-bound proteins on most cells in blood and tissue. Curiously, the numbering of complement components does not fully correlate with the sequence of their activation in the complement cascade (e.g. C4 before C2 and C3), but follows the chronology of their discovery.

An overview of the complement system is presented in **Figure 3**.

5.4.1 Initial complement pathways

Three separate pathways can initiate activation of the complement cascade; the classical (CP), lectin (LP), and alternative (AP) pathway, each merging at C3 and leading to activation of the terminal pathway **Figure 3.**

The classical pathway is typically activated by antibody-antigen immune complexes via C1q binding to IgM or IgG clusters. Moreover, the versatile PRM C1q can initiate the activation of complement by recognizing a variety of other surfaces and molecules such as a C-reactive protein (CRP), serum amyloid P component (SAP), long pentraxin 3 (PTX3), and apoptotic or necrotic cell elements including DNA, histones, and annexins presented on cell membranes [17, 18]. The surface binding of C1q initiates activation of proteases C1r and C1s and further formation of C1 complex (C1qr2s2). The C1s component subsequently cleaves C4 to C4a, C4b, and C2 to C2a, C2b forming the C3 convertase C4bC2a of the classical pathway [19].

The lectin pathway (LP) is activated when soluble recognition molecules such as collectins (MBL, CL-10 or CL-L1, CL-11 or CL-K1) and ficolins (FCN-1, FCN-2, FCN-3) bind to carbohydrates patterns on the surface of bacteria, virus, and fungi. After the binding of the recognition molecules, the MBL-associated serine proteases (MASP-1 -2, and -3) get activated. MASP-2 cleaves C4 while both MASP-1 and MASP-2 cleave C2. As a result, the C3 convertase C4bC2a of the lectin pathway is formed.

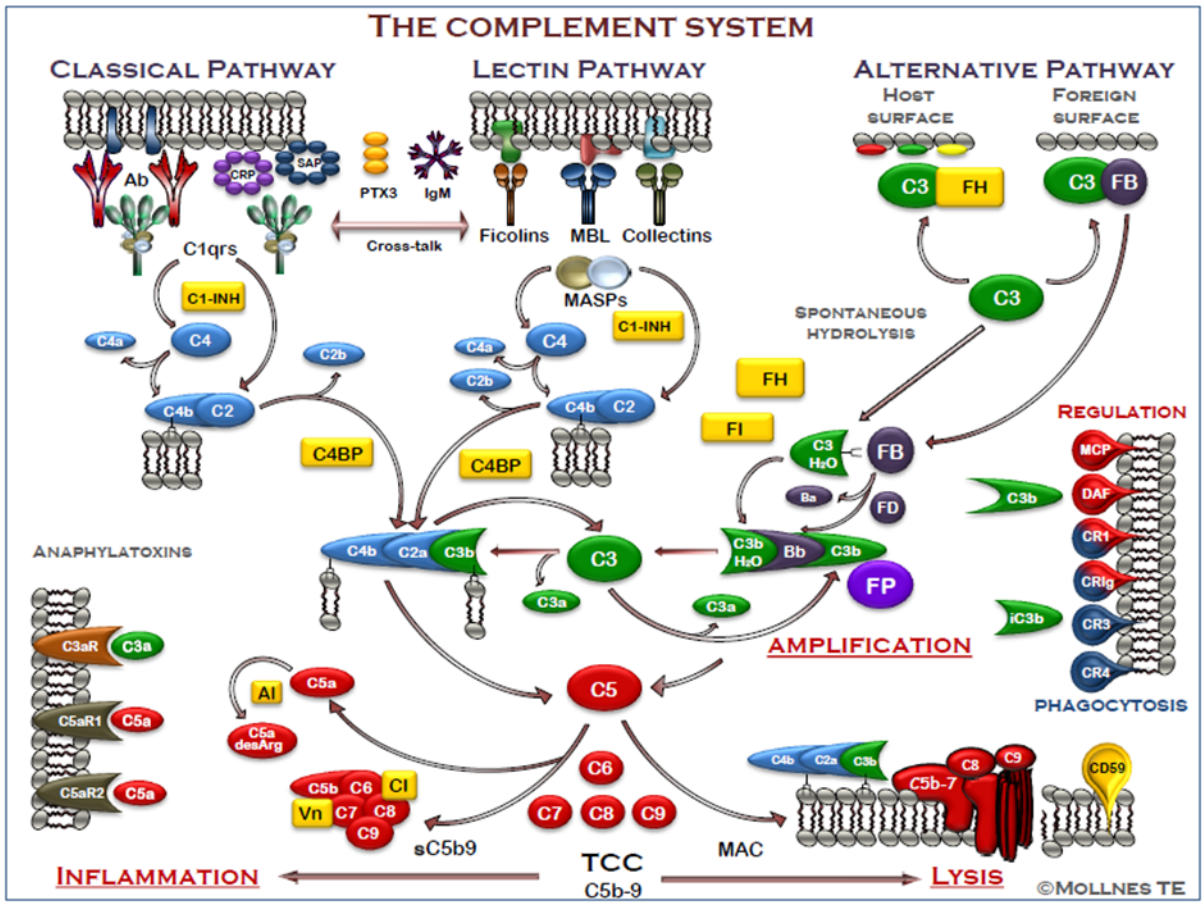


Figure 4. Overview of the complement system The complement system is composed of three pathways: the classical (CP), the lectin (LP), and the alternative (AP). The activation of each pathway leads to cleavage of the common central component C3 to C3a and C3b by the C3-convertases C4bC2a (from CP and LP) and C3bBbP (from AP). C3b assembles with the C4bC2a and C3bBbP convertases and forms the C5 convertase (C4bC2a3b/C3bBbPC3b). C5 gets cleaved into C5a and C5b. The anaphylatoxins C3a and C5a bind to the receptors C3aR and C5aR1/C5aR2 and induce further inflammatory reactions as degranulation, cytokine release, and oxidative burst. C5b initiates the formation of the terminal complement complex (TCC), which is termed membrane attack complex (MAC) when inserted into a membrane or soluble C5b-9 as a plasma marker of complement activation. C3b is converted to iC3b, a strong opsonin, which is recognized by complement receptor 3 (CR3, also known as Mac-1, CD11b/CD18) and 4 (CR4; CD11c/CD18). The complement cascade is closely controlled by soluble inhibitors including C1 inhibitor (C1-INH), factor H (FH), factor I (FI), C4-binding protein (C4BP). Moreover, host cells express complement regulators membrane cofactor protein (MCP/CD55) and CR1/CD35 [15]. *Published with permission of T.E. Mollnes.*

The alternative pathway (AP) initiation differs from classical and lectin pathways. The AP sustains a low level of constant activation of complement in the blood by a process termed "tick over". "Tick-over" is characterized by spontaneous hydrolysis of C3 and production of the bioactive C3 form C3(H_2O). It is a "C3b-like" form of C3 but still contains the C3a moiety in

the fluid phase [20]. The level of hydrolysis of C3 depends on the interplay of C3 with other biological and artificial components including gas bubbles, biomaterial or lipid surfaces and complexes. C3(H2O) binds to factor B. Factor D cleaves factor B to Ba and Bb. Bb in combination with C3b forms the alternative pathway C3 convertase, C3bBb, which is stabilized by properdin. Properdin stabilizes C3bBb by binding to C3b, which prevents its cleavage by factors H and I [21].

5.4.2 The central C3 molecule and the terminal complement pathway

The classical, lectin, and alternative pathways merge at the level of C3 and C5 proteins, which makes them the central components of the complement cascade. C3 can be cleaved by two convertases: C4b2a and C3BbP to C3b and C3a. C3b acts as an opsonin and together with C3 convertases, it forms the C5 convertases C4bC2aC3b and C3bBbC3b. Subsequently, the C5 convertases cleave C5 to C5a and C5b. C5b recruits C6 and C7 proteins in the fluid phase and forms the C5bC6C7 complex that can insert into the cell membranes. If inserted into cell membranes, the binding of C8 and multiple C9 molecules leads to the formation of the membrane attack complex (C5b-9, MAC). The MAC complex can form lytic pores, which lead to cell lysis through water and ion influx to the cell. Sublytic levels of C5b-9 can indirectly interact with several essential intracellular signaling pathways, leading to activation of the inflammasome [22]. The terminal complement complex also has a soluble form known as sC5b-9, which cannot attack the cell membrane, but it is very useful as a fluid phase indicator of the degree of complement activation [23].

The cleavage products C3a and C5a, frequently named anaphylatoxin, bind to their specific G-protein-coupled receptors C3aR, C5aR1, and C5aR2. C3aR is expressed on granulocytes, monocytes, macrophages, subsets of pulmonary and intestinal DCs and activated human T cells and has both pro- and anti-inflammatory effects dependent on the local condition [24]. C5aR1 (CD88) is present on granulocytes, monocytes, macrophages, natural killer (NK) and NK T cells, subsets of DCs, endothelial cells, epithelial and human T cells. C5aR1 is one of the most potent pro-inflammatory receptors in the inflammatory response upon binding of C5a (the C5a-C5aR1 axis), whereas the more recently described C5aR2 has first been thought to act as a decoy receptor but is now regarded as an anti-inflammatory receptor counteracting the C5aR1 induced pro-inflammatory effects [24].

5.5 The effector molecules of the innate immune system

5.5.1 Cytokines - the messengers of the immune system

The three R's in the innate immune response are 1) Recognition, 2) Response, and 3) Resolution.

After the recognition of danger has occurred through the complement system and the TLRs, the response starts immediately to produce the secondary response molecules, which are the "second line" defenders. The major group of these are the cytokines and it will take a few hours to have them synthesized and released since most of these are not pre-formed.

Cytokines comprise a large group of signaling molecules, most being in the range of 10-40 kDa. These act as mediators or 'messengers' that provide the communication between cells in an autocrine, paracrine, and endocrine way and contribute to immune cell homeostasis [25, 26]. Cytokines are produced by virtually all cell types, though the main contribution is from immune cells. Depending on their function and activities, cytokines can be categorized into different groups.

Interleukins are the classical cytokines, including TNF and the largest family of molecules termed interleukins (IL-) before the number; here are those included in this thesis: IL1-ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, and IL-17. All are responsible for the activation and regulation of immune cells. They are released by several cells, including leukocytes. Interleukins are classical examples of autocrine and paracrine signaling, i.e., that they activate the same cells that have produced the interleukin or nearby cells. However, interleukins are not limited to these signaling actions as they are released into the systemic blood circulation [27].

Chemokines are a group of cytokines with chemotactic activities, which promote leukocyte migration towards the source of chemokine production, i.e., into a damaged area. They bind to receptors positioned at the target cell surface, mainly leucocytes [27]. Chemokines are defined by their very consistent gene structure and are divided into subgroups according to this structure. They are homogenous with respect to the molecular weight and are typically 10-15 kDa. The following chemokines are assessed in this thesis: CXCL8 (IL-8), CXCL10 (IP-10), CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), and CCL11 (eotaxin).

Growth factors also belong to the cytokine family and include cellular stimulating factors and proangiogenic factors. In the studies of this thesis the following growth factors are included: G-CSF, GM-CSF, VEGF, bFGF and PDGF-BB.

Interferons play a main role in defense against viral infections as they interfere with virus replication by enabling surrounding cells to increase anti-viral defense mechanisms [28]. Interferons include several families with INF-α, -β, and -γ of which INF-γ is included in this thesis.

5.5.2 Reactive oxygen species (ROS)

Molecular oxygen (dioxygen; O_2) is the vital component for all living organisms, which rely on aerobic metabolism. The result of aerobic respiration is the formation of ATP molecules - the main source of energy generated entirely in mitochondria.

During the last stage of cellular respiration, the electron receptor oxygen is reduced, and water is produced. If this O_2 reduction is only partially performed, highly reactive metabolites of O_2 will be formed. The main three oxygen compounds are superoxide anion ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2), or hydroxyl radicals ($OH\cdot$). These oxygen compounds are often referred to as "reactive oxygen species" (ROS) due to their higher reactivity relative to molecular O_2 and they are pleiotropic including harmful, protective, and signaling effects [29]. During inflammation, ROS are made during phagocytosis and have direct cytotoxic effects, mediate cell signaling and can induce apoptosis.

Uncontrolled levels of ROS can have harmful effects on important cell structures including lipids, proteins, and nucleic acids [30]. Oxidative stress is caused when an imbalance appears between free radical formation and the capability of cells to clear them. Oxidative stress may lead to malondialdehyde and conjugated diene compound formation, which are known to be cytotoxic as well as mutagenic. Being a radical chain reaction, lipid peroxidation spreads very quickly affecting many lipidic molecules [25]. If not strictly controlled, oxidative stress can contribute to several disease processes, both chronic and degenerative, as well as causing tissue damage in acute pathologies including trauma and systemic (cardiac arrest) as well as local (stroke) ischemia/reperfusion injury.

5.5.3 Arachidonic acid metabolites

Arachidonic acid is present as one of the main elements of membrane phospholipids in all cells and is also one of the essential sources in the synthesis of biologically active mediators of inflammation, vasodilation, and vasoconstriction [5]. The stimulation of specific cell-surface receptors activates phospholipase A2 leading to the release of arachidonic acid from the cell membrane. The arachidonic acid is then rapidly converted into an active metabolite by two main classes of enzymes, the cyclooxygenases, and the lipoxygenases.

The cyclooxygenases produce prostaglandins, prostacyclins, and thromboxanes, while lipoxygenases produce leukotrienes. Among these, prostaglandins and leukotrienes are widely studied given their recognized role in human disease conditions as well as physiological and/or pathophysiological activities. They play a major role in inflammation, where they particularly contribute to pain, redness, and swelling [6]

5.6 Therapeutic possibilities targeting the innate immune system

The innate immune system as a first-line defence system plays a crucial role in the body's defence against various pathogens, such as bacteria, viruses, and parasites and sterile stimuli. The 'pre-programmed' responses elicited by the innate immune system are hallmarked by "rapidity" with no need for individual "memory". The consequence of triggering the innate immune system thus results in the initiation of many signaling cascades with the aim to regain homeostasis and clear the imminent threat.

However, loss of control over the innate immunity can lead to uncontrolled activation leading to damaging effects to the host. Thus, the innate immune system, in particular the "first-line" effectors, are important candidates for therapeutic targeting in a number of pathophysiological conditions.

5.6.1 Drugs reducing inflammation

Inhibition of unspecific innate immune processes. The most commonly used drugs for reducing inflammatory processes and managing pain are non-steroidal anti-inflammatory drugs. The major therapeutic actions of non-steroidal anti-inflammatory drugs are primarily enacted by their ability to block the synthesis of certain prostaglandins through inhibition of the

cyclooxygenase enzymes (see above under "Arachidonic acid metabolites"). Among the non-steroidal anti-inflammatory drugs aspirin, acetaminophen, and ibuprofen have been shown to interfere with antiviral immune functions influencing the duration of viral shedding in rhinovirus infected humans. The mechanism of action seems to affect also very basic cellular antiviral functions as in mouse models both aspirin and paracetamol decreased the interferon-induced antiviral responses against influenza virus of cultured mammalian cells [9].

Inhibition of specific molecules of the innate immune system. The molecules of the innate immune system that are targets for therapy are related to the first two of the three R's as discussed above: the molecules of the "Recognition" systems, and the effector molecules released as the "Response" to recognition. This is a field in "therapeutic explosion" and is out of the scope of this thesis, so only the principles and a few examples will be mentioned here. The molecules of recognition are regarded as the "up-stream" part as they are the first to recognize danger. They are limited in numbers and when blocked, will efficiently lead to reduced formation of the "down-stream" effector molecules, which are innumerable in amounts.

Among the "up-stream" targets, the complement component C5 is the only target with FDA and EMA approved drugs for clinical use and C5 inhibition is used to treat a few rare diseases. However, the potential for the treatment of complement-driven diseases in the future is substantial with a broad spectrum of inhibitors, as recently reviewed [31]. Risks associated with blocking of C5 seem to be very low, as patients have been treated for 15 years without serious adverse effects, except for a low amount getting Neisseria infections, which occur during long-term treatment only and are preventable by vaccination. Other inhibitors of "up-stream" targets have been tested in clinical trials and e.g. the TLR4 inhibitor Eritoran was promising until phase III in sepsis, where it failed to increase survival. CD14 is another important TLR molecule, being a co-receptor for several TLRs including TLR4 and TLR2 as discussed above. Preclinical trials in septic baboons are promising, but clinical studies are missing. Interestingly, our group has postulated a dual-blockade of combining inhibition of C5 and CD14, which has shown promising results on survival in both mice and pig polymicrobial sepsis [32, 33], and this inhibition of a major part of the innate immunity should be forwarded to the clinic in cases of systemic inflammatory reasons like sepsis and major trauma. These are diseases that can be treated for a short time, under intensive care and antibiotic protection, and the risk of blocking

most of the innate immune systems for a short time is probably less harmful than leaving the "cytokine storm" in place with a potential to kill the host.

The "down-stream" molecules are innumerable, including the cytokines, and thus it seems intuitively difficult to block one of these and hope for reversing an overwhelming innate immune response. This was also the case when studies started using inhibitors of TNF, IL-6 and others to treat sepsis [15]. This said, a few monoclonal antibodies are currently approved for clinical treatment, like TNF, IL-6, and IL-1R antibodies, but these are limited to mostly chronic inflammatory diseases or specific acute diseases where the disease is driven by special cell populations (e.g. anti-IL-6 treatment in CAR-T induced cytokine release syndrome), where these particular molecules are of importance, but not indicated when the innate immune system is systemically broadly over-activated [34].

5.6.2 Targeting innate immunity by temperature regulation

Temperature may have a significant effect on inflammation and thermotherapy, i.e., moderate lowering of temperature, may reduce local and systemic inflammation in situations like cardiac arrest [35, 36]. Thus, patients undergoing hypothermia and an *ex vivo* study where we investigated the effect of temperature on the inflammatory reaction over a large range from 4°C to 41°C are parts of this thesis.

Hypothermia impairs immune function and inhibits various inflammatory responses. This is inherent to the treatment, and impairment of harmful inflammatory reactions may be one of the mechanisms through which hypothermia can exert protective effects [37]. In clinical studies, hypothermia has been linked to infection risk in the context of accidental hypothermia; controlled therapeutic cooling appears to carry a lower risk, especially if hypothermia is used for limited periods of time (<48 hours) [38]. Prophylactic antibiotics may be considered in high-risk patients who are cooled for prolonged periods.

Hyperthermia is typically seen in infectious diseases and in the first period of sepsis, where fever is typically associated with an increase in the C-reactive protein and the pyrogenic cytokine IL-6, but also in inflammatory, autoimmune and cerebral conditions without infection, where there is a disturbance in the hypothalamic temperature regulatory center [39]. Fever is a double-edged sword and there is a continuous discussion on whether fever is beneficial, detrimental, or just an inert sign reflecting the ongoing condition. To this discussion belongs

the question of whether fever should be treated with antipyretics. It is one of the most used medication groups with probably the less documented effects, except for the clear indication in children suffering from fever convulsions and for the general well-being of the patient. The evidence of beneficial and harmful effects of fever has been reviewed extensively including the evaluation of the evidence versus clinical practice of fever management [40, 41].

5.7 Ischemia/Reperfusion Injury (IRI)

Ischemia/reperfusion injury (IRI) is defined as the tissue damage that follows restoration of blood flow to previously ischemic tissues. Re-establishment of blood flow and oxygenation is essential to salvage ischemic tissues. Paradoxically, reperfusion itself causes further damage, threatening function and viability of the ischemic organ and whole body. IRI can occur in all organs and is particularly dangerous when affecting vital organs like the heart, brain, lungs and kidneys, but may also be severe when abdominal organs and skeletal muscle are involved. Local IRI in a certain organ like the gut, may cause damage of remote organs, e.g. the lung leading to induction of respiratory failure. Finally, IRI may affect the whole body after cardiac arrest or severe trauma and induce a systemic multiorgan failure leading to severe morbidity or death.

The pathophysiology of IRI is a multi-factorial process [42]. Tissue destruction occurs when the oxygen supply is less than the minimal demand required for cellular homeostasis. Derangements in metabolic function begin during the ischemic phase. Initially, glycogen breakdown by anaerobic glycolysis produces two molecules of adenosine triphosphate (ATP) along with lactic acid, resulting in a decrease in tissue pH, which then acts by negative feedback to inhibit further ATP production. ATP is then sequentially broken down into adenosine diphosphate (ADP), adenosine monophosphate (AMP), and inosine monophosphate (IMP) and then further into adenosine, inosine, hypoxanthine, and xanthine. At the cellular level, a lack of ATP production causes ATP-dependent ionic pumps, including the Na+/K+ and Ca2+ pumps, to fail and the transmembrane ionic gradients are lost.

Due to the increasing supply of oxygen during reperfusion, reactive oxygen species are formed and may have a destructive role in mediating tissue damage causing IRI. Specifically, the influx of molecular oxygen catalyzes xanthine oxidase to degrade hypoxanthine to uric acid and thereby liberating the highly reactive superoxide anion (O2-). Superoxide is subsequently converted to hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH•) [43].

Complement activation and deposition also contributes significantly to the pathogenesis of IRI as reviewed in [44]. The anaphylatoxins, in particular C5a, bind to anaphylatoxin receptors on leukocytes and endothelial cells and induce a substantial secondary release of inflammatory mediators. The terminal C5b-9 complex is deposited into the endothelial cell membrane after IRI, either leading to an inflammatory response in sub-lytic doses, or to cell lysis and death by penetrating the membrane.

Moreover, as a result of the release of DAMPs the whole innate immune system recognizes these ligands by the PRRs and a "down-stream" response led by several cytokines is triggered as described above. Importantly, these cytokines are released systemically and are thus important in the development of the systemic inflammatory response and multi-organ failure.

5.7.1 Local and systemic IRI

Depending on the size and extension of the ischemic area and the observed consequences, IRI can be divided into systemic and local. Systemic IRI is typically caused by cardiac arrest or major trauma, while local IRI is classically caused by heart or cerebral stroke. A common clinical observation is that blood flow to an ischemic organ is often not fully restored after the release of vascular occlusion. Mechanisms of this IR-associated "no-reflow" phenomenon include increased leukocyte-endothelial cell adhesion, platelet–leukocyte aggregation, interstitial fluid accumulation, and decreased endothelium-dependent vasorelaxation, which, together, result in mechanical blood flow obstruction. Clinically, this may manifest as continued organ dysfunction in the post-reperfusion period (*e.g.* myocardial stunning), or increased infarct size. The interaction between activated endothelial cells and leukocytes leads to the phenomenon of rolling by integrins, followed by firm adhesion by the intercellular adhesion molecules (ICAMs/VCAMs) ending by penetration of the leukocytes through the endothelium to the tissue is a fundamental process in the pathophysiology of both local and systemic IRI and has been extensively review in several papers, e.g. [45].

5.7.2 The mechanisms of endothelial damage during IRI

The vascular endothelium is a single layer of cells that lines the entire circulatory system. Although a single layer, the total weight in adults is 1 kg and it thus might get recognized as an 'organ'[46] . Endothelial cells participate in hemostasis, immune, and inflammatory reactions.

These cells both produce and react to a wide variety of mediators including cytokines, growth factors, adhesion molecules, vasoactive substances, and chemokines, with effects on many different cells. The endothelium produces a number of vasodilator and vasoconstrictor substances that regulate vasomotor tone, the recruitment and activity of inflammatory cells, and coagulation.

Regarding inflammation, endothelial cells produce and react to various cytokines and adhesion molecules. As endothelium is an important barrier to the free passage of molecules and cells from the blood to the underlying interstitial space, tight junctions between endothelial cells are crucial for paracellular transport and they can get altered in the course of cell activation through complement and cytokines. Leukocyte migration through this endothelial barrier can lead to interruption of this barrier, extravascular fluid leakage, and edema formation[47].

Leukocyte migration is difficult to assess in blood samples. However, leukocyte rolling on the endothelium, firm adherence to the endothelium and endothelial transmigration are described in detail in the review mentioned above [45]. This process is initiated by IRI-induced increases in endothelial P-selectin (CD62P) surface expression, which interacts with its leukocyte counter-receptor P-selectin glycoprotein 1 (PSGL-1). Firm leukocyte adherence results from the subsequent interaction of the leukocyte β2-integrins CD11a/CD18 and CD11b/CD18 with endothelial intercellular adhesion molecule 1 (ICAM-1). Leukocyte transmigration into the interstitial compartment is facilitated by platelet–endothelial cell adhesion molecule 1 (PECAM-1) constitutively expressed along endothelial cell junctions. Upon reaching the extravascular compartment, activated leukocytes release toxic ROS, proteases, and elastases, resulting in increased microvascular permeability, edema, thrombosis, and parenchymal cell death. PMN accumulation in the extravascular compartment is also facilitated by the anaphylatoxin C5a and the chemokine IL-8 released from hypoxic tissues, resulting in a chemotactic gradient that directs neutrophils from the intravascular space towards the hypoxic interstitium. Thus, the measurement of endothelial activation markers in plasma can be used to assess indirectly endothelial cell activation and damage, which is used in this thesis.

5.7.3 Traumatic IRI

Acute trauma is one of the leading causes of morbidity and mortality in the world. Trauma varies from minor trauma, easily coped with, to extensive multi-trauma with a major challenge

to save the patient due to vast disturbance of normal physiology including the innate immune system.

In acute major traumatic lesions, most pathophysiologic processes top during the first few hours and days [48]. The pathophysiological changes are characterized by a local IRI leading to consumption of oxygen and nutrients that generate ischemic and metabolic disturbance. Once reperfusion is established, local DAMPs are released into the systemic circulation and can cause a systemic inflammatory reaction leading to remote organ injury and failure.

In this thesis, we are focusing on the effects of local trauma on systemic inflammation and how systemic IRI after a whole-body trauma affects outcome.

5.7.3.1 Brain injury due to mild trauma – a local IRI

Traumatic brain injury (TBI) represents an insult to the brain from an external mechanical force, leading to permanent or temporary impairment of cognitive, physical, and psychosocial functions, with an associated diminished or altered state of consciousness [49]. TBI is classified by severity and in this thesis - mild traumatic brain injury due to trauma. Although termed "mild", mild TBI represents a major social, economic, and health burden world-wide and the World Health Organization estimates that between 70 and 90% of head injuries that receive treatment are mild [50, 51]. While inflammation in the injured brain, i.e. neuroinflammation, with spill-over to the systemic circulation has been acknowledged in moderate and severe TBI, it is not known if and how long systemic inflammation persists after mild, non-sport related TBI [52].

5.7.3.2 Cardiac arrest as a cause IRI – a systemic IRI

A cardiac arrest is a situation where the heart immediately stops pumping blood. Cardiac arrest is often fatal if appropriate resuscitation is not started immediately. The most common cause of cardiac arrest is coronary artery disease with acute myocardial infarction. Less common causes include major blood loss, lack of oxygen, electrolyte imbalances, heart failure, and intense physical exercise. However, most often highly individualized sets of risk-factors are present [53]. Reveral inherited disorders may also increase the risk including long QT syndrome and various cardiomyopathies. The initial heart rhythm is most often ventricular fibrillation.

Treatment for cardiac arrest includes immediate cardiopulmonary resuscitation and if possible local defibrillation. Targeted temperature management in the resuscitated post-cardiac arrest patient may improve outcome [54].

In Europe, approximately 535,000 cardiac arrests occur each year. About 326,000 (61%) experience cardiac arrest outside of a hospital setting, while 209,000 (39%) occur within a hospital. Cardiac arrest becomes more common with age. It affects males more often than females. The percentage of people who survive out-of-hospital cardiac arrest with treatment by emergency medical services is about 8% and 50% in those successfully resuscitated [55]. Especially the latter patient group is of particular interest as systemic IRI might play a decisive role for outcome.

6 Aims of the study

The main aim of this thesis was to assess the role of the innate immune system with a focus on complement activation and cytokines released during IRI. IRI is a challenge in the course of a number of clinical conditions, where we focused on two; local (traumatic brain injury) and systemic (cardiac arrest) IRI. We performed one methodological study to evaluate if activation products or the ratio of activation products to their un-split proteins is superior in evaluating complement system activation during IRI. This thesis was concluded with a related experimental study where we aimed to investigate the role of temperature on blood-derived inflammation.

Thus, the specific aims were:

1. To study the innate immune response as measured by cytokines in patients with mild traumatic brain injury from admission to 12-month after injury.
2. To evaluate the degree of complement activation and soluble endothelial activation markers associated with long-term cerebral outcome and death in patients with successful resuscitation after cardiac arrest.
3. To assess if the ratios between complement activation product and its native component, by using C3bc/C3 and sC5b-9/C5 as ratios, are superior to complement activation markers C3bc and sC5b-9 alone, for detecting complement activation in patients with IRI.
4. To assess *ex vivo* the effect and mechanism of temperature on complement activation, cytokine release, and cell viability in a broad range of temperatures ranging from 4^0C to 41^0C in a human whole blood model.

7 Methodological considerations

7.1 Study populations in the observational patient studies

The research study design can vary based on the nature of the research question and the opportunity of resources and budget. In studies I-III, we included biobank material from three different patient populations to study the role of the innate immune system. Study I focused on cytokines with the aim to answer if a mild local injury might affect long-term systemic inflammation. Study II and III focused on complement system activation and if complement activation is associated with patient outcome (study II) as well as methodological issues on how to best assess complement activation. All three studies are sub-studies of already performed clinical prospective cohort observations with the aim to generate the basis and hypothesis for future clinical interventional studies. Only paper III included data from paper II and previously published data from one randomized clinical trial. This setup enabled a bigger patient group with the aim to elucidate the important methodological question if complement activation should be displayed as the ratio of the activation product to its un-split protein or solely as an activation product. Both prospective cohort studies included large sample materials with follow-up for six months (paper II) or one year (paper I), which allows prediction of long-term outcomes in these patient groups based on the level of cytokine release or complement activation. However, there are drawbacks to prospective cohort studies. Patients might get non-protocolized interventions, risk factors are not corrected for, results are usually valid for the centers the study was performed at only, and no answers about future treatments can be made [56, 57]. The benefits of observational studies are that they are easier to perform, less cost-intensive, and give an answer about the "real-time status" of the investigated patient group in comparison to protocolized randomized clinical trials. Prior to initiating clinical trials effect and sample size need to be calculated and this is usually done using observational or small pilot clinical trials.

A considerable problem in both observational and randomized studies is that patients are lost to follow-up, i.e., patients although alive, choose to leave the study. This is a known problem in all trials and can affect the power and may thus lead to false-positive results. Guidelines recommend that no more than 20-40% of patients enrolled should be lost to follow-up to avoid biased effects [58]. We had a loss to follow-up of 21.1% (study I) and 10.4% (study II) and

regard the obtained results thus as valid within the above-mentioned limitations of observational studies.

7.1.1 Control groups

All studies included a control group. Although observational studies solely record what happens, control groups are necessary if only one group experiencing an insult is investigated for a certain read-out (here cytokines and complement activation), which is not assessed in a standard and accredited analysis. Thus, the read-out decides if a control group is necessary. Of the investigated read-outs in the studies of this thesis, only complement factors had a standardized reference range, which we adhered to [59]. However, for cytokines and endothelial activation markers, no accepted standard ranges exist. Thus, we needed to include healthy controls to assess the normal ranges of the investigated markers. The collection of controls has the potential to introduce selection bias [60]. Thus, we thrived to overcome selection bias by including age-, gender-, and education-matched controls in study I and age- and gender-matched healthy controls in study II.

7.2 Study population in the experimental study

The experimental study IV was conducted to explore the effect of an intervention (temperature) on innate immune inflammation. A whole blood model was used to assess effects in an *ex vivo* setup. However, this necessitated collecting blood from healthy donors. Healthy donors were recruited among co-workers in the lab only and thus a clear bias was introduced. The bias consisted of the workplace and age (all below pension age, most below the age of 40). However, as the study was truly explorative and should describe mechanisms rather than lead to a new general treatment, this design was accepted.

7.3 Blood sampling

Each project included in this thesis was conducted in whole blood (study IV) or separated parts of whole blood including plasma or cells depending on the purpose (study I-III). The analysis of the levels of complement biomarkers and cytokine release is intricate. The complement system gets continuously activated in the blood and activation can be detected to a low degree

in healthy persons. However, *in vitro,* activation, and degradation of complement proteins start immediately and is affected by storage temperature, time, and type of anticoagulants [4]. Thus, it is critical that samples are collected and stored according to a strict protocol to produce reliable and trustable results. Here, we used rigorous, and most importantly, standardized sampling routines in all studies.

It has previously been shown that complement proteins are more stable in plasma than in serum samples, in combination with ethylene-diamine-tetra-acetic acid (EDTA) [61]. For paper I-III peripheral venous blood was obtained directly in ethylenediaminetetraacetic acid (EDTA) vacutainer tubes, at a final concentration of 10mM for measurements of complement activated products formed *in vivo.* Moreover, the samples were placed immediately on ice and centrifuged within 30 min at 4ºC to separate the plasma. This excluded the possibility of *in vitro* cytokine release from leucocytes and platelets. After that, the collected EDTA plasma was frozen at -80C until further analyses, which ensures no biological activity and enables long-term storage of complement activation products and cytokine proteins.

For paper IV peripheral venosus blood was sampled into sterile 4.5 ml polypropylene tubes containing the anticoagulant lepirudin, (Refludan®) 50 µg/ml. Lepirudin is a thrombin inhibitor and prevents coagulation, while leaving the coagulation cascade functional until thrombin activation, enabling thus complement-coagulation factor interaction [62]. Blood was collected by venous puncture of the forearm vein from healthy donors using an 18 G needle without tourniquet to prevent venous stasis and potential activation of coagulation and complement. Blood samples were immediately aliquoted and incubated at different temperatures with and without stimulation of *E. coli* in order to reduce the need for storage or preservation and the risk of spontaneous activation and loss of cell counts. In the end of incubation time, 10mM EDTA was added to the samples to prevent further complement activation after the desired experimental period. The samples were centrifuged, and plasma obtained for the required readouts, or the entire sample was analysed by flow cytometry to detect the activation of monocytes and granulocytes populations at different temperatures.

7.4 Antibody-mediated assays

Antibody-mediated assays are highly sensitive analyses based on antibody-antigen interaction designed for detecting and quantifying antigens, e.g., proteins, immune complexes, or cell

populations. In these assays, the antigen can be detected in a solid phase or expressed on the cells.

Depending on the principle of analyses used in our projects the assays can be divided into enzyme-linked immunosorbent assay (ELISA) or immunofluorescence-based assay (multiplex and flow cytometry).

7.4.1 Enzyme-linked immunosorbent assay (ELISA)

In all articles included in this thesis, we performed ELISA, which was either in-house developed or by commercial manufacturers. The principle of all in-house developed ELISA is based on pre-coating a plate with 'capture' antibodies specific to neoepitopes of activated biomarkers (C3bc, C3bBbP, and sC5b-9). This is followed by steps of a 'detection' antibody that recognizes a different domain or protein of the activated biomarker and at last with an antibody detecting the Fc-region of the 'detection' antibody and which is an enzyme-conjugated antibody. Here, this enzyme was horseradish peroxidase, which reacts with a substrate (hydrogen peroxide) to develop a color through oxidation [63].

The intensity of the color is read as an optical density (OD) value, which is used to determine the amount of the measured antigen. This is done by comparing the OD of the antigen with a generated standard curve. The standard curve was prepared by dilutions of a sample with a known high concentration of the specific biomarker. Serial dilutions led a dose-dependent curve read as OD and this curve was used as standard curve for the assay.

The soluble terminal complement complex (sC5b-9) was measured in EDTA-plasma by an in-house sandwich-ELISA using monoclonal antibody (clone aE11) as a capture antibody and a monoclonal antibody against C6 (clone 9C4) as detection antibody [63]. For the standard, a zymosan-activated normal human serum was used. C3bc concentration was evaluated by an mouse anti-human C3bc antibody, mAB clone bH6 reacting with a neoepitope exposed in C3b and C3c after C3 activation [59]. C3bBbP was detected based on the monoclonal anti-factor P clone no. 2 (clone #2, Quidel, San Diego, CA) binding the C3bBbP complex and detected by anti-C3c (Behring, Marburg GmbH, Germany) upon activation. Endothelial activation markers in study II were assessed using the same principle, but with commercial DUO ELISAs according to the instruction from the manufacturer (R&D Systems, Minneapolis, MN).

The sandwich ELISA has a lot of advantages, including high specificity (the antigen is specifically captured and detected), flexibility, and sensitivity. However, variations between assays can be problematic and to assure reliable results the coefficient of variation (CV) was assessed. The inter-assay CV was determined by assessing the deviation of technical controls to the total mean of all technical controls over all plates analysed in a project. The intra-assay CV was assessed for each plate. Here, we either run all time points from one patient on the same plate (study I) or samples were run in triplicates (study II). However, one of the limitations in this method, that it is time-consuming, in comparison to other assays, and that it gives only one read-out per sample.

7.4.2 27-plex bead-based immunoassay

For detecting the cytokines in paper I and IV we used a Bio-Plex Pro™ Human Cytokine 27-plex Assay #M500KCAF0Y from Bio-Rad Laboratories Inc. (Hercules, CA) analysed by Luminex MAGPIX technology. In brief, fluorescence-coloured beads coupled to specific detection antibodies as provided by the manufacturer were incubated with plasma samples. Magnetic washing removed all irrelevant plasma proteins. Afterward, biotinylated-detection antibodies were added, and this was followed up with exposure to streptavidin-PE and read on the multiplex.

A flow cytometer excites the beads and the reporter molecules, respectively, leading to separation of the beads in a plot, and measurements of the fluorophore intensity reflecting the amount of the biomarker. The 27-plex kit was used to detect 27 different cytokines including chemokines and growth factors. Human standard I (Bio Rad) was used for all assays.

The same control measures to detect CV as described above were used here as well.

7.4.3 Flowcytometry

In paper IV, we used multi-colour flow cytometry to detect and measure physical and chemical characteristics of cell populations by Attune NxT Acoustic Focusing cytometer (Thermo Fisher Scientific, MA) with multiple excitation lasers.

Prior staining, erythrocytes were lysed with erythrocyte lysing solution and fixed with 0.1% paraformaldehyde in PBS containing 0.1% bovine serum albumin. The extracellular staining of whole blood cells was carried out with fluorophore-marked antibodies including anti-CD11b-

PE (#333142) and anti-CD14-FITC (#345784) purchased from BD Biosciences (San Jose, CA); anti-human CD15 (#48015842) and anti-CD45-PO (#1912804B) purchased from Thermo Fisher Scientific. The flow cytometry analyses were used to detect the expression of CD11b on monocytes and granulocytes in whole blood. The sample was analyzed on a flow cytometer with a forward scatter threshold to remove debris and small particles from the sample collection.

Data analysis was performed using FlowJo (Version10.7.1) software. First, the leukocyte population was identified with anti-CD45, and then monocytes (CD14+) and granulocytes (CD15+) were gated by the side scatter with CD11b expression. The data was presented as the median fluorescence intensity (MFI) of each population and the cell count for granulocytes and monocytes.

This technique is useful when examining mixed populations of cells, such as blood and tissue cells as it allows for simultaneous identification of multiple cell type.

Apoptotic and necrotic population of HUVEC cells were analysed by Dead Cell Apoptosis Kit with Annexin V FITC and PI from Invitrogen™ (V13242, Thermo Fisher Scientifi, MA). The viability of HUVEC cultured in growth media at 37°C in a 5% CO_2 and 95% humidified atmosphere served as control for the experiment. The viability analyses were performed according to the manufacturer's instructions.

7.5 Models of inflammation

7.5.1 Whole-Blood Model of Inflammation

In paper IV, whole blood was used as an *ex vivo* model to evaluate the effect of temperature on activation of the complement cascade, release of cytokines and further investigations of temperature effect on granulocyte and monocyte cell populations.

Lepirudin anticoagulated human whole blood from ten healthy donors was incubated in the presence of *E. coli*$\times 10^7$*/ml* or absence in the range of clinically relevant temperatures for 15 min, 30 min and 120 min.

The advantage of this model is that it gives the opportunity to study several branches of the inflammatory network including complement, the changes in leukocyte and platelet cell surface markers, the release of their granular proteins, and cytokine production [62]. However, blood

interacts constantly with endothelial cells *in vivo*, a condition the whole blood model does not address. Thus, we also wanted to assess the effect of temperature on endothelial cells.

7.5.2 HUVEC model

In paper III, to examine the mechanism of endothelial cell activation we used the Human Umbilical Vein Endothelial Cell (HUVEC) model. Previous studies showed that endothelial cells are not only a passive barrier but can contribute actively to the inflammation process [64]. HUVECs, in concentration 25 000 cells/well, were seeded in 48-wells plates, coated with 1% gelatine. HUVECs were cultured in a specific endothelial growth media. HUVECs were incubated at 37°C in a 5% CO_2, 95% humidified atmosphere until 90%-95% of confluence (2-3 days). All cells were used in passage 4–6 only to avoid differentiation and different activities based on the age of the cells. HUVEC cells were exposed to different temperatures over 24h in ambient atmosphere.

7.6 Statistical considerations

A core element in biomedical research is hypothesis testing. Typically, the null hypothesis states no difference between two samples, whereas the complementary alternative hypothesis is stating the opposite. A probability value or p-value is used to test the null hypothesis. A p-value of ≤ 0.05 indicates evidence against the null hypothesis leading to rejection of the null-hypothesis. Importantly, statistical testing can either reject or fail to reject the null hypothesis, but never prove that it is true.

In our studies, all analyses were performed using IBM SPSS Statistics for Macintosh (IBM Cooperation, Armonk, NY) or GraphPad Prism 9 (GraphPad Software, San Diego, CA)statistical software. All data was tested for normality and based on these results, parametric or non-parametric tests were performed. Likewise, descriptive statistics were presented as mean with standard deviation (SD) or confidence interval (CI) for normally distributed data, and median with interquartile range (IQR) for skewed data.

In study I, data was often normally distributed, while data in study II and III was mostly the data not normally distributed. This might be due to both the sample size (bigger sample size in

study I) and patient-to-patient variation: Patients in study I were mostly young, previously healthy adults experiencing a classified trauma, i.e., mild TBI. In contrast, patients in study II and III were older, had varying degree of underlying diseases and experienced unclassifiable severe trauma due to cardiac arrest and heart failure.

We used the Mann-Whitney test for the comparison of two non-paired groups and the Wilcoxon test for the comparison of two paired groups. We used the Kruskal-Wallis or the Friedman test for the comparison of three or more unpaired or paired groups, respectively. All multiple comparisons were post-hoc corrected for multiple testing to minimize the likelihood of making a type I error (rejecting the null hypothesis on a false basis).

Disadvantages of using nonparametric methods include lack of power as compared with parametric methods. This is a particular concern if the sample size is small, and results should thus be verified in bigger studies to check if the corresponding parametric analysis leads to the same significant differences.

Regression analyses were performed to determine the association of clinical outcome variables to the level of measured biomarkers accounted for clinical variables that might have affected the outcome as well (confounders).

Mixed model analyses were performed in papers I and IV to compare the time course of cytokine or complement levels versus a control group. This approach was chosen because mixed model analysis takes into account baseline variations and accepts missing data points, which a more traditional analysis like analysis of variance repeated measurements would not [65].

7.7 Ethical considerations

Modern biomedical research requires the inclusion of human participants or human biological material to investigate medical interventions' effect on health outcomes. All clinical studies conducted on humans must be pre-approved according to the Health Research Act by the Regional Committees for Medical and Health Research Ethics. Additionally, clinical studies and sampling from volunteers require the collection of informed consent from each included subject.

Obtaining informed consent in clinical research is always one of the most sensitive ethical challenges. Patients are in a vulnerable situation especially the patients included in studies of this thesis as all had experienced acute trauma prior inclusion. Thus, patients were carefully approached, and information was given both orally and through written brochures. If patients were not able to consent due to the trauma itself or because they were minors, consent of their legally acceptable representative, preferably guardians was obtained. However, consent of the patient itself was always collected if a patient regained ability to consent, e.g. the patient woke up after post cardiac arrest treatment. Additionally, the collection of all clinical records, questionnaires, and paramedic records should be done in a standardized way to enable systematic reviews and comparisons between studies. Thus, the collection of data in study II was based on the Utstein criteria, a consensus-based guideline on collecting and reporting data in cardiac arrest patients [66].

All studies included in our project were approved by the Regional Committee for Medical Research Ethics of Norway. Written consent was obtained from all participants or their relatives or guardians. In paper I, the inclusion age was ≥ 16 years old, which is below the eligible year of 18 years in Norway. However, according to the Norwegian Health Research Act [66], minors aged 16 and over can give legal consent to participate in medical and health research, but in addition consent from a guardian (usually parents) also needs to be obtained.

In paper II, the patient inclusion age was ≥ 18 years old. However, included subjects were unconscious due to brain damage caused by hypoxia period after out-of-hospital cardiac arrest. As a result, receiving the written consent from them was not possible at that time point. Thus, written informed consent was obtained from close relatives or guardians within 24 hours after hospitalization and from all patients regaining consciousness and decision-making capacity within six months.

8 Summary of results

Paper I: Systemic Inflammation Persists the First Year after Mild Traumatic Brain Injury: Results from the Prospective Trondheim Mild TBI Study

Hypothesis: In patients with mild brain trauma, innate immunity activation can be detected until one-year post-injury.

Background: Mild traumatic brain injury has a low mortality rate (< 1%). However, it may cause long-term consequences known as post-concussion syndrome, which is mainly characterized by cognitive, emotional, and neurobehavioral problems. Activation of the innate immune system has been suggested to be an important mechanism of injury and driver of the long-term consequences. Nevertheless, the persistence and role of the innate immune response is unknown.

Method: We investigated the concentration- and time-profile of plasma cytokines in hospitalized and non-hospitalized patients with mild brain injury (n=207) over 12 months after injury. EDTA plasma was obtained at admission (within 72 h post-injury), after 2 weeks, 3 and 12 months. The associations of plasma cytokines to injury-related and demographic variables at admission were studied and compared to community controls (n=82). Cytokines were analyzed by a fluorescence magnetic 27-plex bead-based immunoassay.

Results:

1. Twelve cytokines (Fig. 4A, B) were reliably detected including IFN-γ , IL-8, eotaxin , MIP-1b, MCP-1, IL-17A, IL-9, TNF, FGF-basic, IP-10, PDGF, and IL-1ra. The first eight listed cytokines were significantly elevated at all time-points compared to controls, while IP-10, PDGF and IL-10 were not. IL-17A and FGF-basic showed significantly increases in patients from admission to follow-up at 3 months and remained increased at 12 months compared with admission.
2. MRI findings were negatively associated with four cytokines: eotaxin, MIP-1b, IL-9, and IP-10.
3. TNF increased in patients with other injuries.
4. Age was positively associated with nine cytokines: IL-8, eotaxin, MIP-1b, MCP-1, IL-17A, IL-9, TNF, FGF-basic, and IL-1ra.

Conclusion: Mild brain injury activates an innate immune inflammatory response persistently at low grade detected by cytokines released up to 1-year post-injury. Activation increased with age, but was negatively associated with MRI findings of brain injury

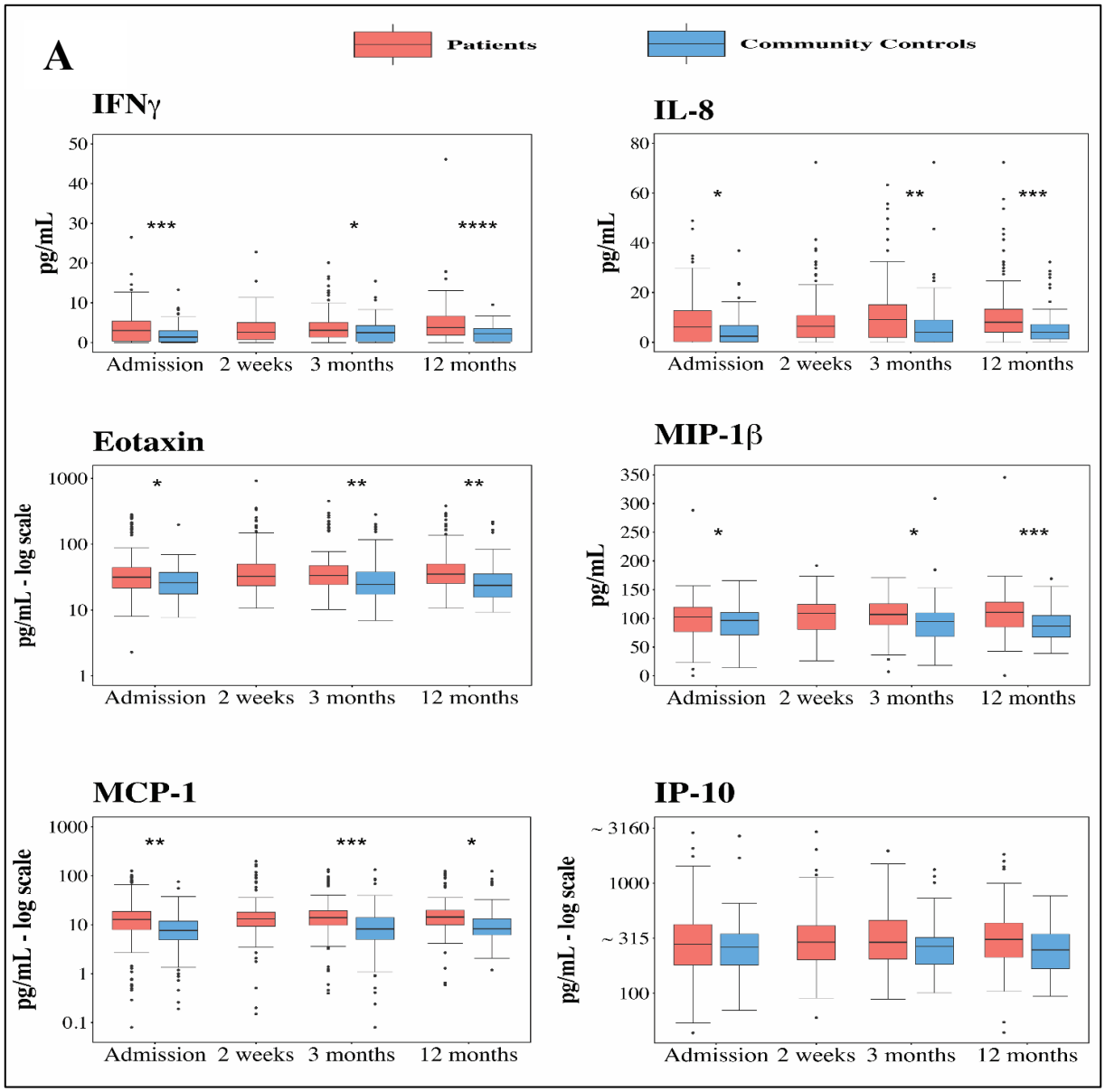
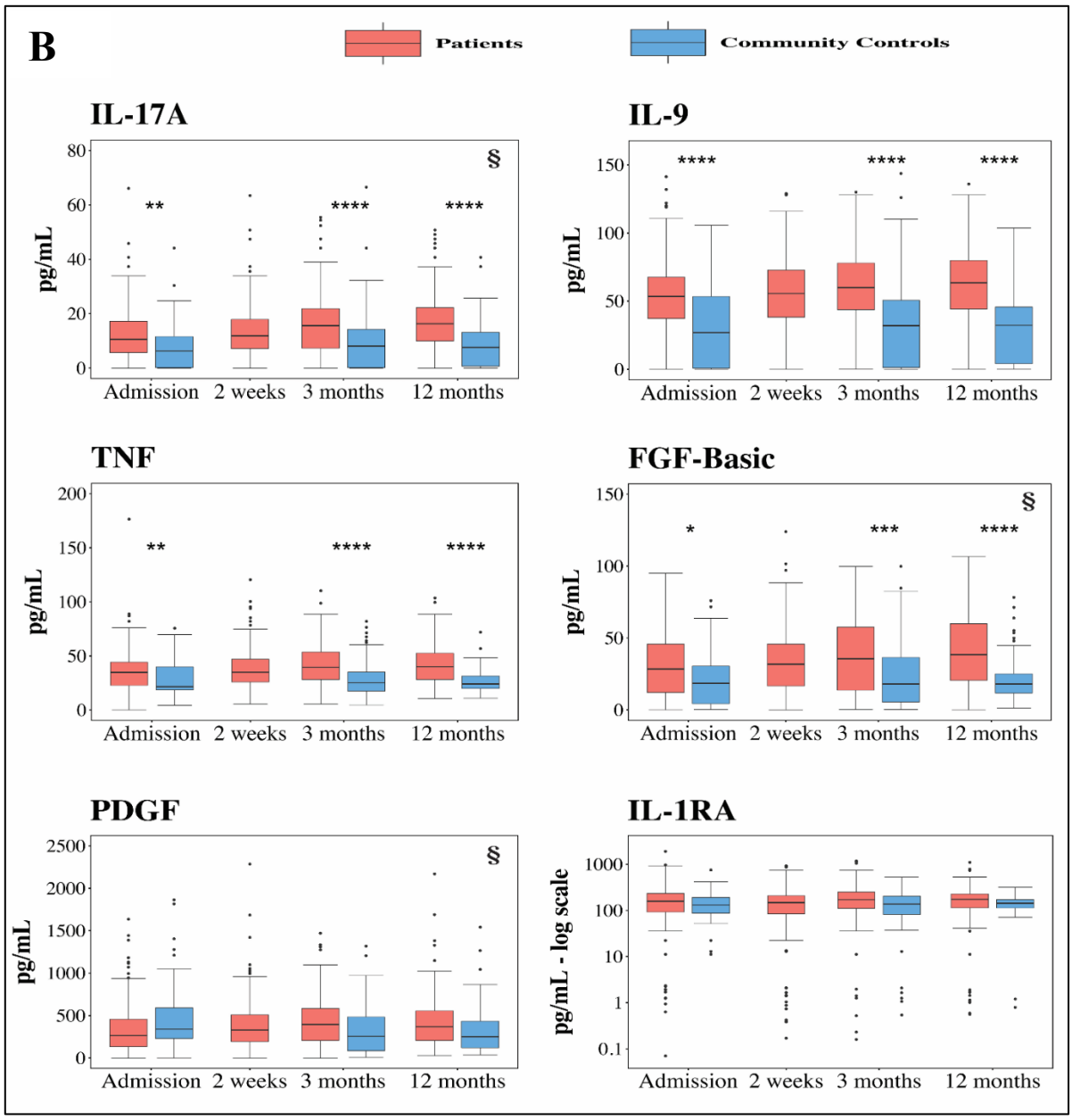


Figure 4A and B. Levels of twelve cytokines for patients with mild traumatic brain injury and controls over time. Data are presented as box plots with median as line, borders, 25th and 75th percentile, and whiskers (value of the 25th and 75th percentile +1.5 interquartile range). Points above and below the whiskers represent outliers. Asterisks (*) indicate significant group difference between patients and controls at a particular time-point in the linear effect model. The p-value level is represented as follows:*<0.05,**<0.01, *** <0.001, **** <0.0001


B
Patients
Community Controls
IL-17A
IL-9
TNF
FGF-Basic
PDGF
IL-1RA
pg/mL
pg/mL - log scale
Admission
2 weeks
3 months
12 months
§

Paper II: Complement activation is associated with poor cerebral outcome after out-of-hospital cardiac arrest

Hypothesis: In patients with out-of-hospital cardiac arrest, the level of complement activation products and release of markers of endothelial activation are associated with cerebral outcome and death.

Background: Cardiac arrest causes whole-body ischemia, and subsequent resuscitation inflicts reperfusion injury, triggering the activation of the innate immune system. Patients surviving cardiac arrest frequently suffer from permanent brain damage independent of the length of cardiac arrest.

Methods: In this study, we investigated the level of complement activation and the following release of soluble endothelial activation markers and their association with long term cerebral outcome and death. A total of 232 patients with out-of-hospital cardiac arrest and successful resuscitation were included in the study. EDTA blood samples at admission and day three were analysed by in-house ELISA for the complement activation products sC5b-9 and C3bc. Furthermore, soluble endothelial activation markers, (E-selectin, thrombomodulin, and syndecan-1) and the co-receptor of toll-like receptors (CD14) were analysed by commercial ELISA. Complement results were compared to an international standard, CD14 and endothelial markers to age-matched healthy controls. Outcome was evaluated after 6 months by neurologists blinded to all parameters of cardiac arrest. Cerebral Performance Category score (CPC, 1-2 good outcome, 3-5 bad outcome with 5 including death) was used for evaluation of cerebral outcome.

Findings:

1. The levels of sC5b-9 and C3bc at admission were significantly higher in patients with bad cerebral outcome compared to those with good cerebral outcome (Fig. 5).
2. High level of sC5b-9 was significantly associated with bad cerebral outcome regardless of time-to-return of spontaneous circulation (ROSC). Post hoc analysis of the interaction term showed that odds for poor outcome were increasing with increasing values of sC5b-9 when time-to-ROSC was below 25 minutes.

3. All endothelial markers, as well as CD14, were significantly increased in patients compared to the control group. E-selectin and thrombomodulin were significantly higher in patients with poor versus good outcome.

Conclusion: After out-of-hospital cardiac arrest and successful cardiopulmonary resuscitation, the complement system is activated, leading to endothelial cell activation. Levels of sC5b-9 were associated with outcome after 6-months.

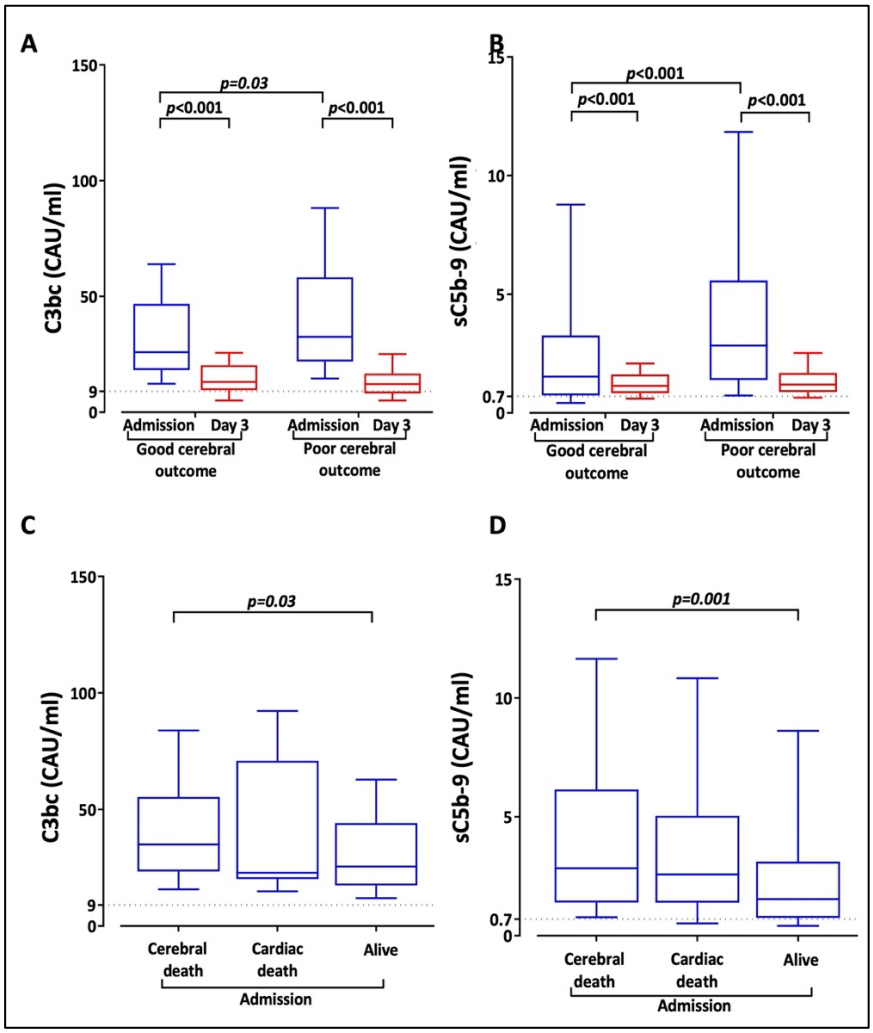


Figure 5: The level of complement actiavtion products at admission (blue) is higher than at day 3 (red) in all patients and higher in patients with poor compared to good cerebral outcome (panel A and B). At admission, patietns who later died of cerebral causes showed significantly higher complement activation than patients that survived or deceased during the observation period due to cardiac disease. Data is shown as box plots with median as line and box indicating 25th – 75th percentile and whiskers representing 10th – 90th percentiles. Wilcoxon signed-rank paired test and non-paired Mann-Whitney U test.

Paper III: Complement ratios C3bc/C3 and sC5b-9/C5 do not increase the sensitivity of detecting complement activation as compared to C3bc and sC5b-9 alone

Hypothesis: We hypothesized that using the ratio of C3bc/C3 and sC5b-9/C5 levels may offer a more accurate assessment of complement activation compared to the activation products alone.

Background: The evaluation of complement activation in clinical settings may be challenging. Acute activation of the complement cascades *in vivo* leads to the consumption of complement proteins. However, complement protein production is also increased during an acute phase reaction. Thus, some reports suggest that the ratio between an activation product and its parent protein (e.g. C3bc/C3 and sC5b-9/C5) is a more sensitive indicator of activation compared to the level of the activation product alone.

Methods: Serial EDTA plasma samples obtained from two clinical studies including patients with acute heart failure following ST-elevated myocardial infarction (n=60 patients) and patients with out-of-hospital cardiac arrest (n=207 patients) were used. Healthy controls (n=20) served to determine reference cut-off values for activation products and ratios, defined as two SD above the mean. C3 was measured at the Routine Immunological Laboratory at Oslo University Hospital. C5 was analyzed by a commercially available enzyme-linked immunosorbent assay (Abcam, Cambridge, UK). C3bc and sC5b-9 were analyzed prior to this study in two already published studies (study II in this thesis and Orrem HL et al. [67]).

Findings:

1. Increased C3bc/C3 and sC5b-9/C5 ratios were vastly dependent on the activation product alone (99.5% and 99.1%, respectively).
2. Strong correlations between C3bc and C3bc/C3-ratio and between sC5b-9 and sC5b9/C5-ratio were found in both patients' cohorts (sC5b-9 shown here, Fig. 6).
3. sC5b-9 identified worse outcome groups better than sC5b-9/C5-ratio
4. As compared to the activation products, the ratios were not more sensitive, rather less, in detecting systemic complement activation as compared to the activation products alone.

Conclusion: Complement ratios C3bc/C3 and sC5b-9/C5 do not increase the sensitivity of detecting complement activation as compared to the activation products C3bc and sC5b-9 alone

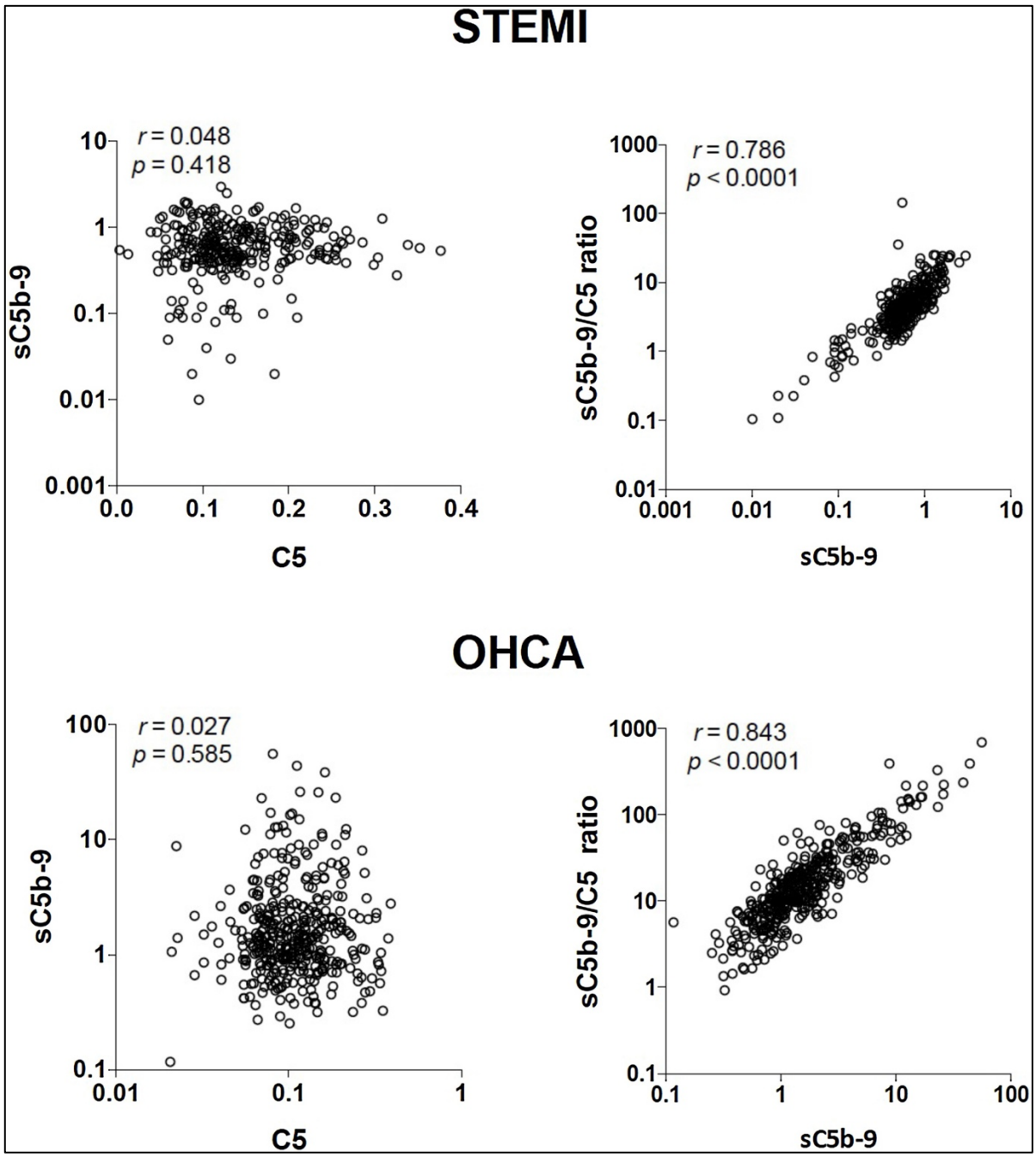


Figure 6. No correlation was found between sC5b-9 and C5 (left panels), while there was high and significant correlation between sC5b-9 and the sC5b-9/C5 ratio (right panels), which was consistent in both the STEMI (upper panel) and OHCA (lower panel) patient population. Data presented log transformed. Spearman-rank correlation. STEMI; ST-elevation myocardial infarction, OHCA; Out-of hospital cardiac arrest.

Paper IV: *Escherichia coli*-induced inflammatory responses are temperature-dependent in human whole blood *ex vivo*

Hypothesis: We hypothesized that temperatures above and below the baseline of normal 37°C have an impact on the complement system, granulocyte and monocyte activation, cytokine release in human whole blood, and endothelial cells viability *in vitro* alone or in the presence of bacteria.

Background: Changes in body temperature from normothermia (37ºC) may be caused by environmental forces, diseases, or therapeutic measures. Hyperthermia may be caused by inflammatory reactions and fever. Hypothermia is usually instigated by external and therapeutic causes. Hyper- and hypothermia have both beneficial, i.e., protective, and harmful effects. Hyperthermia may stimulate immune responses while hypothermia might increase infection susceptibility. In this study, we selected a broad range of temperatures to investigate their effect on the innate immune response.: hypothermia (4°C, 12°C, 20°C, 33°C), normothermia (37°C), and hyperthermia (39°C and 41°C)

Methods: EDTA plasma was collected from Lepirudin anticoagulated whole blood from 10 healthy donors after incubation for 15, 30, or 120 minutes in the presence or absence of *E. coli* (1×10^7 bacteria/ml) at the above-mentioned temperatures. The complement activation products C3bBbP and sC5b-9 were analysed by in-house ELISA. Analysis of cytokines were performed by a fluorescence magnetic bead-based immunoassay (Bio-Plex Human Cytokine 27-Plex, Bio-Rad Laboratories, Inc., Hercules CA, USA) after incubation for 120 min.

After storage for 24 hours at the different temperatures, the viability of human umbilical vein endothelial cells (HUVEC) was analysed by flow cytometry using a Dead Cell Apoptosis Kit with Annexin V FITC and Propidium Iodide (PI). Granulocyte and monocyte activation was analysed by flow cytometry based on the expression of adhesion molecule CD11b on their surfaces and stained with anti-CD11b, anti-CD14, anti-CD15, and anti-CD45 fluorochromes. The analysis was performed using fluorescence-activated cell sorting on a flow cytometer.

Findings:

1. Complement activation was time- and temperature-dependent (Fig.7). *E. coli* induced stronger and faster formation of C3bBbP and sC5b-9, but showed the same temperature dependency.

2. Release of IL-1β, IL-2, IL-6, IL-8, and TNF occurred in the presence of *E. coli* only and cytokine release increased gradually from 33°C to 39°C. However, at 41°C cytokine release decreased and was at the same level compared to 37°C, except for IL-1β which was significantly lower than at 37°C.
3. Granulocyte and monocyte activation measured as CD11 expression increased significantly between 37°C and 41°C after *E. coli* stimulation. Activation did not show important temperature dependency, although there was an increase from 37°Cto 41°C in non-stimulated cells.
4. After exposing HUVEC cells to the different temperatures, percentage of Annexin V and PI negative cells were highest at the lower temperatures and dropped markedly above 37°C. Corresponding to this finding, the number of necrotic and apoptotic cells was lowest at the lowest temperatures and increased strongly at temperatures above 37°C.

Conclusion:

Complement activation and cytokine production are temperature-dependent and increase with increasing temperatures within the temperature range studied. While increasing complement activation was observed over the whole temperature range, cytokine release decreased at the highest temperature (41°C). Activation of granulocytes and monocytes showed none or minor temperature-dependency. Cell viability was preserved at lower temperatures, while cell death and apoptosis increased strongly above 37°C.

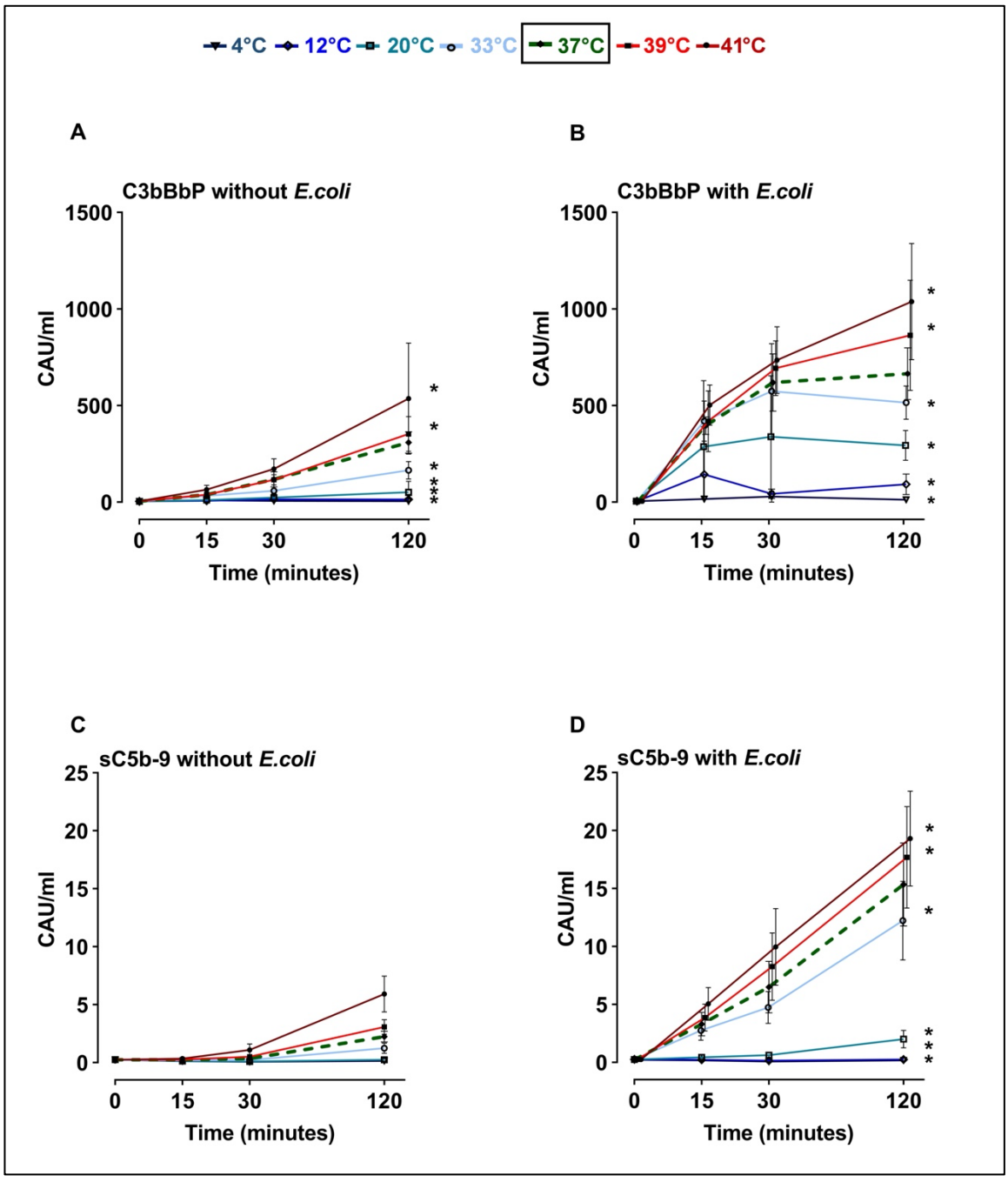


Figure 7. Complement activation complement activation in human whole blood measured by the formation of C3bBbP (A and B) and sC5b-9 (C and D) with and without the presence of *Escherichia coli (E. coli)* is temperature-dependent. Results are expressed as CAU/ml (*y*-axis). Data are given as mean values ± 95% CI for n=10 donors. Time 0 represents the baseline activation measured immediately after collecting the blood while times 15, 30, and 120 represent the incubation period in minutes. Statistical significance was estimated using the mixed model analysis by multiple comparisons of each temperature along the incubation time compared to 37°C with sequential Sidac post-hoc test. ∗; P ≤0.05

9 Discussion

Inflammation is critically involved in the reperfusion phase of IRI and may exacerbate ischemia induced injury. Thus, the studies in this thesis were designed to examine the multifaceted interplay of inflammation in diseases with characteristics of IRI and focus on the innate immune response – mainly on the complement system. This thesis investigated the impact of innate immunity and complement activation in local and systemic IRI as well as its role in the prognosis of patients' outcome. In addition, the feasibility of complement activation measurement in patient samples was assessed to elucidate if concentration of a complement activation product or a ratio to its un-split parent protein is superior when complement activation is assessed in clinical patient samples. Finally, the possible approaches in treatment strategies targeting temperature with the aim to attenuate the innate immune inflammatory response by a general lowering of metabolic processes were assessed.

9.1 Long-term effects of ongoing sterile inflammation

Sterile inflammation occurs in various diseases, including those associated with I/R injuries such as acute myocardial infarction, traumatic brain injury, organ transplantation, and the most severe condition - cardiac arrest. The rapid inflammatory reaction triggered by sterile stimuli initiates activation of a network of defense systems involving the complement system and endothelial cells leading to migration of immune cells and release of signaling molecules such as cytokines [9]. If the sterile stimulus cannot get eliminated or the inflammatory reaction is not stopped, inflammation persists long-term [68]. Long-term persisting inflammation may lead to the development of chronic inflammatory conditions like atherosclerosis and subsequent cardiovascular diseases, neuro-generative diseases (e.g., Alzheimer's disease), autoimmune diseases (e.g., rheumatoid arthritis), cancer, and many more [69]. One of the main clinical goals over several decades has been to identify reliable inflammatory biomarkers that can be used in the long-term clinical prognosis in patients with ongoing sterile inflammation. Until now, most markers have shown modest value due to low sensitivity and specificity in predicting prognosis. Therefore, we focused our studies on investigating biomarkers of innate immune system activation at the time of the incident, over time, and their association with long-term outcomes.

In paper I, we found supporting evidence that after traumatic brain injury, which leads to local IRI, a low-level activated systemic immune response can persist over a long time. Out of 27 cytokines, twelve were reliably identified in the patient's blood. Nine out of these twelve

cytokines were significantly elevated in patients with mild TBI during follow-up. These findings imply that although the initial result is local and minor in severity, systemic inflammation is caused over a long time, supporting the hypothesis that inflammation is not stopped in these patients.

Paper II showed that sterile inflammation during the first three days after severe whole body IRI induced by cardiac arrest, was related to long-term cerebral outcome. We found that the activated complement proteins C3bc and sC5b-9 at admission were associated with poor cerebral outcome if the time to re-establishment of circulation was short (less than 25 minutes). This supports the hypothesis that reperfusion injury plays an important role for long-term outcome when the initial ischemic injury is not dominating the cell injury with an immediate bad outcome.

9.1.1 The causes of inflammation in acute trauma

Acute trauma elicits an acute inflammatory response that lasts from minutes to days and is characterized by three stages: *recognition, response,* and *resolution.* The first stage, which immediately starts after injury, is characterized by the appearance of an arsenal of DAMPs that are recognized by receptors of immune proteins and cells [70] via PRRs such as TLRs, NOD-like receptors, RAGE, purinergic receptors or complement receptors [9, 71]. The main source of proinflammatory "particles" after local acute trauma are necrotic cells and their debris [72]. The dying cells release or expose proinflammatory molecules that are normally intracellular and "hidden" by the plasma membrane, which includes e.g. ATP, DNA, HMGB-1, SAP130, cytokines, and other intracellular proteins [72].

The endothelium is an important contributor to inflammation during acute trauma. Trauma-generated DAMPs, such as mitochondrial DNA and ROS lead to endothelial expression of adhesion molecules that facilitate leukocyte adhesion [73]. Moreover, disturbed endothelial cells lead to an endothelial release of cytokines. The activation of endothelial-associated innate leukocytes, particularly neutrophil granulocytes, by complement and pro-inflammatory cytokines creates an overall pro-inflammatory microenvironment.

In paper I, we focused on cytokines in the inflammatory process caused by mild brain trauma, which is localized trauma of one part of the body. The brain and the central nervous system have traditionally been considered sites of immunological individuality in the sense of

shortened or weakened responses. This theory has been based on the relative impermeability of the blood-brain barrier (BBB) to cellular and molecular components of the immune and inflammatory responses during normal physiological conditions. However, it is now evident that the passage of inflammatory mediators through the BBB is an active process under conditions with an inflammatory reaction in place. Thus, selectivity is actually decreased, leading to increased transfer of inflammatory mediators to the systemic circulation [74]. This applies especially to small proteins, like cytokines [75]. However, many more proteins are released from the CNS to the systemic circulation including (brain-specific) DAMPs that may stimulate systemic inflammation like e.g., S100, HMGB-1, and Tau-protein to name a few [52]. Indeed, even mild TBI has been shown to change genome-expression in peripheral blood mononuclear cells, which change to a more pro-inflammatory type [76].

Trauma to the whole body was assessed in study II. While traumatic brain injury is localized to one organ, cardiac arrest affects homeostasis of the whole body. Although origin of cardiac arrest is most often a cardiac event, e.g., myocardial infarction or primary arrhythmia, cessation of circulation affects the whole body. When resuscitated successfully, activation of the immune system is thus postulated to be immediate, broad, and systemic [77]. The damage caused by this activation has been thought to develop over hours and days after cardiac arrest and we showed that indeed complement activation precedes endothelial cell activation, which is a step to organ damage. Various DAMPs have been proposed to be responsible for the immune system activation, especially HMGB1, which is elevated over days after cardiac arrest, and cerebral inhibition thereof has been shown to diminish brain injury in mice [78].Thus, these findings demonstrate that remote organs act in a pro-inflammatory manner on cardiac arrest. The complement system might get activated directly by HMGB-1 [78], which may be a reason for the increased complement levels we found in our study.

9.1.2 Causes of ongoing systemic inflammation – spill-over from local inflammation vs. persistent systemic inflammation

In paper I, we determined the prolonged release of cytokines over a 1-year follow-up period in patients with mild brain trauma. Pro-inflammatory interleukins including TNF, IL-8, IL-17A and IL-9 were detected and significantly increased in patients during the whole observation period. These cytokines are believed to be involved in the neuroinflammation process and cause stimulation and recruitment of neutrophils [48, 79]. The neuroinflammatory process includes

activation of microglial cells, which results in the production of pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF [80]. A previously performed clinical study determined that microglial activation could persist for years following TBI [81]. The analyses of microglia and associated pathology in TBI patients revealed clusters of activated microglia (evidenced by CR3 and CD68 immunoreactivity) in 28% of patients that survived for more than one year after a single brain injury. Based on this knowledge, we can hypothesize that in study I, the detected prolonged release of cytokines to the peripheral blood were the result of spill-over from the local area of inflammation in the brain through the BBB and produced by microglial cells. However, the main number of cytokines are produced by lymphocytes and macrophages and have relatively short half-life *in vivo* [82]. Moreover, the source of cytokine production can be far from the brain as spilled-over cytokines through the BBB to the circulatory system can lead to systemic activation of leukocytes and endothelial cells and, thus, peripheral cytokine production with ongoing low grade sterile inflammation.

In study II, we showed that cardiac arrest directly led to systemic complement activation. We did not investigate cytokine response here, as this has long been acknowledged before [78]. We were not able to determine the persistence of the systemic inflammatory reaction in this study as the samples were obtained only during the first days after cardiac arrest. However, it is known that cardiac arrest leads to disruption of the BBB and influx of peripheral immune cells also leading to activation of microglia [83]. We showed that complement activation was associated to endothelial cell activation immediately after cardiac arrest and this activation was associated with cerebral outcome after 6 months, which might imply that cardiac arrest initiates an inflammatory cascade that leads to long-term inflammation also locally, i.e., in the brain. While no study has investigated remote organ inflammation over long-term after cardiac arrest, this is an emerging field in heart failure research where effects of circulating cytokines on kidneys, gut microbiota composition and skeletal muscle catabolism are described (reviewed in) [84].

9.1.3 Consequences of long-term inflammation for the patient – advantages vs. disadvantages

Accumulated previous experimental evidence supports the theory that an ongoing inflammatory reaction has both harmful and beneficial effects.

The advantages of sterile long-term inflammation caused by local and systemic injuries involve the clearance of tissue debris, restoring homeostasis of the tissue, and wound repair as described previously, but mainly in experimental systems [85].

In study I, we assessed cytokines over a long period of time after the initial trauma in a clinical cohort of patients. Interestingly, basic fibroblast growth factor (FGF-basic) was elevated in patients compared to controls at all time-points, with a significant increase in patient levels from the admission phase to 3 months. FGF-basic has been shown to increase neurogenesis, preserve BBB integrity and enhance blood vessel proliferation, while suppressing autophagy [35]. Of note, the continuing increase of FGF-basic towards 3 months after injury evidenced in this study suggests that repair may continue longer than previously expected [36]. Moreover, an increased level of platelet-derived growth factor (PDGF), a pro-angiogenetic and vascular repair cytokine, was increased in our patients, consistent with previous observations [37].

Complement activation per se is short-lived and pro-inflammatory. However, C3a has been shown to promote neurogenesis and thus improve recovery after stroke, i.e., brain IRI [86]. Likewise, C3 and C5 have been shown to be of utter importance for liver regeneration and removal of damaged tissue [87, 88].

We cannot conclude if the observed prolonged inflammation in the studies of this thesis has beneficial effects, but our observations add that activation of known protective cytokines is present in clinical patient cohorts and exists longer than previously expected.

Disadvantages of ongoing inflammatory reaction include extension of the area of inflammation, tissue injury, defective organ remodelling and reduction of organ function [7] Persistence of inflammatory processes leads to infiltration of primary inflammatory cells such as macrophages, lymphocytes, and plasma cells into the traumatized tissue, production of inflammatory cytokines, growth factors, enzymes and hence contribution to progression of tissue damage and secondary repair, including fibrosis, leading to tissue and organ dysfunction [89].Likewise, ongoing sterile inflammation leads to constant activation of the coagulation system and clot formation, eventually leading to thrombosis [85].

In study I, chemokine IL-8 (CXCL8), eotaxin (CCL11) and MIP-1β (CCL4) were significantly increased at all timepoints. Previously, all have been reported to be of significance in TBI; IL-8 as a key-mediator of neuroinflammation in severe TBI [90], eotaxin associated with chronic traumatic encephalopathy [91] and MIP-1β with early inflammation leading to recruitment and

activation of immunological cells [92]. All of these cytokines have been shown to induce production of the pro-inflammatory cytokine IFN-γ [93]. Notably, IFN-γ was also increased at all time-points in our study. IFN-γ has been found to play an important role in regulating immune cell recruitment to the injured brain[94].

In addition, IL-17A increased further from admission to 3 months post-injury. In rodent models of severe focal TBI, IL-17A was associated with secondary brain injury, microglial autophagy and inflammation [95, 96]. To our knowledge, study I is the first clinical study that describes an increase in IL-17A over months in mild TBI patients, which may imply that a pro-inflammatory milieu is maintained in the brain, inducing long-lasting inflammatory processes.

We cannot conclude if these systemically observed cytokines generate from the brain, but as these patients had a mild TBI only, it is tempting to speculate that the origin of prolonged cytokine stimulation indeed was brain centralized. Long-term inflammatory reaction in the brain has been described to be a major contributor to neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis [97]. As a result of long-term neuroinflammation, activated microglia can trigger neuronal dysfunction, structural damage, ultimately cell death and formation of β-amyloid fibrils. Interestingly, the major pathological hallmark of Alzheimer's disease is the activation of the NLRP3 inflammasome and generation of IL-1β and IL-8 in microglia through activation of various PRRs [97]. We found long-term increase of IL-8 in TBI patients also at 3 and 12 months after the trauma. The findings from our study may thus prove mechanistically important assessing the proposed link of TBI to Alzheimer’s disease [98].

Deleterious effects of complement activation are difficult to assess as complement activation is mainly local. However, inhibition of the central component C3 has been shown to reduce time to recovery after brain IRI in stroke and intracerebral hemorrhage at least in mice [99, 100]. In this thesis, long-term neurological outcome was assessed. Initial systemic complement activation was found to be associated with poor neurological outcome and death 6 months after cardiac arrest. While previous studies focused on 30-day survival [101], we added that this effect also determines long-term outcome. Treating complement activation in the course of cardiac arrest, might thus both have short- and long-term beneficial and neuroprotective consequences [102].

To conclude, chronic inflammation is a double-edged sword that has both beneficial and deleterious effects, and each treatment should get thoroughly evaluated prior clinical studies are planned and performed.

9.2 Possible reasons for prolonged systemic inflammation

Prolonged systemic inflammation might be a result of an impaired resolution of acute inflammation, dysregulation of endothelial cells or the sympathetic-parasympathetic nervous system. Common for all is the failure to produce adequate amounts of anti-inflammatory and pro-resolving mediators or a failure these mediators to bind to their receptors. Result is nonetheless prolonged release of pro-inflammatory agents to the bloodstream [69].

The molecules, which qualify as pro-resolving mediators are diverse and include specialized lipid mediators (lipoxin LXA_4), resolvins and protectins [103], proteins and peptides (annexin A1, adrenocorticotropic hormone, chemerin peptides, and galectin-1) [104], purines (adenosine) [105], as well as neuromodulators (acetylcholine and other neuropeptides) [106]. These mediators will not be further discussed here. However, the underlying mechanisms leading to imbalance of this intricate system of pro- and anti-inflammatory stimuli are closely related to the induction of prolonged systemic inflammation and are discussed in the following section.

9.2.1 Impaired resolution of acute inflammation

The resolution phase, the last step in the inflammatory response, involves the limitation or termination of complement activation and neutrophil tissue infiltration; the return to homeostatic regulation of chemokines and cytokines; the return of non-apoptotic immune cells to the blood vasculature, and the decrease in number of immune cells including macrophages and dendritic cells at the site of inflammation [69]. The final step of resolution is the induction of tissue repair, i.e., return to homeostasis without fibrosis or scar formation.

An impaired resolution of acute inflammation is characterized by progression from non-resolving acute inflammation to persistent chronic inflammation leading to excessive tissue damage. Phagocytosis plays a central role in resolution of sterile inflammation. The macrophages ensure clearance of apoptotic cells in a non-inflammatory manner, whereas necrosis induces inflammation leading to cellular debris that is cleared by e.g., the classical

complement system. However, macrophages have a limited phagocytic capacity, and recent work has demonstrated that macrophages can reach a point of saturation (or so-called 'exhaustion') beyond which their phagocytic activity is substantially impaired [107].

In papers I and II, the pathological condition was caused by an acute IRI that stimulates the release of DAMPs and led to complement (study II) and cytokine (study I) activation. In study I, this cytokine release was substantial during the whole observation period of 12 months and might thus lead to phagocyte exhaustion and failure of the resolution program of acute inflammation. However, we did not investigate cytokine concentration with patient outcome at 12-months and it would thus be worthwhile to investigate cytokine response and macrophage activity in patients suffering from long-term consequences of a mild TBI.

9.2.2 Dysregulation of endothelial cell activation

Endothelial cells represent a single monolayer of cells that covers the entire vascular system and separates the blood from the tissues [108]. The endothelium works as a receptor-effector organ and responds to physical and chemical stimuli to release relevant substances to maintain vasomotor balance and vascular-tissue homeostasis. Acute local or whole-body IRI causes endothelial activation because of complement activation and cytokine release, with accompanying increase in endothelial cell surface markers, including adhesion molecules like P- and E-selectins, ICAM-I and VCAM-I, attracting leukocytes [109]. The early release of pro-inflammatory cytokines, including TNF , IL-1β and IL-6 in patients after myocardial infarction or TBI, both representing local I/R injury, has been associated with endothelial activation [52, 110]. In paper I, we determined a systemic increased release of twelve cytokines over a one-year period in the patient with mild TBI. We cannot exclude that this would lead to a low-grad systemic endothelial activation although we have no data on endothelial markers in this study.

During acute IRI in cardiac arrest patients, as described in paper II, complement- and endothelial activation markers were increased. Complement activation occurred immediately, followed by release of soluble endothelial markers (e.g., sE-selectin), suggesting that complement was the primary event. This is consistent with previous findings that E-selectin expression on porcine aortic endothelial cells incubated with normal human blood is dependent on C5b-9 [111]. Of interest, the same study showed that C5a was responsible for leukocyte activation. Thus, an effect of complement system on endothelial activation is likely and was confirmed in a clinical material of cardiac arrest patients.

9.2.3 Dysregulation of the sympathetic and parasympathetic nervous systems affect the inflammatory response

Another possible factor that may play an important role in unresolved long-term inflammation is dysregulation of the sympathetic and parasympathetic nervous systems, which have an effect on the inflammatory reaction via the hypothalamic-pituitary-adrenal axis and the vagal nervous system [112]. During stress situations, the hypothalamus produces corticotropin-releasing hormone, which stimulates the pituitary gland to secrete an adrenocorticotropic hormone that stimulates the adrenal cortex. As a result, the adrenal glands produce catecholamines (adrenaline and noradrenaline) and glucocorticosteroids (cortisol) which interact with immune cells. Cortisol, together with noradrenaline, modulates the release of cytokines and thus inflammation through the glucocorticoid receptor, also known as NR3C1, and adrenergic receptors, primarily the β2-adrenergic receptor on immune cells [113]. Moreover, catecholamines have additional anti-inflammatory effects on several human immune cell populations, however this is outside the scope of this thesis and reviewed in [113].

The vagal nervous system affects the immune response via a cholinergic anti-inflammatory pathway. The release of acetylcholine following vagal activation has been shown to inhibit LPS-induced production of systemic pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-18) in human macrophages [114]. Tracey and colleagues have documented a powerful anti-inflammatory pathway mediated by the vagal nerve via acetylcholine, which acts on α7 nicotinic cholinergic receptors to suppress release of TNF-α, IL-1β, IL-6, IL-8, and HMGB1 by macrophages in visceral organs [115]. Because this pathway is activated in the brain in response to infection, injury, ischemia, and inflammatory cytokines, it constitutes an "inflammatory reflex" and electrical stimulation of the vagal nerve or administration of cholinergic agonists may exert a therapeutic effect in inflammatory states [115]. Thus, dysregulation of these two systems may lead to an un-balanced immune system and increased inflammation, which could contribute to the increased cytokines in plasma one year after brain trauma as we found in paper I.

9.3 Initiation of the innate immuneresponse in IRI

9.3.1 The recognition systems of the innate immune response induced by IRI

IRI induces exposure or release of endogenous innate immune activators as described above. These DAMPs are detected by several important classes of receptors, called the PRRs, that induce an inflammatory response upon detection.

TLRs are, among others, an important group of receptors recognising DAMPs [116, 117]. Animal studies have shown significantly increased expression of TLR2 mRNA in postischemic mouse brain, in lesion-associated microglia, and also in endothelial cells, neurons, and astrocytes [118, 119]. TLR2 knockout mice, in comparison to wild-type, had decreased proliferative capacity of the microglia in the ischemic tissue, reduced MCP-1 level, and decreased the concentration of the $CD45^{+}/CD11b^{+}$ cells at the site of cerebral ischemic injury [120].

TLR4, most known as the LPS receptor, is promiscuous, binds a number of DAMPs, and plays an important role in innate immunity and ischemic injury [121]. TLR4-knockout mice undergoing whole cerebral ischemia had significantly decreased cerebral damage in comparison to wild-type [122]. Clinical studies have further confirmed that TLR4 expression in neutrophils was independently associated with prognosis in cerebral stroke patients [123]. A common co-receptor for TLR2 and TLR4, and probably several other TLRs, is the CD14 molecule which is present both on the cell surface and as soluble sCD14 [14]. In paper II we found sCD14 to correlate with soluble endothelial markers. CD14 has been postulated to be an interesting target to attenuate the TLR-induced cytokine response on a broad basis, both to attenuate IRI in transplantation [124], and together with a complement inhibitor to attenuate the sepsis-induced cytokine storm and has been shown in experimental sepsis models to improve survival [15]. It remains to be shown whether inhibition of CD14 will be efficient in clinical therapy of local or systemic IRI.

9.3.2 Triggering the complement system

9.3.2.1 DAMPS

Both whole body- and local IRI cause cell damage and further cell death with release of DAMPs. One of the first systems to sense this danger is the complement system. It is generally accepted that the innate immune system has evolved mechanisms to recognize some of these molecules that are hidden form recognition in non-injured states.

Complement proteins have been demonstrated to deposit onto dead cells in tissues, indicating that cell injury and death can trigger the complement cascade [125]. The classical pathway is typically activated by antibodies, but also by e.g., serum amyloid P and C-reactive protein when bound to surfaces. Of particular interest with respect to complement activation in IRI has been the discovery of naturally occurring IgM antibodies that react with structures exposed from sub-endothelial matrix when the endothelium disintegrates [126, 127]. These antibodies activate complement, probably through the lectin pathway [128], and then worsen the endothelial damage in I/R injury.

9.3.2.1 Crosstalk between coagulation and the complement system

The crosstalk between the complement and the coagulation system may contribute to the development of postischemic capillary no-reflow phenomenon caused by microvascular occlusion as a result of possible thrombo-inflammatory response occurring during reperfusion [129]. In my work, we did not focus on the interplay between complement and coagulation, but the important role of complement-coagulation interaction needs to be considered when discussion the role of complement in IRI [130].

Endothelial damage during IRI causes the exposure of subendothelial tissue factor initiating the extrinsic coagulation cascade, generation of thrombin and platelet activation. Platelets, through interactions of several platelet receptors with extracellular ligands and soluble proteins, adhere and aggregate to the damaged site. These platelet alpha granules contain Factor D that cleaves Factor B of the alternative pathway to its active form. Furthermore, platelets express binding sites for C1q.

Many of the studies showing activation of complement by coagulation factors have been done in purified systems not representative for the physiologic situation. For example, thrombin has been postulated to cleave C5 directly. We recently showed in a physiologic model that thrombin could not cleave native C5 [131]. However, when C5 underwent a conformational change, as evident in the purified form or by acidification of plasma to pH ≤6.8, this thrombin induced cleavage was observed. Thus, we cannot exclude that at a very low pH locally in a tissue, might lead to direct C5 activation by thrombin.

9.3.2.2 Reactive oxygen species

The first line of harmful factors formed in ischemic tissues within seconds to minutes following the restoration of blood flow are reactive oxygen species (ROS) and activated complement

proteins. Both induce further inflammatory processes, extending the injured area. The reactive oxygen species are one of the of the most toxic products, which are formed as a natural by-product of the normal aerobic metabolism of oxygen generation during mitochondrial electron transport [132].

From previous experiments, like the reactive lysis test, it has been acknowledged that treatment of purified human C5 with hydrogen peroxide (H_2O_2) leads to activation of the C5 protein, without cleavage of C5a but rendering C5 able to bind C6 and induce reactive lysis by C5-9 [133]. Another study performed in serum showed C5a-like chemotactic activity for human neutrophils, indicating formation of a C5a opsonin [134]. Moreover, the source of production of the oxygen radicals, including superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), can derive not only from the result of "oxidative stress", but also from activated leukocytes i.e., macrophages and neutrophils [135]. These studies were performed *in vitro* under un-physiological conditions, like those showing thrombin cleavage of C5, and with respect to translation to *the in vivo* situation, should be interpreted with caution. However, activation of complement by ROS and indirect induction of ROS production by complement is very likely.

9.4 The role of complement activation in sterile inflammation

Inflammation is a complex pathophysiologic process that engages mediators and different cell types and tissues and gets initiated by any stimulus causing cell injury. Often, inflammation is a response to infection. However, physical injury alone can also induce this reaction and cause a sterile inflammatory response. Nevertheless, sterile inflammation share a near to similar formula with non-sterile inflammation in evoking the innate immune responses. Following cell injury, endogenous DAMPs and exogenous PAMPs are released, resulting in binding to soluble complement receptors [136]. These receptors are PRRs, or more properly called PRMs, since they are soluble proteins, and are particularly found in the lectin pathway [137].The most important ones are the mannose-binding lectin (MBL), ficolins 1, 2 and 3, collectin 10 and 11, in addition to the classical pathway PRM C1q. Many sterile DAMPs are recognized by these PRMs, all leading to complement activation, with subsequent down-stream activation of virtually the whole inflammatory network. This has become gradually apparent from experimental and clinical studies where specific complement inhibitors have been used in several inflammatory conditions [31].

9.5 The assessment of complement activation in the clinic

In this work, I found supporting evidence that complement activation products, in particular sC5b-9, were associated with patient outcome. Detection of complement in the clinic has challenges, and for a more comprehensive review, I refer to Harboe et al [23]. Thus, a ccomparison of the sensitivity of detecting systemic complement activation by the activation products alone vs. the ratios between the activation product and its parent protein was performed.

9.5.1 Activation product vs. ratio of activation product/un-split product

Many complement activation products are produced during complement activation and are used for quantification of complement activation in general or activation of specific complement activation pathways in particular, like C1rs-C1-INH, C4a, C4d, C3a, C3bc, Ba, Bb, C3bBbP, C5a and sC5b-9 (TCC). Traditionally, levels of C3, C4 and C1-Inh have been used to assess complement. However, recent years have shown that activation products and especially sC5b-9 (plasma TCC) are most valuable indicators of complement activation [138].

In the clinical setting, it may be difficult to assess the presence of complement activation. Low levels of C3 and C4 could be interpreted as an increased consumption and thus increased activation but could also be explained by reduced synthesis due to liver failure or dilution of the sample. Increased values can be seen during an acute phase reaction, and in case of simultaneous consumption, the values can be within the normal range. The measurement of complement activation products is therefore required for safe documentation of *in vivo* activation. However, activation of complement occurs primarily locally, and a slightly increased formation of an activation product can easily be interpreted as insignificant. Thus, different reports have suggested that the ratio between an activation product and its parent protein (e.g. C3dg/C3, C3bc/C3 or C4bc/C4) is a more sensitive indicator of activation compared to the measurement of the activation product alone [139-141].

In paper III we challenged this view as we could not find it sufficiently documented for large clinical trials with substantial complement activation. Thus, we investigated complement activation in patient material from two large clinical trials including patients with myocardial infarction (LEAF-study) [142] and cardiac arrest (NORCAST-study) [143], which is the same

patient population presented in paper II. The parent molecules C3 and C5 were quantified and compared to their respective activation products C3bc and sC5b-9. The ratios C3bc/C3 and sC5b-9/C5 were solely dependent on the increase in activation products in >98% of the patients and sC5b-9 alone identified worse outcome groups better than sC5b-9/C5 ratio. Thus, this study documents that the ratio of activation products, C3bc/C3 and sC5b-9/C5, did not improve sensitivity in detecting systemic complement activation when compared to the activation product itself. While previous studies were derived from small patient cohorts, our study comprised large patient cohorts. However, we cannot exclude that a difference between activation product and the ratio might occur in other populations with chronic diseases, less degree of complement activation and long-term complement protein consumption.

9.5.2 Activation product to be used in routine and in research

While all complement activation products indicate activation, specific research questions need to get formulated to decide on the activation product that describes the question in-mind best [23]. For routine use, sC5b-9 (plasma TCC) is now established as a standard world-wide. The reasons for this are as follows; sC5b-9 is the final product of complement activation and indicates that the whole cascade is activated to its very end. Although the protein itself hardly has any function in fluid-phase, it is a good surrogate marker for C5 activation and thus also C5a release. There are no indications in the literature that sC5b-9 is formed *in vivo* without release of C5a. However, C5a is more difficult to detect since it binds to the C5a receptor immediately and the half-life is approximately one minute [144], whereas that for the sC5b-9 is 40-50 minutes [145, 146]. Finally, sC5b-9 has been shown to be one of the most stable complement activation products *in vitro,* tolerating repetitive freeze-thaw cycles [59]. Therefore, most clinical studies published the last couple of years have used sC5b-9 as indicator of complement activation.

For scientific purposes several assays are available for dissecting mechanisms of activation. In paper II, we measured C3bc in addition to sC5b-9. C3bc gives important information with respect to the relative degree of initial vs. terminal activation and is the second choice on the list. Then, if detailed information on which of the initial pathway(s) are activated, a number of activation products can be studied and here, C3bBbP was measured to assess alternative pathway activation (study IV). The classical and alternative pathway have products to be quantified, but the lectin pathway has so far no reliable activation products published. There

are, however, some promising assay under development, but so far only been presented as abstract as congresses.

9.6 Treatment

9.6.1 The role of temperature on sterile inflammation

The effects of temperature on the inflammatory response, the metabolic rate and other aspects of the human body have been investigated for many decades. It has been hypothesized that hypothermia has a protective mechanism in cases of uncontrolled inflammation, occurring in patients experiencing systemic or local IRI [147, 148]. Temperatures from extreme (4.2°C; 9°C) to moderate hypothermia (26°C-32°C) to treat patients during cardiovascular surgery or comatose cardiac arrest patients after successful resuscitation have been evaluated, however these patients had low survival and neither complement activation nor inflammation was assessed [149-151]

More recent studies investigating resuscitated cardiac arrest patients who were treated with prolonged (72 hours) hypothermia (32-34°C) showed that complement activation was suppressed during the whole hypothermia period and increased modestly during rewarming [152]. In contrast, cytokine and chemokine release remained stable during hypothermia and decreased during rewarming in the same study.

Two big randomized controlled multicenter trials, performed on unconscious adults after out-of-hospital cardiac arrest with targeted temperature management (TTM trial 1 and 2) at either 33°C versus 36°C and 33°C versus <37.8°C for 28 hours showed that hypothermia at a targeted temperature of 33°C did not confer a survival benefit [153]. Inflammation has been assessed in sub-groups of the TTM1 trial and no difference between target temperatures was found for the cytokines assessed [154] .

In paper IV, we used a broad range of temperatures (4°C, 12°C, 20°C, 33°C, 37°C, 39°C and 41°C) to investigate the effect of both hypothermia and hyperthermia on the inflammatory response. In general, the data were consistent with the *in vivo* findings referred to above. Spontaneous complement activation increased modestly in a temperature dependent manner, and markedly after incubation with *E. coli* over the whole temperature range. An abrupt increase was seen between 20°C and 33°C. The release of the cytokines IL-1β, IL-2, IL-6, IL-8, and

TNF differed from complement activation as there was no spontaneous release at any temperature. *E. coli* induced an increase in the cytokines starting at 33°C, peaking at 39°C and then decreased at 41°C. This was accompanied by an increase in apoptotic cells with temperature, and a marked increase in necrotic cells at 41°C. These findings may indicate that cells are not able to continue cytokine production at higher temperatures although complement activation was still increasing. This extreme situation may be found in patients experiencing extreme temperatures, when the inflammatory response seems exhausted while the complement system is still uncontrolled activated.

Thus, the results from the paper IV indicate that low temperature reduce complement activation and cytokine release. However, temperatures below 33°C seem to be necessary to obtain this effect, which cannot be used for whole body hypothermic treatment due to the risk of severe adverse complications like cardiac arrest. Nevertheless, very low temperatures are used for storage of transplantable organs and the optimal temperature for organ storage is discussed constantly [155]. The findings from this thesis might be of use as we show that inflammation is decreased also at higher temperatures than 4°C. Thus, from the inflammation point-of view, storage of transplantable organs could be possible at higher temperatures as well.

9.6.2 Complement therapeutics in sterile inflammation

Complement inhibition has been used as routine clinical treatment for paroxysmal nocturnal haemoglobinuria (PNH) for 15 years. Later, three other diseases have been approved by the FDA for treatment with complement inhibitors: atypical haemolytic-uremic syndrome (aHUS), myasthenia gravis (MG) and neuromyelitis optica spectrum diseases (NMOSD). Until recently, only one drug has been available for clinical use; the monoclonal anti-C5 antibody eculizumab. New drugs are currently coming to the marked and several new diseases are suggested for complement therapy. It is far beyond the scope of the current thesis to discuss this topic in general and we refer to a newly published comprehensive review on clinical application of complement drugs [31]. Here, we limit the discussion for a possible application of complement inhibitors in IRI.

9.6.2.1 Pre-clinical and clinical findings with complement inhibitors in IRI

The effect of complement inhibition on post-transplant heart IRI was studied in C57BL/6 mice by using isografts to avoid any rejection [156]. All three initial pathways were inhibited level of C3 using CR2-Crry and the alternative pathway using CR2-fH immediately post

transplantation. Both inhibitors significantly reduced myocardial I/R injury. Moreover, compared with untreated controls, both inhibitors reduced graft complement deposition, neutrophil and macrophage infiltration, adhesion molecule expression (P-selectin, E-selectin, and I-CAM-1), and proinflammatory cytokine expression (TNF, IL-1β, KC (the human analogue to IL-8), and MCP-1). Eculizumab has been evaluated in kidney donation where it was given systemically to the recipient just prior reperfusion. Study results have not been published as a scientific article, yet, but are available on clinicaltrials.gov (NCT02145182) and as a pilot study [157]. No beneficial effect was found. However, complement activation has been shown to be initiated during preservation already [158] and damage can be initiated after both C3 deposition and C5 activation. Thus, complement inhibition in transplantation related IRI should be evaluated further with the aim to identify the correct timing and inhibitor.

A substantial effect on myocardial infarction was observed in rats using the C3 inhibitor sCR1, with a reduction of the infarction size by 44% [159]. Similar results were documented in experimental myocardial infarction in pigs using the C5 inhibitor coversin [160]. Large clinical trials investigated the effect of the C5 complement inhibitor pexelizumab, a precursor of eculizumab, which also inhibits the cleavage of C5. However, this trial failed to show any beneficial effect in patients with acute myocardial infarction as measured by 30 days mortality [161]. However, a study in patients undergoing coronary bypass surgery showed that treatment with pexelizumab reduced the mortality in a subgroup of patients with multiple risk factor who underwent prolonged cross-clamping during coronary artery bypass surgery [162].

A possible effect of complement inhibition in patients with global IRI as seen in cardiac arrest has not been investigated. In a previous study in patients undergoing high aortic clamping due to thoracic aortic aneurysm surgery, we showed that complement was early activated, and the secondary cytokine response was most likely complement-dependent [163]. These patients were not undergoing cardiopulmonary bypass i.e., complement activation was not influenced by any foreign surface, and the IRI was due to a substantial loss of blood supply to all organs below the diaphragm during a 40 min period. The IRI seen after cardiac arrest, as presented in paper II, is even more global. In both cases, complement activation started immediately and in the cardiac arrest patients this activation was followed by endothelial cell activation. Therefore, we suggest a rationale for complement inhibition for patients undergoing cardiac arrest, provided that the treatment can be started shortly after the event i.e., at the prehospital stage.

9.7 Future implications

One of the central findings in this thesis was that levels of the complement activation products C3bc and sC5b-9 were associated with adverse cerebral outcome in patients with out-of-hospital cardiac arrest. Based on our findings, targeting the complement cascade seems promising since complement is a recognition system acting upstream, in the frontline to fight danger, and because it is known to subsequently induce numerous inflammatory mediators contributing to IRI, leading to organ damage and failure, and ultimately to death.

Importantly, complement treatment in acute medical conditions, including cardiac arrest, polytrauma and sepsis, all characterized by a severe IRI, would need to be treated immediately. The way "to the point of no return" is short. This contrasts with many chronic diseases that are current candidates for complement treatment.

We also showed that in acute situations, the activation product sC5b-9 is the most sensitive and reliable test for evaluation of the degree of complement activation. Thus, a single bed-side test for sC5b-9 would be highly desired in the aforementioned acute situations to identify patients who could benefit of complement inhibitory therapy. Finally, the results from our temperature study imply that inflammation should always be regarded as a phenomenon that is influenced by many factors. Temperature should thus be regarded as an intervention and monitored closely also regarding inflammation. The need for future experimental studies on complement inhibition, regarding organ preservation for transplantation is obvious.

In conclusion, there is an urgent need for clinical studies to evaluate the effect of complement inhibition in acute medical care, involving both local and systemic IRI. The main challenge will be to select the correct patient populations and the optimal time to start treatment and the correct complement inhibitor to ultimately prove the hypothesis that complement inhibition is beneficial for patients experiencing IRI.

10 References

1. Du, C., Bhatia, M., Tang, S.C.W., Zhang, M., and Steiner, T., *Mediators of Inflammation: Inflammation in Cancer, Chronic Diseases, and Wound Healing.* Mediators of Inflammation, 2015. 2015: p. 570653.
2. Rocha e Silva, M., *A brief survey of the history of inflammation.* Agents Actions, 1978. 8(1-2): p. 45-9.
3. Cavaillon, J.-M., *Once upon a time, inflammation.* Journal of Venomous Animals and Toxins including Tropical Diseases, 2021. 27: p. e20200147.
4. Rankin, J.A., *Biological mediators of acute inflammation.* AACN Clin Issues, 2004. 15(1): p. 3-17.
5. Netea, M.G., Kullberg, B.J., and Van der Meer, J.W.M., *Circulating Cytokines as Mediators of Fever.* Clinical Infectious Diseases, 2000. 31(Supplement_5): p. S178-S184.
6. Černý, J. and Stříž, I., *Adaptive innate immunity or innate adaptive immunity?* Clin Sci (Lond), 2019. 133(14): p. 1549-1565.
7. Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., . . . Zhao, L., *Inflammatory responses and inflammation-associated diseases in organs.* Oncotarget, 2017. 9(6): p. 7204-7218.
8. Singer, M., Deutschman, C.S., Seymour, C.W., Shankar-Hari, M., Annane, D., . . . Angus, D.C., *The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3).* Jama, 2016. 315(8): p. 801-10.
9. Chaplin, D.D., *Overview of the immune response.* J Allergy Clin Immunol, 2010. 125(2 Suppl 2): p. S3-23.
10. Matzinger, P., *The danger model: a renewed sense of self.* Science, 2002. 296(5566): p. 301-5.
11. Matzinger, P., *Tolerance, danger, and the extended family.* Annu Rev Immunol, 1994. 12: p. 991-1045.
12. Tang, D., Kang, R., Coyne, C.B., Zeh, H.J., and Lotze, M.T., *PAMPs and DAMPs: signal 0s that spur autophagy and immunity.* Immunol Rev, 2012. 249(1): p. 158-75.
13. Takeuchi, O. and Akira, S., *Pattern recognition receptors and inflammation.* Cell, 2010. 140(6): p. 805-20.
14. Lee, C.C., Avalos, A.M., and Ploegh, H.L., *Accessory molecules for Toll-like receptors and their function.* Nat Rev Immunol, 2012. 12(3): p. 168-79.
15. Barratt-Due, A., Pischke, S.E., Nilsson, P.H., Espevik, T., and Mollnes, T.E., *Dual inhibition of complement and Toll-like receptors as a novel approach to treat inflammatory diseases-C3 or C5 emerge together with CD14 as promising targets.* J Leukoc Biol, 2017. 101(1): p. 193-204.
16. Nesargikar, P.N., Spiller, B., and Chavez, R., *The complement system: history, pathways, cascade and inhibitors.* Eur J Microbiol Immunol (Bp), 2012. 2(2): p. 103-11.
17. Pilely, K., Fumagalli, S., Rosbjerg, A., Genster, N., Skjoedt, M.O., . . . Garred, P., *C-Reactive Protein Binds to Cholesterol Crystals and Co-Localizes with the Terminal Complement Complex in Human Atherosclerotic Plaques.* Front Immunol, 2017. 8: p. 1040.
18. Martin, M., Leffler, J., and Blom, A.M., *Annexin A2 and A5 serve as new ligands for C1q on apoptotic cells.* J Biol Chem, 2012. 287(40): p. 33733-44.
19. Goicoechea de Jorge, E., López Lera, A., Bayarri-Olmos, R., Yebenes, H., Lopez-Trascasa, M., and Rodríguez de Córdoba, S., *Common and rare genetic variants of complement components in human disease.* Mol Immunol, 2018. 102: p. 42-57.
20. Merle, N.S., Church, S.E., Fremeaux-Bacchi, V., and Roumenina, L.T., *Complement System Part I - Molecular Mechanisms of Activation and Regulation.* Front Immunol, 2015. 6: p. 262.

21. Harboe, M., Johnson, C., Nymo, S., Ekholt, K., Schjalm, C., . . . Nilsson, P.H., *Properdin binding to complement activating surfaces depends on initial C3b deposition.* Proc Natl Acad Sci U S A, 2017. 114(4): p. E534-e539.
22. Xie, C.B., Jane-Wit, D., and Pober, J.S., *Complement Membrane Attack Complex: New Roles, Mechanisms of Action, and Therapeutic Targets.* Am J Pathol, 2020. 190(6): p. 1138-1150.
23. Harboe, M., Thorgersen, E.B., and Mollnes, T.E., *Advances in assay of complement function and activation.* Adv Drug Deliv Rev, 2011. 63(12): p. 976-87.
24. Reis, E.S., Mastellos, D.C., Hajishengallis, G., and Lambris, J.D., *New insights into the immune functions of complement.* Nat Rev Immunol, 2019. 19(8): p. 503-516.
25. *Cytokines in the balance.* Nat Immunol, 2019. 20(12): p. 1557.
26. Parkin, J. and Cohen, B., *An overview of the immune system.* Lancet, 2001. 357(9270): p. 1777-89.
27. Zhang, J.-M. and An, J., *Cytokines, inflammation, and pain.* International anesthesiology clinics, 2007. 45(2): p. 27-37.
28. Schroder, K., Hertzog, P.J., Ravasi, T., and Hume, D.A., *Interferon-gamma: an overview of signals, mechanisms and functions.* J Leukoc Biol, 2004. 75(2): p. 163-89.
29. Sies, H. and Jones, D.P., *Reactive oxygen species (ROS) as pleiotropic physiological signalling agents.* Nat Rev Mol Cell Biol, 2020. 21(7): p. 363-383.
30. Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., . . . Bitto, A., *Oxidative Stress: Harms and Benefits for Human Health.* Oxidative Medicine and Cellular Longevity, 2017. 2017: p. 8416763.
31. Garred, P., Tenner, A.J., and Mollnes, T.E., *Therapeutic Targeting of the Complement System: From Rare Diseases to Pandemics.* Pharmacol Rev, 2021. 73(2): p. 792-827.
32. Huber-Lang, M., Barratt-Due, A., Pischke, S.E., Sandanger, Ø., Nilsson, P.H., . . . Mollnes, T.E., *Double blockade of CD14 and complement C5 abolishes the cytokine storm and improves morbidity and survival in polymicrobial sepsis in mice.* J Immunol, 2014. 192(11): p. 5324-31.
33. Skjeflo, E.W., Sagatun, C., Dybwik, K., Aam, S., Urving, S.H., . . . Mollnes, T.E., *Combined inhibition of complement and CD14 improved outcome in porcine polymicrobial sepsis.* Crit Care, 2015. 19: p. 415.
34. Tedesco, V.E.t. and Mohan, C., *Biomarkers for Predicting Cytokine Release Syndrome following CD19-Targeted CAR T Cell Therapy.* J Immunol, 2021. 206(7): p. 1561-1568.
35. Beom, J.H., Kim, J.H., Seo, J., Lee, J.H., Chung, Y.E., . . . You, J.S., *Targeted temperature management at 33°C or 36°C induces equivalent myocardial protection by inhibiting HMGB1 release in myocardial ischemia/reperfusion injury.* PLoS One, 2021. 16(1): p. e0246066.
36. Hong, D.K., Park, Y.S., Woo, J.S., Kim, J.H., Beom, J.H., . . . Suh, S.W., *Transient Global Ischemia-Induced Brain Inflammatory Cascades Attenuated by Targeted Temperature Management.* Int J Mol Sci, 2021. 22(10).
37. Bisschops, L.L., Hoedemaekers, C.W., Mollnes, T.E.P., and van der Hoeven, J.G., *Rewarming after hypothermia after cardiac arrest shifts the inflammatory balance.* Critical care medicine, 2012. 40(4): p. 1136-1142.
38. Kim, J.H., Nagy, Á., Putzu, A., Belletti, A., Biondi-Zoccai, G., . . . Landoni, G., *Therapeutic Hypothermia in Critically Ill Patients: A Systematic Review and Meta-Analysis of High Quality Randomized Trials.* Crit Care Med, 2020. 48(7): p. 1047-1054.
39. Niven, D.J. and Laupland, K.B., *Pyrexia: aetiology in the ICU.* Crit Care, 2016. 20(1): p. 247.
40. Doyle, J.F. and Schortgen, F., *Should we treat pyrexia? And how do we do it?* Crit Care, 2016. 20(1): p. 303.
41. Aronoff, D.M. and Neilson, E.G., *Antipyretics: mechanisms of action and clinical use in fever suppression.* The American Journal of Medicine, 2001. 111(4): p. 304-315.
42. Collard, C.D. and Gelman, S., *Pathophysiology, clinical manifestations, and prevention of ischemia-reperfusion injury.* Anesthesiology, 2001. 94(6): p. 1133-8.

43. Kalogeris, T., Baines, C.P., Krenz, M., and Korthuis, R.J., *Cell biology of ischemia/reperfusion injury*, in *Int Rev Cell Mol Biol*. 2012. p. 229-317.
44. Gorsuch, W.B., Chrysanthou, E., Schwaeble, W.J., and Stahl, G.L., *The complement system in ischemia-reperfusion injuries.* Immunobiology, 2012. 217(11): p. 1026-33.
45. Vestweber, D., *How leukocytes cross the vascular endothelium.* Nat Rev Immunol, 2015. 15(11): p. 692-704.
46. Khan, F., Pharo, A., Lindstad, J.K., Mollnes, T.E., Tønnessen, T.I., and Pischke, S.E., *Effect of Perfusion Fluids on Recovery of Inflammatory Mediators in Microdialysis.* Scand J Immunol, 2015. 82(5): p. 467-75.
47. McEver, R.P., *Selectins: initiators of leucocyte adhesion and signalling at the vascular wall.* Cardiovasc Res, 2015. 107(3): p. 331-9.
48. Huber-Lang, M., Lambris, J.D., and Ward, P.A., *Innate immune responses to trauma.* Nature Immunology, 2018. 19(4): p. 327-341.
49. Menon, D.K., Schwab, K., Wright, D.W., and Maas, A.I., *Position statement: definition of traumatic brain injury.* Arch Phys Med Rehabil, 2010. 91(11): p. 1637-40.
50. Maas, A.I.R., Menon, D.K., Adelson, P.D., Andelic, N., Bell, M.J., . . . Yaffe, K., *Traumatic brain injury: integrated approaches to improve prevention, clinical care, and research.* Lancet Neurol, 2017. 16(12): p. 987-1048.
51. Bazarian, J.J., McClung, J., Shah, M.N., Cheng, Y.T., Flesher, W., and Kraus, J., *Mild traumatic brain injury in the United States, 1998--2000.* Brain Inj, 2005. 19(2): p. 85-91.
52. Simon, D.W., McGeachy, M.J., Bayır, H., Clark, R.S., Loane, D.J., and Kochanek, P.M., *The far-reaching scope of neuroinflammation after traumatic brain injury.* Nature Reviews Neurology, 2017. 13(3): p. 171-191.
53. Zimmerman, D.S. and Tan, H.L., *Epidemiology and risk factors of sudden cardiac arrest.* Curr Opin Crit Care, 2021. 27(6): p. 613-616.
54. Sandroni, C., Nolan, J.P., Andersen, L.W., Böttiger, B.W., Cariou, A., . . . Morley, P.T.J.I.C.M., *ERC-ESICM guidelines on temperature control after cardiac arrest in adults.* 2022. 48(3): p. 261-269.
55. Gräsner, J.T., Herlitz, J., Tjelmeland, I.B.M., Wnent, J., Masterson, S., . . . Perkins, G.D., *European Resuscitation Council Guidelines 2021: Epidemiology of cardiac arrest in Europe.* Resuscitation, 2021. 161: p. 61-79.
56. Boyko, E.J., *Observational research--opportunities and limitations.* J Diabetes Complications, 2013. 27(6): p. 642-8.
57. Hess, A.S. and Abd-Elsayed, A., *Observational Studies: Uses and Limitations*, in *Pain: A Review Guide*, A. Abd-Elsayed, Editor. 2019, Springer International Publishing: Cham. p. 123-125.
58. Kristman, V., Manno, M., and Côté, P., *Loss to follow-up in cohort studies: how much is too much?* Eur J Epidemiol, 2004. 19(8): p. 751-60.
59. Bergseth, G., Ludviksen, J.K., Kirschfink, M., Giclas, P.C., Nilsson, B., and Mollnes, T.E., *An international serum standard for application in assays to detect human complement activation products.* Mol Immunol, 2013. 56(3): p. 232-9.
60. Henderson, M. and Page, L., *Appraising the evidence: what is selection bias?* Evid Based Ment Health, 2007. 10(3): p. 67-8.
61. Mollnes, T.E., Garred, P., and Bergseth, G., *Effect of time, temperature and anticoagulants on in vitro complement activation: consequences for collection and preservation of samples to be examined for complement activation.* Clin Exp Immunol, 1988. 73(3): p. 484-8.
62. Mollnes, T.E., Brekke, O.L., Fung, M., Fure, H., Christiansen, D., . . . Lambris, J.D., *Essential role of the C5a receptor in E coli-induced oxidative burst and phagocytosis revealed by a novel lepirudin-based human whole blood model of inflammation.* Blood, 2002. 100(5): p. 1869-77.

63. Mollnes, T.E., Lea, T., Frøland, S.S., and Harboe, M., *Quantification of the terminal complement complex in human plasma by an enzyme-linked immunosorbent assay based on monoclonal antibodies against a neoantigen of the complex.* Scand J Immunol, 1985. 22(2): p. 197-202.
64. Xiao, L., Liu, Y., and Wang, N., *New paradigms in inflammatory signaling in vascular endothelial cells.* Am J Physiol Heart Circ Physiol, 2014. 306(3): p. H317-25.
65. Fitzmaurice, G.M. and Ravichandran, C., *A primer in longitudinal data analysis.* Circulation, 2008. 118(19): p. 2005-10.
66. Idris, A.H., Bierens, J., Perkins, G.D., Wenzel, V., Nadkarni, V., . . . Hazinski, M.F., *2015 Revised Utstein-Style Recommended Guidelines for Uniform Reporting of Data From Drowning-Related Resuscitation: An ILCOR Advisory Statement.* Circ Cardiovasc Qual Outcomes, 2017. 10(7).
67. Orrem, H.L., Nilsson, P.H., Pischke, S.E., Grindheim, G., Garred, P., . . . Andersen, G.Ø.J.E.h.f., *Acute heart failure following myocardial infarction: complement activation correlates with the severity of heart failure in patients developing cardiogenic shock.* 2018. 5(3): p. 292-301.
68. Lintermans, L.L., Stegeman, C.A., Heeringa, P., and Abdulahad, W.H., *T cells in vascular inflammatory diseases.* Front Immunol, 2014. 5: p. 504.
69. Sugimoto, M.A., Sousa, L.P., Pinho, V., Perretti, M., and Teixeira, M.M., *Resolution of Inflammation: What Controls Its Onset?* Front Immunol, 2016. 7: p. 160.
70. Neher, M.D., Weckbach, S., Flierl, M.A., Huber-Lang, M.S., and Stahel, P.F., *Molecular mechanisms of inflammation and tissue injury after major trauma--is complement the "bad guy"?* J Biomed Sci, 2011. 18(1): p. 90.
71. Billiar, T.R. and Vodovotz, Y., *Time for trauma immunology.* PLoS Med, 2017. 14(7): p. e1002342.
72. Rock, K.L., Latz, E., Ontiveros, F., and Kono, H., *The sterile inflammatory response.* Annual review of immunology, 2010. 28: p. 321-342.
73. Sun, S., Sursal, T., Adibnia, Y., Zhao, C., Zheng, Y., . . . Itagaki, K., *Mitochondrial DAMPs increase endothelial permeability through neutrophil dependent and independent pathways.* PLoS One, 2013. 8(3): p. e59989.
74. Cederberg, D. and Siesjö, P., *What has inflammation to do with traumatic brain injury?* Childs Nerv Syst, 2010. 26(2): p. 221-6.
75. Pan, W., Stone, K.P., Hsuchou, H., Manda, V.K., Zhang, Y., and Kastin, A.J., *Cytokine signaling modulates blood-brain barrier function.* Current pharmaceutical design, 2011. 17(33): p. 3729-3740.
76. Merchant-Borna, K., Lee, H., Wang, D., Bogner, V., van Griensven, M., . . . Bazarian, J.J., *Genome-Wide Changes in Peripheral Gene Expression following Sports-Related Concussion.* J Neurotrauma, 2016. 33(17): p. 1576-85.
77. Patil, K.D., Halperin, H.R., and Becker, L.B., *Cardiac arrest: resuscitation and reperfusion.* Circ Res, 2015. 116(12): p. 2041-9.
78. Jou, C., Shah, R., Figueroa, A., and Patel, J.K., *The Role of Inflammatory Cytokines in Cardiac Arrest.* J Intensive Care Med, 2020. 35(3): p. 219-224.
79. Russo, M.V. and McGavern, D.B., *Inflammatory neuroprotection following traumatic brain injury.* Science, 2016. 353(6301): p. 783-5.
80. Smith, J.A., Das, A., Ray, S.K., and Banik, N.L., *Role of pro-inflammatory cytokines released from microglia in neurodegenerative diseases.* Brain Res Bull, 2012. 87(1): p. 10-20.
81. Johnson, V.E., Stewart, J.E., Begbie, F.D., Trojanowski, J.Q., Smith, D.H., and Stewart, W., *Inflammation and white matter degeneration persist for years after a single traumatic brain injury.* Brain, 2013. 136(Pt 1): p. 28-42.
82. Gräsner, J.T., Wnent, J., Herlitz, J., Perkins, G.D., Lefering, R., . . . Bossaert, L., *Survival after out-of-hospital cardiac arrest in Europe - Results of the EuReCa TWO study.* Resuscitation, 2020. 148: p. 218-226.

83. Zhang, C., Brandon, N.R., Koper, K., Tang, P., Xu, Y., and Dou, H., *Invasion of Peripheral Immune Cells into Brain Parenchyma after Cardiac Arrest and Resuscitation.* Aging Dis, 2018. 9(3): p. 412-425.
84. Li, H., Chen, C., and Wang, D.W., *Inflammatory Cytokines, Immune Cells, and Organ Interactions in Heart Failure.* Front Physiol, 2021. 12: p. 695047.
85. Chamorro, A. and Hallenbeck, J., *The harms and benefits of inflammatory and immune responses in vascular disease.* Stroke, 2006. 37(2): p. 291-293.
86. Stokowska, A., Atkins, A.L., Morán, J., Pekny, T., Bulmer, L., . . . Pekna, M., *Complement peptide C3a stimulates neural plasticity after experimental brain ischaemia.* Brain, 2017. 140(2): p. 353-369.
87. Thorgersen, E.B., Barratt-Due, A., Haugaa, H., Harboe, M., Pischke, S.E., . . . Mollnes, T.E., *The Role of Complement in Liver Injury, Regeneration, and Transplantation.* Hepatology, 2019. 70(2): p. 725-736.
88. Strey, C.W., Markiewski, M., Mastellos, D., Tudoran, R., Spruce, L.A., . . . Lambris, J.D., *The proinflammatory mediators C3a and C5a are essential for liver regeneration.* J Exp Med, 2003. 198(6): p. 913-23.
89. Pahwa, R., Goyal, A., Bansal, P., and Jialal, I., *Chronic inflammation.* 2018.
90. Kushi, H., Saito, T., Makino, K., and Hayashi, N., *IL-8 is a key mediator of neuroinflammation in severe traumatic brain injuries.* Acta Neurochir Suppl, 2003. 86: p. 347-50.
91. Cherry, J.D., Stein, T.D., Tripodis, Y., Alvarez, V.E., Huber, B.R., . . . McKee, A.C., *CCL11 is increased in the CNS in chronic traumatic encephalopathy but not in Alzheimer's disease.* PLOS ONE, 2017. 12(9): p. e0185541.
92. Ciechanowska, A., Popiolek-Barczyk, K., Pawlik, K., Ciapała, K., Oggioni, M., . . . Mika, J., *Changes in macrophage inflammatory protein-1 (MIP-1) family members expression induced by traumatic brain injury in mice.* Immunobiology, 2020. 225(3): p. 151911.
93. Zlotnik, A. and Yoshie, O., *The Chemokine Superfamily Revisited.* Immunity, 2012. 36(5): p. 705-716.
94. Kunis, G., Baruch, K., Rosenzweig, N., Kertser, A., Miller, O., . . . Schwartz, M., *IFN-γ-dependent activation of the brain's choroid plexus for CNS immune surveillance and repair.* Brain, 2013. 136(11): p. 3427-3440.
95. Li, T., Zhang, Y.M., Han, D., Hua, R., Guo, B.N., . . . Xu, T., *Involvement of IL-17 in Secondary Brain Injury After a Traumatic Brain Injury in Rats.* Neuromolecular Med, 2017. 19(4): p. 541-554.
96. Shi, H., Wang, J., Wang, J., Huang, Z., and Yang, Z., *IL-17A induces autophagy and promotes microglial neuroinflammation through ATG5 and ATG7 in intracerebral hemorrhage.* J Neuroimmunol, 2018. 323: p. 143-151.
97. Amor, S., Puentes, F., Baker, D., and van der Valk, P., *Inflammation in neurodegenerative diseases.* Immunology, 2010. 129(2): p. 154-169.
98. Kokiko-Cochran, O.N. and Godbout, J.P., *The Inflammatory Continuum of Traumatic Brain Injury and Alzheimer's Disease.* Front Immunol, 2018. 9: p. 672.
99. Mocco, J., Mack, W.J., Ducruet, A.F., Sosunov, S.A., Sughrue, M.E., . . . Connolly, E.S., Jr., *Complement component C3 mediates inflammatory injury following focal cerebral ischemia.* Circ Res, 2006. 99(2): p. 209-17.
100. Rynkowski, M.A., Kim, G.H., Garrett, M.C., Zacharia, B.E., Otten, M.L., . . . Connolly, E.S., *C3a receptor antagonist attenuates brain injury after intracerebral hemorrhage.* J Cereb Blood Flow Metab, 2009. 29(1): p. 98-107.
101. Jenei, Z.M., Zima, E., Csuka, D., Munthe-Fog, L., Hein, E., . . . Merkely, B., *Complement activation and its prognostic role in post-cardiac arrest patients.* Scand J Immunol, 2014. 79(6): p. 404-9.

102. Zacharia, B.E., Hickman, Z.L., Grobelny, B.T., DeRosa, P.A., Ducruet, A.F., and Connolly, E.S., *Complement inhibition as a proposed neuroprotective strategy following cardiac arrest.* Mediators Inflamm, 2009. 2009: p. 124384.
103. Serhan, C.N., Chiang, N., Dalli, J., and Levy, B.D., *Lipid mediators in the resolution of inflammation.* Cold Spring Harb Perspect Biol, 2014. 7(2): p. a016311.
104. Perretti, M. and D'Acquisto, F., *Annexin A1 and glucocorticoids as effectors of the resolution of inflammation.* Nat Rev Immunol, 2009. 9(1): p. 62-70.
105. Haskó, G. and Cronstein, B., *Regulation of inflammation by adenosine.* Front Immunol, 2013. 4: p. 85.
106. Mirakaj, V., Dalli, J., Granja, T., Rosenberger, P., and Serhan, C.N., *Vagus nerve controls resolution and pro-resolving mediators of inflammation.* J Exp Med, 2014. 211(6): p. 1037-48.
107. Mukundan, L., Odegaard, J.I., Morel, C.R., Heredia, J.E., Mwangi, J.W., . . . Chawla, A., *PPAR-delta senses and orchestrates clearance of apoptotic cells to promote tolerance.* Nat Med, 2009. 15(11): p. 1266-72.
108. Yang, Q., He, G.-W., Underwood, M.J., and Yu, C.-M., *Cellular and molecular mechanisms of endothelial ischemia/reperfusion injury: perspectives and implications for postischemic myocardial protection.* American journal of translational research, 2016. 8(2): p. 765-777.
109. Deisher, T.A., Haddix, T.L., Montgomery, K.F., Pohlman, T.H., Kaushansky, K., and Harlan, J.M., *The role of protein kinase C in the induction of VCAM-1 expression on human umbilical vein endothelial cells.* FEBS Lett, 1993. 331(3): p. 285-90.
110. Ong, S.-B., Hernández-Reséndiz, S., Crespo-Avilan, G.E., Mukhametshina, R.T., Kwek, X.-Y., . . . Hausenloy, D.J., *Inflammation following acute myocardial infarction: Multiple players, dynamic roles, and novel therapeutic opportunities.* Pharmacology & Therapeutics, 2018. 186: p. 73-87.
111. Saethre, M., Baumann, B.C., Fung, M., Seebach, J.D., and Mollnes, T.E., *Characterization of natural human anti-non-gal antibodies and their effect on activation of porcine gal-deficient endothelial cells.* Transplantation, 2007. 84(2): p. 244-50.
112. Pongratz, G. and Straub, R.H., *The sympathetic nervous response in inflammation.* Arthritis Res Ther, 2014. 16(6): p. 504.
113. Scanzano, A. and Cosentino, M., *Adrenergic regulation of innate immunity: a review.* Front Pharmacol, 2015. 6: p. 171.
114. Zila, I., Mokra, D., Kopincova, J., Kolomaznik, M., Javorka, M., and Calkovska, A., *Vagal-immune interactions involved in cholinergic anti-inflammatory pathway.* Physiol Res, 2017. 66(Suppl 2): p. S139-s145.
115. Tracey, I. and Mantyh, P.W., *The cerebral signature for pain perception and its modulation.* Neuron, 2007. 55(3): p. 377-91.
116. Lu, L., Zhou, H., Ni, M., Wang, X., Busuttil, R., . . . Zhai, Y., *Innate Immune Regulations and Liver Ischemia-Reperfusion Injury.* Transplantation, 2016. 100(12): p. 2601-2610.
117. Wang, P.-F., Xiong, X.-Y., Chen, J., Wang, Y.-C., Duan, W., and Yang, Q.-W., *Function and mechanism of toll-like receptors in cerebral ischemic tolerance: from preconditioning to treatment.* Journal of Neuroinflammation, 2015. 12(1): p. 80.
118. Lehnardt, S., Lehmann, S., Kaul, D., Tschimmel, K., Hoffmann, O., . . . Weber, J.R., *Toll-like receptor 2 mediates CNS injury in focal cerebral ischemia.* J Neuroimmunol, 2007. 190(1-2): p. 28-33.
119. Ziegler, G., Harhausen, D., Schepers, C., Hoffmann, O., Röhr, C., . . . Trendelenburg, G., *TLR2 has a detrimental role in mouse transient focal cerebral ischemia.* Biochem Biophys Res Commun, 2007. 359(3): p. 574-9.
120. Timmers, L., Pasterkamp, G., de Hoog, V.C., Arslan, F., Appelman, Y., and de Kleijn, D.P., *The innate immune response in reperfused myocardium.* Cardiovasc Res, 2012. 94(2): p. 276-83.

121. Lee, S.M., Hutchinson, M., and Saint, D.A., *The role of Toll-like receptor 4 (TLR4) in cardiac ischaemic-reperfusion injury, cardioprotection and preconditioning.* Clin Exp Pharmacol Physiol, 2016. 43(9): p. 864-71.
122. Caso, J.R., Pradillo, J.M., Hurtado, O., Lorenzo, P., Moro, M.A., and Lizasoain, I., *Toll-like receptor 4 is involved in brain damage and inflammation after experimental stroke.* Circulation, 2007. 115(12): p. 1599-608.
123. Brea, D., Blanco, M., Sobrino, T., Ramos-Cabrer, P., and Castillo, J., *The levels of expression of toll-like receptors 2 and 4 in neutrophils are associated with the prognosis of ischaemic stroke patients.* Rev Neurol, 2011. 52(1): p. 12-9.
124. Own, J., Ulevitch, R., and McKay, D., *CD14 blockade to prevent ischemic injury to donor organs.* Transpl Immunol, 2022. 72: p. 101580.
125. Ricklin, D., Hajishengallis, G., Yang, K., and Lambris, J.D., *Complement: a key system for immune surveillance and homeostasis.* Nature immunology, 2010. 11(9): p. 785-797.
126. Zhang, M. and Carroll, M.C., *Natural IgM-mediated innate autoimmunity: a new target for early intervention of ischemia-reperfusion injury.* Expert Opin Biol Ther, 2007. 7(10): p. 1575-82.
127. Sihag, S., Haas, M.S., Kim, K.M., Guerrero, J.L., Beaudoin, J., . . . Allan, J.S., *Natural IgM Blockade Limits Infarct Expansion and Left Ventricular Dysfunction in a Swine Myocardial Infarct Model.* Circ Cardiovasc Interv, 2016. 9(1): p. e002547.
128. Zhang, M., Takahashi, K., Alicot, E.M., Vorup-Jensen, T., Kessler, B., . . . Carroll, M.C., *Activation of the lectin pathway by natural IgM in a model of ischemia/reperfusion injury.* J Immunol, 2006. 177(7): p. 4727-34.
129. Kalogeris, T., Baines, C.P., Krenz, M., and Korthuis, R.J., *Chapter Six - Cell Biology of Ischemia/Reperfusion Injury*, in *International Review of Cell and Molecular Biology*, K.W. Jeon, Editor. 2012, Academic Press. p. 229-317.
130. Markiewski, M.M., Nilsson, B., Ekdahl, K.N., Mollnes, T.E., and Lambris, J.D., *Complement and coagulation: strangers or partners in crime?* Trends Immunol, 2007. 28(4): p. 184-92.
131. Nilsson, P.H., Johnson, C., Quach, Q.H., Macpherson, A., Durrant, O., . . . Mollnes, T.E., *A Conformational Change of Complement C5 Is Required for Thrombin-Mediated Cleavage, Revealed by a Novel Ex Vivo Human Whole Blood Model Preserving Full Thrombin Activity.* J Immunol, 2021. 207(6): p. 1641-1651.
132. Turrens, J.F., *Mitochondrial formation of reactive oxygen species.* J Physiol, 2003. 552(Pt 2): p. 335-44.
133. von Zabern, W.V., Hesse, D., Nolte, R., and Haller, Y., *Generation of an activated form of human C5 (C5b-like C5) by oxygen radicals.* Immunol Lett, 1987. 14(3): p. 209-15.
134. Shingu, M. and Nobunaga, M., *Chemotactic activity generated in human serum from the fifth component of complement by hydrogen peroxide.* The American journal of pathology, 1984. 117(2): p. 201-206.
135. Fantone, J.C. and Ward, P.A., *Role of oxygen-derived free radicals and metabolites in leukocyte-dependent inflammatory reactions.* Am J Pathol, 1982. 107(3): p. 395-418.
136. Köhl, J., *The role of complement in danger sensing and transmission.* Immunol Res, 2006. 34(2): p. 157-76.
137. Garred, P., Genster, N., Pilely, K., Bayarri-Olmos, R., Rosbjerg, A., . . . Skjoedt, M.O., *A journey through the lectin pathway of complement-MBL and beyond.* Immunol Rev, 2016. 274(1): p. 74-97.
138. Mollnes, T.E., Jokiranta, T.S., Truedsson, L., Nilsson, B., Rodriguez de Cordoba, S., and Kirschfink, M., *Complement analysis in the 21st century.* Mol Immunol, 2007. 44(16): p. 3838-49.
139. Nurnberger, W. and Bhakdi, S., *Plasma C3d/C3 quotient as a parameter for in vivo complement activation.* J Immunol Methods, 1984. 74(1): p. 87-91.

140. Nielsen, E.W., Johansen, H.T., Hogasen, K., Wuillemin, W., Hack, C.E., and Mollnes, T.E., *Activation of the complement, coagulation, fibrinolytic and kallikrein-kinin systems during attacks of hereditary angioedema.* Scand J Immunol, 1996. 44(2): p. 185-92.
141. Kim, A.H.J., Strand, V., Sen, D.P., Fu, Q., Mathis, N.L., . . . Atkinson, J.P., *Association of Blood Concentrations of Complement Split Product iC3b and Serum C3 With Systemic Lupus Erythematosus Disease Activity.* Arthritis Rheumatol, 2019. 71(3): p. 420-430.
142. Husebye, T., Eritsland, J., Arnesen, H., Bjørnerheim, R., Mangschau, A., . . . Andersen, G.Ø., *Association of Interleukin 8 and Myocardial Recovery in Patients with ST-Elevation Myocardial Infarction Complicated by Acute Heart Failure.* PLOS ONE, 2014. 9(11): p. e112359.
143. Nakstad, E.R., Stær-Jensen, H., Wimmer, H., Henriksen, J., Alteheld, L.H., . . . Sunde, K., *Late awakening, prognostic factors and long-term outcome in out-of-hospital cardiac arrest - results of the prospective Norwegian Cardio-Respiratory Arrest Study (NORCAST).* Resuscitation, 2020. 149: p. 170-179.
144. Wagner, J.L. and Hugli, T.E., *Radioimmunoassay for anaphylatoxins: a sensitive method for determining complement activation products in biological fluids.* Anal Biochem, 1984. 136(1): p. 75-88.
145. Deppisch, R., Schmitt, V., Bommer, J., Hänsch, G.M., Ritz, E., and Rauterberg, E.W., *Fluid phase generation of terminal complement complex as a novel index of bioincompatibility.* Kidney Int, 1990. 37(2): p. 696-706.
146. Mollnes, T.E., *Early- and late-phase activation of complement evaluated by plasma levels of C3d,g and the terminal complement complex.* Complement, 1985. 2(2-3): p. 156-64.
147. Arrich, J., Holzer, M., Havel, C., Müllner, M., and Herkner, H.J.C.d.o.s.r., *Hypothermia for neuroprotection in adults after cardiopulmonary resuscitation.* 2016(2).
148. Dallan, L.A.P., Giannetti, N.S., Rochitte, C.E., Polastri, T.F., Bernoche San Martin, C.Y., . . . Timerman, S., *Cooling as an Adjunctive Therapy to Percutaneous Intervention in Acute Myocardial Infarction: COOL-MI InCor Trial.* Ther Hypothermia Temp Manag, 2020. 11(3): p. 135-144.
149. Zafren, K., Paal, P., Brugger, H., and Lechner, R., *Induced Hypothermia to 4.2°C with Neurologically Intact Survival: A Forgotten Case Series.* Wilderness Environ Med, 2020. 31(3): p. 367-370.
150. Williams, G.R., Jr. and Spencer, F.C., *The clinical use of hypothermia following cardiac arrest.* Ann Surg, 1958. 148(3): p. 462-8.
151. *Mild therapeutic hypothermia to improve the neurologic outcome after cardiac arrest.* N Engl J Med, 2002. 346(8): p. 549-56.
152. Bisschops, L.L., Van Der Hoeven, J.G., Mollnes, T.E., and Hoedemaekers, C.W.J.C.C., *Seventy-two hours of mild hypothermia after cardiac arrest is associated with a lowered inflammatory response during rewarming in a prospective observational study.* 2014. 18(5): p. 546.
153. Nielsen, N., Wetterslev, J., Cronberg, T., Erlinge, D., Gasche, Y., . . . Friberg, H., *Targeted temperature management at 33°C versus 36°C after cardiac arrest.* N Engl J Med, 2013. 369(23): p. 2197-206.
154. Bro-Jeppesen, J., Kjaergaard, J., Wanscher, M., Nielsen, N., Friberg, H., . . . Hassager, C., *The inflammatory response after out-of-hospital cardiac arrest is not modified by targeted temperature management at 33 °C or 36 °C.* Resuscitation, 2014. 85(11): p. 1480-7.
155. Cameron, A.M. and Barandiaran Cornejo, J.F., *Organ preservation review: history of organ preservation.* Curr Opin Organ Transplant, 2015. 20(2): p. 146-51.
156. Atkinson, C., He, S., Morris, K., Qiao, F., Casey, S., . . . Tomlinson, S., *Targeted complement inhibitors protect against posttransplant cardiac ischemia and reperfusion injury and reveal an important role for the alternative pathway of complement activation.* J Immunol, 2010. 185(11): p. 7007-13.

157. Schröppel, B., Akalin, E., Baweja, M., Bloom, R.D., Florman, S., . . . Heeger, P.S., *Peritransplant eculizumab does not prevent delayed graft function in deceased donor kidney transplant recipients: Results of two randomized controlled pilot trials.* Am J Transplant, 2020. 20(2): p. 564-572.
158. Lo, S., Jiang, L., Stacks, S., Lin, H., and Parajuli, N., *Aberrant activation of the complement system in renal grafts is mediated by cold storage.* Am J Physiol Renal Physiol, 2021. 320(6): p. F1174-f1190.
159. Weisman, H.F., Bartow, T., Leppo, M.K., Marsh, H.C., Jr., Carson, G.R., . . . Fearon, D.T., *Soluble human complement receptor type 1: in vivo inhibitor of complement suppressing post-ischemic myocardial inflammation and necrosis.* Science, 1990. 249(4965): p. 146-51.
160. Pischke, S.E., Gustavsen, A., Orrem, H.L., Egge, K.H., Courivaud, F., . . . Mollnes, T.E., *Complement factor 5 blockade reduces porcine myocardial infarction size and improves immediate cardiac function.* Basic Res Cardiol, 2017. 112(3): p. 20.
161. Armstrong, P.W., Granger, C.B., Adams, P.X., Hamm, C., Holmes, D., Jr., . . . Van de Werf, F., *Pexelizumab for acute ST-elevation myocardial infarction in patients undergoing primary percutaneous coronary intervention: a randomized controlled trial.* Jama, 2007. 297(1): p. 43-51.
162. Smith, P.K., Carrier, M., Chen, J.C., Haverich, A., Levy, J.H., . . . Verrier, E., *Effect of pexelizumab in coronary artery bypass graft surgery with extended aortic cross-clamp time.* Ann Thorac Surg, 2006. 82(3): p. 781-8; discussion 788-9.
163. Fiane, A.E., Videm, V., Lingaas, P.S., Heggelund, L., Nielsen, E.W., . . . Mollnes, T.E., *Mechanism of complement activation and its role in the inflammatory response after thoracoabdominal aortic aneurysm repair.* Circulation, 2003. 108(7): p. 849-56.

Article I

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Systemic Inflammation Persists the First Year after Mild Traumatic Brain Injury: Results from the Prospective Trondheim Mild Traumatic Brain Injury Study

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Abstract

Innate immune activation has been attributed a key role in traumatic brain injury (TBI) and successive morbidity. In mild TBI (mTBI), however, the extent and persistence of innate immune activation are unknown. We determined plasma cytokine level changes over 12 months after an mTBI in hospitalized and non-hospitalized patients compared with community controls; and examined their associations to injury-related and demographic variables at admission. Prospectively, 207 patients presenting to the emergency department (ED) or general practitioner with clinically confirmed mTBI and 82 matched community controls were included. Plasma samples were obtained at admission, after 2 weeks, 3 months, and 12 months. Cytokine levels were analysed with a 27-plex beads-based immunoassay. Brain magnetic resonance imaging (MRI) was performed on all participants. Twelve cytokines were reliably detected. Plasma levels of interferon gamma (IFN-γ), interleukin 8 (IL-8), eotaxin, macrophage inflammatory protein-1-beta (MIP-1β), monocyte chemoattractant protein 1 (MCP-1), IL-17A, IL-9, tumor necrosis factor (TNF), and basic fibroblast growth factor (FGF-basic) were significantly increased at all time-points in patients compared with controls, whereas IFN-γ-inducing protein 10 (IP-10), platelet-derived growth factor (PDGF), and IL-1ra were not. IL-17A and FGF-basic showed significant increases in patients from admission to follow-up at 3 months, and remained increased at 12 months compared with admission. Interestingly, MRI findings were negatively associated with four cytokines: eotaxin, MIP-1β, IL-9, and IP-10, whereas age was positively associated with nine cytokines: IL-8, eotaxin, MIP-1β, MCP-1, IL-17A, IL-9, TNF, FGF-basic, and IL-1ra. TNF was also increased in those with presence of other injuries. In conclusion, mTBI activated the innate immune system consistently and this is the first study to show that several inflammatory cytokines remain increased for up to 1 year post-injury.

Keywords: concussion; cytokines; growth factors; immune system; pathophysiology

Introduction

The World Health Organization (WHO) has identified traumatic brain injury (TBI) as a key societal challenge as it emerges as the number one cause of death and disability from 2020, with 2.5 million cases estimated to occur each year in the European Union.[1] More than 85% of TBIs are classified as mild.[2] Although the majority recover well after mild TBI (mTBI), 10–20% experience long-lasting symptoms such as headache, emotional distress, and problems with concentration and memory.[3,4]

Given that even mild tissue injury can damage cells and release or expose damage associated molecular patterns (DAMPS), we can hypothesize that inflammation in the brain, which is detectable in

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systemic blood, could be an important pathophysiological mechanism following mTBI.[5]

The interplay between central nervous system (CNS)-derived inflammation and systemic inflammation is intricate. Recent evidence suggests that cytokines orchestrate a complex interplay between peripheral leukocytes and chronically activated microglia, especially if the blood–brain barrier is compromised after mechanical damage, as seen in TBI.[6] Although activation of the innate immune system is crucial for recovery after TBI, as it promotes beneficial clearance of injured cells/cell debris, prolonged neuroinflammation has been shown to be detrimental, leading to progressive CNS degeneration.[5,7] In experimental models, it was shown that repeated mTBI caused systemic and neuroinflammation associated with chronic behavioral deficits.[8] In sports-related mTBI an immediate activation of the innate immune system (interleukin 6 [IL-6], IL-12) has been observed with return to baseline after a week.[9] The long-term course of systemic inflammation and type of cytokines present in blood in patients with mTBI has been evaluated sparsely, with one study including 52 patients showing an increase of IL-1β, IL-6, and monocyte chemoattractant protein 1 (MCP-1) over a 3-month period with association of MCP-1 to post-concussion syndrome.[10]

Many patients with mild TBI are not admitted to hospitals and are thus possibly underrepresented in clinical studies.[11] Therefore, the Trondheim Mild TBI study was designed to include patients who were seen in the primary care setting for mTBI, in addition to patients who were admitted to the hospital, with 1-year follow-up of all patients.[12] In addition, age-matched community controls were included for the same length of the follow-up for comparison.

In the present study, we examined plasma levels of a wide range of inflammatory biomarkers in patients with mTBI from admission to 12 months after injury compared with age-, sex-, and education-matched community controls over the same time period. We then examined associations between injury-related and demographic variables and cytokine levels in the acute phase.

We hypothesized that mTBI leads to increases in systemic cytokines over a prolonged time period and that acute-phase inflammatory cytokines are associated with injury-related and demographic factors.

Methods

Ethics, participants, and recruitment

The Trondheim Mild TBI study is a large-scale prospective cohort study with follow-up for 12 months in patients with mTBI and matched controls, all between 16 and 59 years of age (Norwegian National Ethics Approval: REK 2013/754). The upper age limit was chosen due to the higher frequency of comorbidities in the elderly. All participants, and guardians of participants between the age of 16 and 18 years, provided informed consent. The cohort and magnetic resonance imaging (MRI) follow-up have been described in detail previously.[12,13] All data were handled in accordance with the STROBE checklist (Supplementary Fig. S1).

Patients were included in the study between April 1, 2014 and December 5, 2015. Patients were prospectively recruited by continuous screening of computed tomography (CT) referrals and patient lists from two emergency departments (EDs): St. Olavs Hospital (Trondheim University Hospital), a regional Level 1 trauma center in Trondheim, Norway, and Trondheim Municipal Emergency Clinic, a general practitioner-run, outpatient clinic. Patients were included when having sustained a TBI categorized as mild according to the WHO criteria: 1) Glasgow Coma Scale (GCS) score 13–15 at presentation in the ED; 2) witnessed loss of consciousness (LOC) <30 min, confusion, or post-traumatic amnesia (PTA) <24 h, or traumatic lesion on neuroimaging, and did not meet any exclusion criteria. (Supplementary Table S1).[14,15]

Clinical information was obtained from patient interviews and medical records. LOC was rated as present only if observed. Duration of PTA was recorded as the time after injury for which the patient had no continuous memory (< 1 h, or 1–24 h). GCS score was assessed in the ED or inferred from the record. Presence of injuries to parts of the body other than the head was recorded, and dichotomized into ''yes'' or ''no,'' based on self-report and records. Such injuries were cranial fractures; fractures of extremities, clavicles, and ribs; wounds; and sprains, dislocations, and other soft-tissue injuries. Bruises and wounds that did not need suturing were not included. Major trauma was an exclusion criterion in the study.

Community controls, matched on age, sex, and years of education were recruited among staff, friends, and families of staff and patients. The same exclusion criteria as for the TBI patients were used (Supplementary Table S1).

Magnetic resonance imaging protocol

MRI scans were acquired using a 3.0 Tesla Siemens Skyra MRI scanner, software version E11C, with a 32-channel head coil. The same MRI protocol was used for all participants; three-dimensional (3D) volumes were obtained with T1-weighted (magnetization prepared rapid acquisition gradient echo [MPRAGE]), T2-weighted, fluid-attenuated inversion recovery (FLAIR), and susceptibility-weighted imaging (SWI). An axial (2D) diffusion-weighted scan and a diffusion tensor/kurtosis scan were also acquired. The clinical scans were read by an experienced neuroradiologist according to predefined criteria as described in a previous publication.[13]

Blood samples

Blood was acquired from patients with mTBI in the admission phase, defined as within 72 h post-injury; 2 weeks (± 3 days); 3 months (± 2 weeks); and 12 months (± 1 month) after the injury. For the community controls, blood was collected at inclusion corresponding to the admission phase for the patients, and after 3 and 12 months. Plasma samples used in the current study were obtained from whole blood collected in 5-mL tubes containing ethylenediaminetetraacetic acid (EDTA), directly placed on ice, and centrifuged within 30 min at 2000 x *g* for 10 min at 4°C. Aliquoted plasma samples were immediately stored at −80°C.

Cytokine analysis

The EDTA plasma samples were analyzed using a commercial fluorescence magnetic bead-based immunoassay, with high-sensitivity detection range and precision (Bio-Plex Human Cytokine 27-Plex, Bio-Rad Laboratories, Inc., Hercules, CA, USA). The following cytokines were analyzed: IL-1β, IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (C-X-C motif chemokine ligand 8; CXCL8), IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin-1 (C-C motif chemokine ligand 11; CCL11), basic fibroblast growth factor (FGF-basic), granulocyte colony stimulating factor (GCSF), granulocyte-macrophage colony stimulating factor (GM-CSF), interferon gamma (IFN-γ), IFN-γ-inducing protein 10 (IP-10; CXCL10), monocyte chemoattractant protein 1 (MCP-1; CCL2), macrophage inflammatory protein-1-alpha (MIP-1α; CCL3), macrophage inflammatory protein-1-beta (MIP-1β; CCL4), platelet-derived growth factor-BB (PDGF-BB), RANTES (CCL5), tumor necrosis factor (TNF), and vascular endothelial growth factor (VEGF).

The analyses were performed according to the manufacturer's instructions. Briefly, plasma samples were diluted 1:4 in Sample Diluent (Bio-Rad Laboratories, Inc.). A lower detection limit for

the cytokines in the low picogram/milliliter range (<20 pg/mL for all cytokines) was determined automatically by the software based on the standard curve for each cytokine. Based on many years of experience with the multi-plex assay and a low inter-assay coefficient of variation (<11 for all cytokines), the samples were run in single. All samples from this study were analyzed using the same batch of the Bio-Plex assay and on each plate randomly chosen sample sets from patients and healthy controls were analyzed.

Under physiological conditions most of the cytokines in plasma are either not detected or detected in very low amounts.[16] Thus, only cytokines that were present in methodologically and clinically meaningful amounts, according to our previous experience,[16] in more than 75% of all samples during the observation period, were selected for further study ($n = 12$, see Results section). The remaining 15 were regarded as negative and therefore not included in further analyses.

Statistical analysis

Frequencies and percentages of demographic and clinical variables for the total number of participants with data available at a minimum of one time-point were calculated.

Descriptive statistics (mean, standard deviation, median, interquartile range, and range) of non-log transformed cytokine values were calculated.

Cytokine data are presented as box plots. Mixed model analyses were performed to compare the time course of cytokine levels from the admission phase to 12 months after injury for the mTBI group versus the community control group. As there are no available control data at the 2-week time-point, this time-point was removed from the mixed model analyses, although the 2-week patient data are retained in the box plots. Certain cytokine concentrations were log-transformed due to large ranges and non-normal distribution of the data (marked on plot legends and table legends). The mixed model analyses were conducted with time, group, and time-by-group interaction as fixed effects and a subject-specific random intercept to account for within-subject correlations. Mixed model analyses were performed for all cytokines with and without controlling for heterogeneous variances. All model fits were shown to be improved with heterogeneous variances controlled for (Supplementary Table S2), thus all data presented are derived from these models. Post hoc contrast analyses between patient and control groups were performed for all cytokines that showed a main effect of group. For those showing a time-by-group interaction effect, further post hoc contrast analyses were used to assess within-group changes for the mTBI group between successive time-points.

Best-subset regression analyses were performed to determine the combination of demographic (sex and age) and clinical (GCS score, PTA duration, LOC, traumatic MRI findings) variables that best predict admission-phase cytokine levels. The best model was determined based on the lowest Akaike Information Criterion (AIC). The cytokine values were first standardized at each time-point, and then the best-subset regression analyses were performed using the standardized biomarkers as outcome variables. Regression coefficients based on non-standardized biomarker values were also reported. In the patients with mTBI, we also calculated the empirical group means and standard deviations of cytokine levels for each categorical predictor used in the best-subset regression analysis.

All tests were two-sided with significance determined at $p < 0.05$. Post hoc contrast analyses of the mixed models were Bonferroni corrected (significance level of group differences at each time-point: $0.05/3 = 0.017$; significance level of within-group changes for the mTBI group between time-points: $0.05/3 = 0.017$).

All statistical tests were calculated using R version 3.2.2.[17] Descriptive statistics were calculated using R's base package functions. Mixed model analyses were performed using the nlme package.[18] Best-subset multiple regressions were performed using the bestglm package.[19]

Results

Study cohort

In total, 379 patients with mTBI participated in the overall study. Blood samples were provided from 207 patients for at least one time-point and of these, 194 had brain MRI performed within 72 h. (Fig. 1). Of the 86 community controls enrolled, 82 provided blood samples for at least one time-point (Fig. 2).

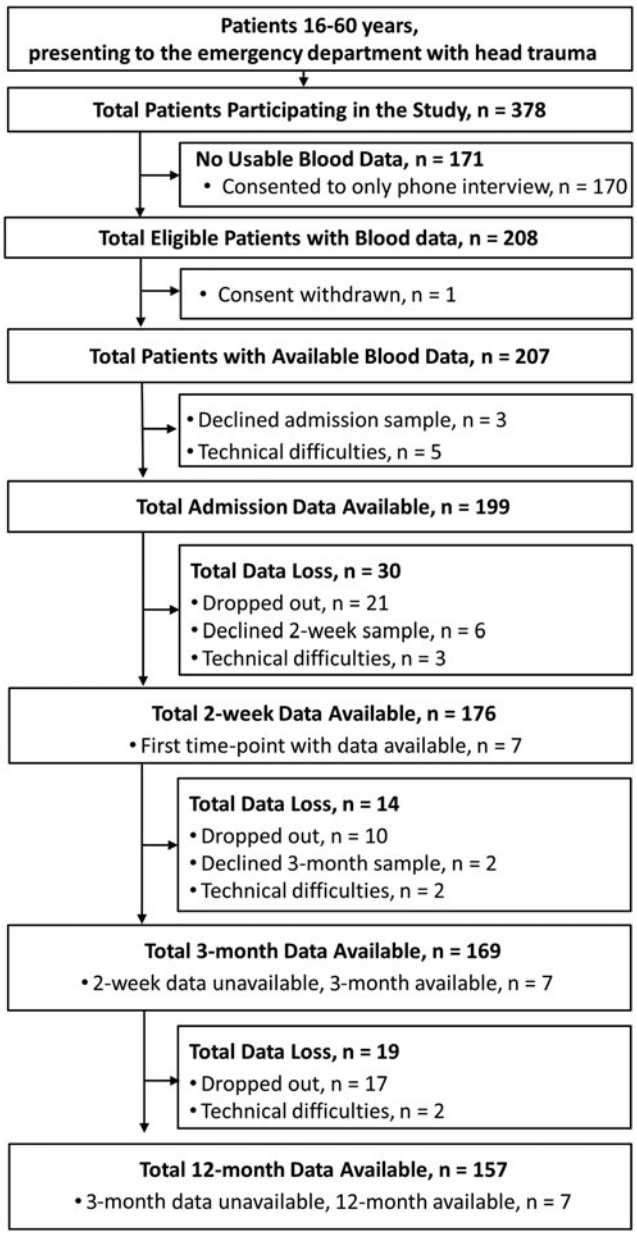

FIG. 1. Identification, enrollment, and follow-up of patients with mild TBI (mTBI). Flow chart of inclusion of patients with mTBI into the Trondheim Mild TBI follow-up study, along with progression of blood data available at each successive time-point. CT, computed tomography; mTBI, mild traumatic brain injury.

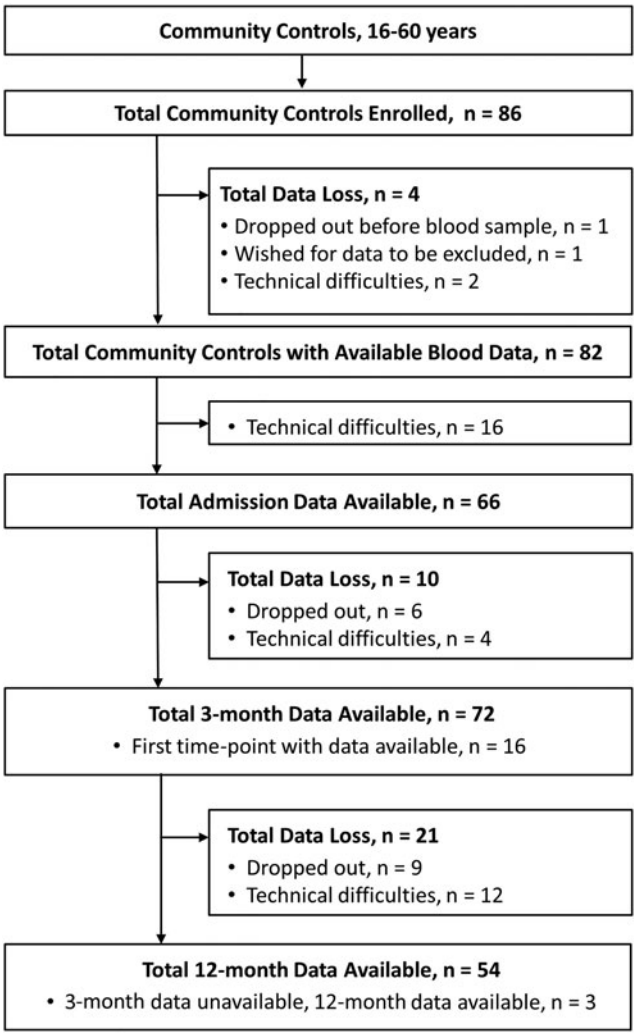

FIG. 2. Identification, enrollment, and follow-up of community controls. Flow chart of inclusion of community controls along with progression of blood data available at each successive time-point. Blood samples were drawn from community controls at enrollment (admission) and 3-month and 12-month time-points (not 2 weeks).

Demographic and injury-related characteristics of the mTBI and control group showed that 63.3% of patients with mTBI were male (Table 1). The majority had very mild injuries, with GCS scores of 15 in 76%; 52.7% were not observed with a LOC, most had PTA of less than 1 h (69.1%), intracranial traumatic findings MRI at 72 h were present in few (11.1%), and the majority (63.3%) did not experience other injuries (Table 1). The most common other injuries were soft-tissue injuries.

Time course of plasma cytokine levels in patients with mTBI from admission to 12 months after injury compared with controls

Cytokines were grouped according to biological function or class; pro-inflammatory IFNγ and chemokines (Fig. 3), pro-inflammatory interleukins (Fig. 4A–C), growth factors (Fig. 4D,E), and anti-inflammatory regulator IL-1ra (Fig. 4F). Nine of the twelve cytokines assessed showed significant differences between the mTBI patient and control groups, whereas IP-10, PDGF, and IL-1ra did not show an effect of group (Table 2). For the cytokines showing a significant effect of group, contrast analyses to assess group differences at each time-point revealed significant differences between patients with mTBI and controls at each time-point assessed (Fig. 3 and 4, Table 3). A time-by-group interaction was evidenced for FGF-basic and IL-17A, indicating that the time course between patients and controls differed for those two cytokines. Five of the cytokines (MIP-1β, MCP-1, TNF, IL-9, and IL-8) showed significant differences of both group and time, but no time-by-group interaction. PDGF showed a significant interaction of time-by-group, but no main effect of group or time. Two cytokines (IP-10 and IL-1ra) demonstrated no effects of group or time, nor a time-by-group interaction (Table 2).

Lastly, for the cytokines IL17-A and FGF-basic, which showed both a significant group effect and a significant time-by-group interaction, post hoc contrast analyses were performed to assess differences between time-points for patients with mild TBI (Table 4). Both increased between admission and 3 months; however, there was no significant increase between 3 and 12 months. Significant differences were observed also between admission and 12 months, indicating patient cytokine levels did not return to control levels at 12 months.

TABLE 1. PATIENT CHARACTERISTICS

	Patients with mild TBI Patients N = *207*	*Controls* N = *82*
Gender (%)		
Males	131 (63.3)	46 (56.1)
Females	76 (36.7)	36 (43.9)
Age at inclusion		
Mean age, years (SD)	32.4 (13.2)	33.02 (12.9)
Age range, years	16 – 60	16 – 60
GCS (%)		
13	5 (2.4)	
14	33 (16.0)	
15	158 (76.0)	
Unknown	11 (5.3)	
LOC (%)		
Unobserved LOC	109 (52.7%)	
Observed LOC	98 (47.3%)	
PTA (%)		
PTA <1 h	143 (69.0)	
PTA 1-24 h	64 (31.0)	
Traumatic intracranial finding on MRI at 72 h (%)		
TAI only	6 (2.9)	
Contusion only	3 (1.4)	
Intracranial hematoma only	3 (1.4)	
TAI and contusion	5 (2.4)	
Contusion and hematoma	6 (2.9)	
No findings	184 (88.9)	
Other injuries (%)		
None	131 (63.3)	
Fractures	35 (16.9)	
Soft-tissue injuries	41 (19.8)	

Total number with percentages in parenthesis presented.

GCS, Glasgow Coma Score; LOC, loss of consciousness; MRI, magnetic resonance imaging; PTA, post-traumatic amnesia; SD, standard deviation, TAI, traumatic axonal injury.

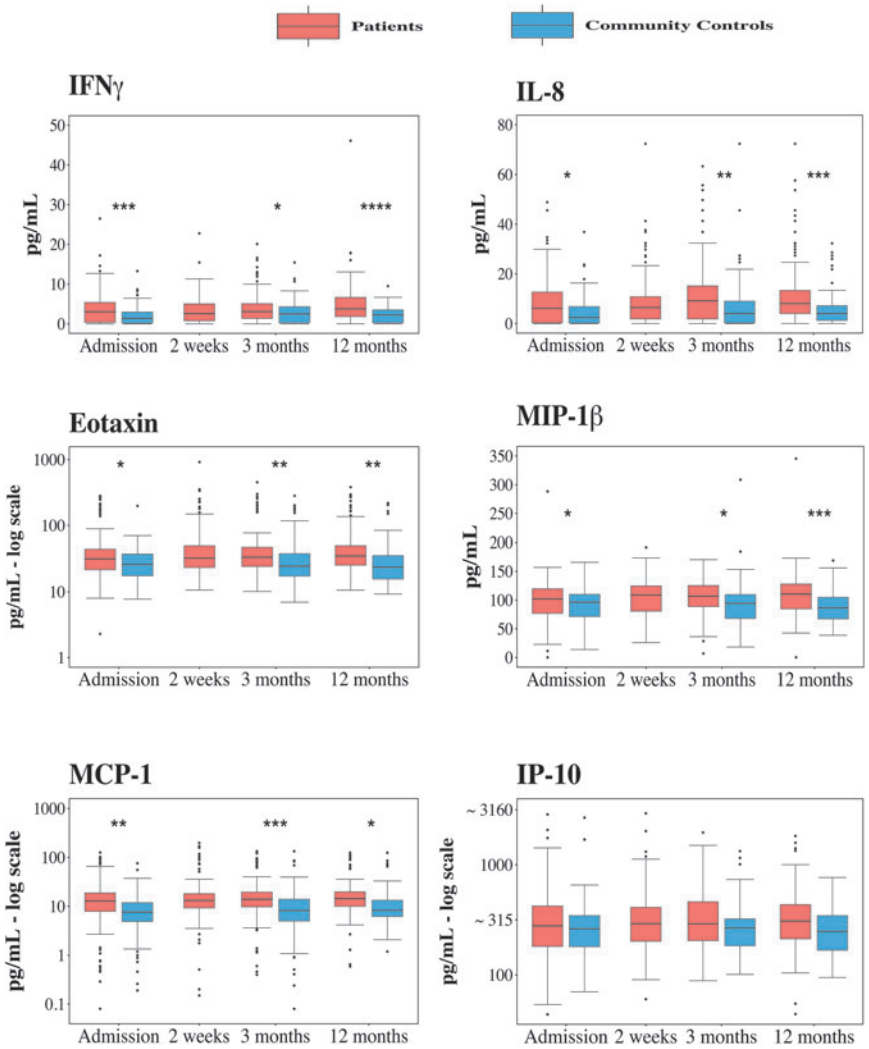

FIG. 3. Levels of IFNγ and five chemokines for patients with mTBI and controls over time. IFNγ, IL-8, eotaxin, MIP-1β, and MCP-1 were significantly higher in patients than in community controls at all time-points. IP-10 was not significantly different between groups at any time-point. Data are presented as box plots with median as line, borders, 25th and 75th percentile, and whiskers (value of the 25th and 75th percentile +1.5 interquartile range). Points above and below the whiskers represent outliers. Asterisks (*) indicate significant group difference between patients and controls at a particular time-point in the linear effect model. The *p*-value level is represented as follows:* <0 .05, ** <0.01, *** <0.001, **** <0.0001. IFN-γ, interferon gamma; IL-8, interleukin 8; IP-10, IFN-γ-inducing protein 10; MCP-1, monocyte chemoattractant protein 1; MIP-1β, macrophage inflammatory protein-1-beta; mTBI, mild traumatic brain injury. Color image is available online.

Association of cytokine levels at admission with demographic and injury-related variables

Associations between the 12 cytokines at time of admission and demographic and clinical predictors were calculated in an all-subset multiple regression analysis with standardized beta coefficients (Fig. 5). Non-standardized beta coefficients and *p*-values are presented in Supplementary Table S3 and exact values of cytokines in Supplementary Table S4. The models of eotaxin, MIP-1β, IL-9, and IP-10 included negative associations of MRI findings, meaning those with MRI findings exhibited lower levels of inflammatory markers than those with no MRI findings.

Age was included in the models for the majority of cytokines, specifically: IL-8, eotaxin, MIP-1β, MCP-1, IL-17A, TNF, IL-1ra, IP-10, and PDGF. The associations were positive for all cytokines, indicating that inflammation markers increase as a function of increasing age.

''Other injuries'' showed a strong positive effect on TNF, which indicates that TNF levels are significantly higher in those with injuries to other parts of the body. To ensure the group difference between mTBI and controls (Fig. 3) was not due to other injuries, we re-ran the time course linear mixed model including mTBI patients without other injuries versus controls. The mTBI group without other injuries had increased TNF levels compared with the controls at 3 and 12 months, and a significant group and time effect was present (Supplementary Tables S5 and S6). The increased TNF level in the mTBI group in the acute phase was thus mainly due to other injuries. But, during the first year TNF levels increased significantly in the mTBI group without other injuries. These results show that mTBI per se was associated with chronically elevated TNF levels.

A negative effect of other injuries was also included in the model for eotaxin. Re-running the time course linear mixed model with the mTBI patients without other injuries versus controls uncovered

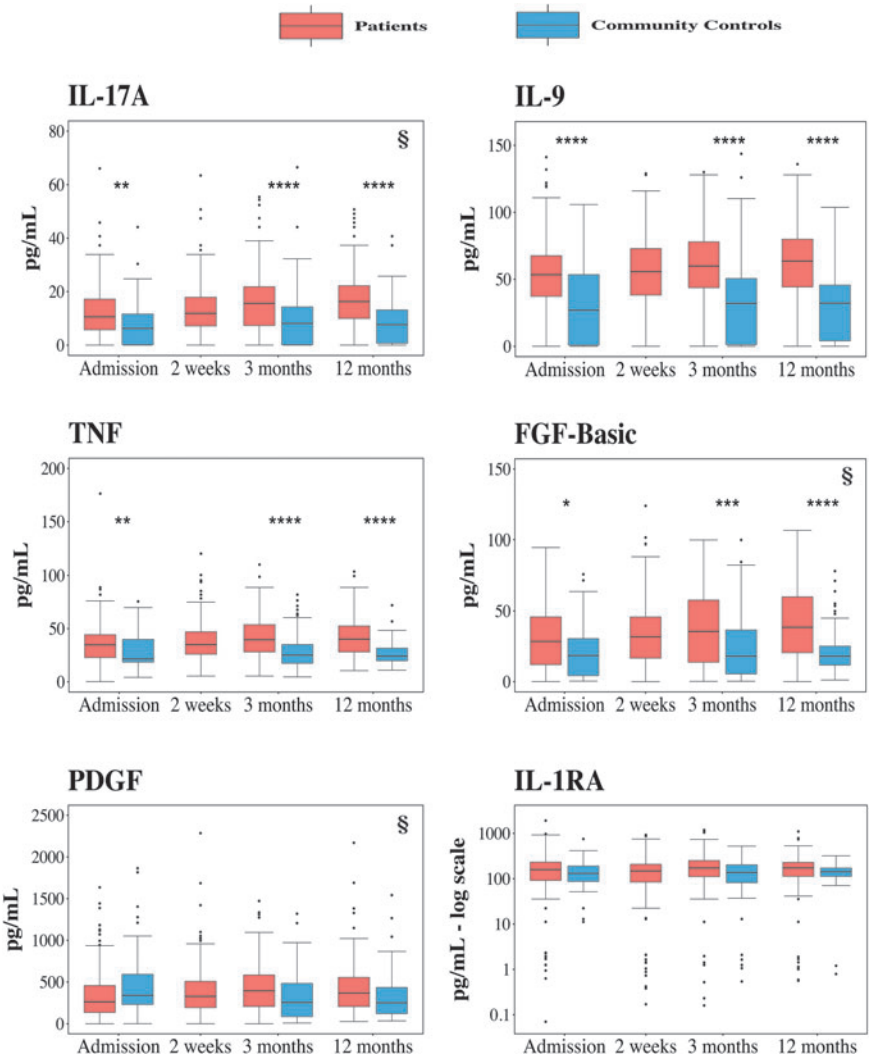

FIG. 4. Levels of interleukins, TNF, growth factors, and IL-1ra for patients with mTBI and controls over time. IL-17A, IL-9, TNF, and growth factor FGF-basic were significantly higher in patients than in community controls at all three time-points. No differences were shown for growth factor PDGF and regulator IL-1ra. For IL-17A, FGF-basic, and PDGF, the group differences between patients with mTBI and community controls increased throughout the 12-month observation period (§). Box plots, outliers, and group differences are presented and calculated in the same manner as in Figure 3. §, significant time x group interaction in the linear effect model; FGF-basic, basic fibroblast growth factor; IL, interleukin; IL-1ra, IL-1 receptor antagonist; mTBI, mild traumatic brain injury; PDGF, platelet-derived growth factor; TNF, tumor necrosis factor. Color image is available online.

that the mTBI group without other injuries contributed most to the increased eotaxin levels in the acute phase. The mTBI patients without other injuries had significantly higher eotaxin levels than controls at all 3 time-points, and a significant group effect was present (Supplementary Tables S5 and S6). The increased eotaxin levels in mTBI can therefore be ascribed to the TBI alone.

LOC was included as a positive association in the IFNγ model, but a negative association in the MIP-1β and eotaxin models, indicating that in those with observed LOC IFNγ concentrations were higher, whereas MIP-1β and eotaxin concentrations were lower. GCS scores were included as negative associations in models of eotaxin and MIP-1β, indicating those with lower GCS score (i.e., more severe injury) exhibited lower cytokine values. Lastly, sex was included as a positive association in only the IFNγ model, and as a negative association in the eotaxin, IL-17A, FGF-basic, and PDGF models. This indicates that female patients with mTBI exhibited higher values of IFNγ and lower values of the other four cytokines mentioned above. PTA was not included in any of the cytokine models.

Discussion

In this unique cohort of patients with mTBI, treated both in the hospital and the primary health care setting, we showed a prolonged increase in cytokines in blood, reflecting an inflammatory response from admission to 1 year after mTBI compared with matched healthy controls.

Prolonged activation of the systemic immune system in mTBI

In contrast to previous studies investigating limited numbers of cytokines, we used an unbiased assessment of 27 cytokines.[10]

Table 2. Summary of Main Effects of Group and Time and the Interaction of Group and Time for Each of the 12 Cytokines

Cytokines	*Group F-value (*p-*value)*	*Time F-value (*p-*value)*	*Interaction (group-by-time) F-value (*p-*value)*
IFN-γ	F = 22.42, ***p* < 0.0001**	F = 1.55, $p = 0.213$	F = 2.99, $p = 0.051$
IL-8	F = 10.64, ***p* = 0.001**	F = 7.83, ***p* = 0.0005**	F = 2.00, $p = 0.136$
Eotaxin[a]	F = 8.90, ***p* = 0.003**	F = 2.66, $p = 0.071$	F = 1.57, $p = 0.210$
MIP-1β	F = 10.91, ***p* = 0.001**	F = 4.34, ***p* = 0.014**	F = 0.96, $p = 0.382$
MCP-1[a]	F = 9.44, $p = 0.$**002**	F = 3.34, $p = 0.$**037**	F = 1.87, $p = 0.155$
IP-10[a]	F = 3.75, $p = 0.054$	F = 0.82, $p = 0.442$	F = 0.05, $p = 0.954$
IL-17A	F = 20.59, ***p* < 0.0001**	F = 14.38, ***p* < 0.0001**	F = 4.06, ***p* = 0.018**
Il-9	F = 29.66, ***p* < 0.0001**	F = 10.00, ***p* = 0.0001**	F = 0.17, $p = 0.842$
TNF	F = 21.47, ***p* < 0.0001**	F = 6.30, ***p* = 0.002**	F = 1.05, $p = 0.351$
FGF-basic	F = 17.40, ***p* < 0.0001**	F = 14.67, ***p* < 0.0001**	F = 4.97, ***p* = 0.007**
PDGF	F = 2.80, $p = 0.095$	F = 1.35, $p = 0.261$	F = 4.99, ***p* = 0.007**
IL-1ra[a]	F = 0.36, $p = 0.698$	F = 0.50, $p = 0.481$	F = 2.45, $p = 0.088$

[a]Log transformed data. Significant differences are bolded.
[a]Linear mixed model analysis.
Group: patients with mild TBI compared with community controls when time is not taken into account; Time: time course of cytokine concentrations when group is not taken into account; Group-by-Time: interaction of Group and Time; a significant effect indicates the time courses of patients with mild TBI and community controls significantly differed.
FGF-basic, basic fibroblast growth factor; IL, interleukin; IL-1ra, IL-1 receptor antagonist; IFN-γ, interferon gamma; IP-10, IFN-γ-inducing protein 10; MCP-1, monocyte chemoattractant protein 1; MIP-1β, macrophage inflammatory protein-1-beta; PDGF, platelet-derived growth factor; TBI, traumatic brain injury; TNF, tumor necrosis factor.

Cytokines form part of a complex inflammatory network,[20] which has been shown to be associated with injury severity in moderate to severe TBI, in both human and experimental studies.[21,22] Our study is, to the best of our knowledge, the first to show significant systemic cytokine elevations persisting for 1 year in mTBI across a pro-inflammatory cytokine network.[23,24]

To date, studies of cytokine expression patterns over time have focused on patients with moderate to severe TBI demonstrating slightly higher plasma concentrations in the acute phase post-injury than observed in our study.[22,24] Whereas long-term plasma cytokine studies, to our knowledge, are lacking in patients with severe TBI, autopsy studies have revealed that intracerebral inflammation leading to microglial activation can persist for years, and we can thus only speculate that this inflammation would be observable in plasma as well, comparable to patients with mTBI in this study.[7] In this study in mTBI, cytokine plasma levels were generally low and lower than observed during severe TBI.[24] However, persistent, low-grade inflammation, comparable to the

Table 3. Group Comparisons between Patients with Mild TBI and Community Controls at Each Time-point, for Each of the Cytokine Concentrations Showing a Significant Group Effect in the Previous Analysis

	Admission estimate[a] *[95% CI]* p-*value*	*3 months estimate*[a] *[95% CI]* p-*value*	*12 months estimate*[a] *[95% CI]* p-*value*
IFN-γ	1.4 [0.6 – 2.2]	1.0 [0.1 – 1.9]	2.3 [1.4 – 3.1]
	***p* = 0.0007**	$p = 0.023$	***p* < 0.0001**
IL-8	2.8 [0.04 – 5.5]	4.2 [1.2 – 7.1]	4.8 [2.2 – 7.4]
	$p = 0.047$	***p* = 0.006**	***p* = 0.0003**
Eotaxin[b]	**0.1** [0.02 – 0.2]	**0.1** [0.05 – 0.2]	**0.1** [0.05 – 0.2]
	***p* = 0.017**	***p* = 0.002**	***p* = 0.002**
MIP-1β	9.3 [0.5 – 18.0]	11.5 [2.6 – 20.4]	14.4 [6.4 – 22.3]
	$p = 0.038$	***p* = 0.012**	***p* = 0.0004**
MCP-1[b]	0.19 [0.06 – 0.32]	0.25 [0.010 – 0.329]	0.14 [0.03 – 0.26]
	***p* = 0.04**	***p* = 0.0008**	***p* = 0.013**
IL-17A	3.7 [1.1 – 6.4]	5.5 [2.8 – 8.2]	6.6 [4.1 – 9.1]
	***p* = 0.006**	***p* < 0.0001**	***p* < 0.0001**
IL-9	19.4 [11.3 – 27.4]	20.2 [12.4 – 28]	20.9 [13.2 – 28.5]
	***p* < 0.0001**	***p* < 0.0001**	***p* < 0.0001**
TNF	8.0 [3.0 – 13.0]	10.7 [6.1 – 15.4]	10.5 [6.0 – 15.1]
	***p* = 0.002**	***p* < 0.0001**	***p* < 0.0001**
FGF-basic	7.3 [1.3 – 13.3]	10.2 [4.4 – 16.0]	14.4 [8.8 – 20.1]
	***p* = 0.017**	***p* = 0.0006**	***p* < 0.0001**

[a]Estimate refers to mean group differences as estimated by the mixed model. CI is the 95% confidence interval of the estimated group difference.
[b]Log transformed data. Post hoc contrast analyses based on the linear mixed model. Significant differences are bolded.
FGF-basic, basic fibroblast growth factor; IL, interleukin; IFN-γ, interferon gamma; MCP-1, monocyte chemoattractant protein 1; MIP-1β, macrophage inflammatory protein-1-beta; TBI, traumatic brain injury; TNF, tumor necrosis factor.

Table 4. Comparisons between Time-Points in Patients with Mild TBI, for Each of the Cytokine Concentrations Showing a Significant Group Effect and a Significant Group-by-Time Interaction

	Admission – 3 months *Estimate*[a] *[95% CI]* p-*value*	*3 months – 12 months* *Estimate*[a] *[95% CI]* p-*value*	*Admission – 12 months* *Estimate*[a] *[95% CI]* p-*value*
IL-17A	3.7 [2.3 – 5.2] **$p < 0.0001$**	-0.2 [-1.8 – 1.4] $p = 0.796$	3.5 [2.0 – 5.0] **$p < 0.0001$**
FGF-basic	7.3 [4.2 – 10.4] **$p < 0.0001$**	2.5 [-1.0 – 5.9] $p = 0.157$	9.8 [6.4 – 13.2] **$p < 0.0001$**

[a]Estimate refers to mean group differences as estimated by the mixed model. CI is the 95% confidence interval of the estimated group difference. Post-hoc contrast analyses based on the linear mixed model.Significant differences are bolded.
FGF-basic, basic fibroblast growth factor; IL, interleukin; TBI, traumatic brain injury.

cytokine levels obtained in this study, has been described in one prior study of patients with mTBI and chronic diseases with inflammatory components such as Alzheimer's and multiple sclerosis, and has been attributed to long-term morbidity, such as fatigue and mortality.[10,25,26]

A hallmark in this study is the prolonged activation of cytokines over the 1-year follow-up period in patients with mTBI, compared with matched community controls. This is in contrast to studies showing acute but transient increase of plasma cytokines in mTBI. In a study of blast-exposed military personnel, cytokines IL-6 and TNF returned to baseline levels within hours,[27] whereas in sports-related concussion cytokines IL-6 and IL-12 returned to baseline within 7 days.[9] In children with concussion, TNF, IL-6, IL-1β, IL-10, and several neuronal damage plasma markers returned to baseline within days, whereas IL-8 remained increased for 3 months.[28] Likewise, a recent study in 52 patients with mTBI showed prolonged increase of IL-1β, IL-6, and MCP-1, combined with a general increase in cytokine load in patients with mTBI in comparison with healthy controls over a period of 3 months.[10] Overall, few clinical studies have been conducted to evaluate the

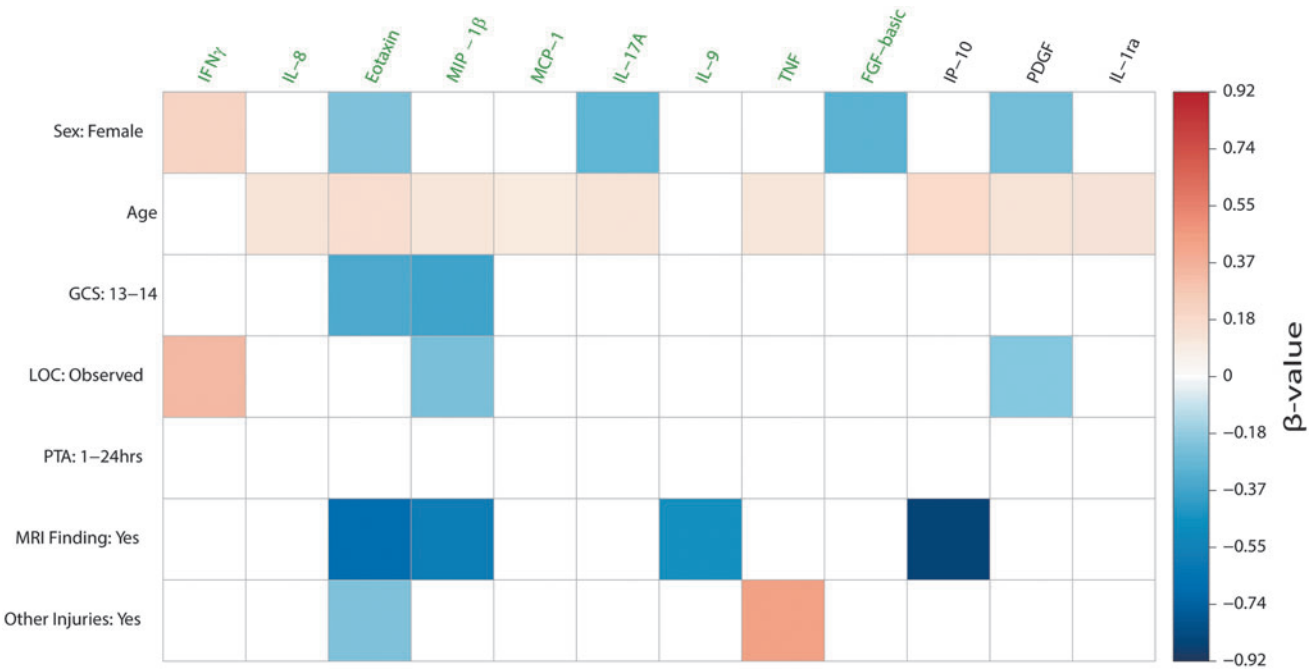

FIG. 5. Cytokines at admission are associated with demographic and clinical variables. Cytokines were input into an all-subsets multiple regression as outcome variables, and all demographic and clinical variables were input as possible predictor variables. Separate regressions were performed for each cytokine, with the associations shown to improve model fit depicted as colored squares. Model fit was determined based on the Akaike Information Criterion. Associations presented are the beta-values of the final model. A blank space indicates that the predictor was not included in the final model. The direction and size of regression coefficient is represented according to the figure legend color scheme, whereby increasingly positive associations are graded to red, and increasingly negative associations are graded to blue. The nine cytokines colored in green correspond to those whose linear mixed models revealed a significant group difference between patients and controls, whereas the three cytokines to the right (black) did not show significant group differences. All beta-values are standardized. Non-standardized beta-values and *p*-values are presented in Supplementary Table S3. Baseline comparison for Sex was male. Baseline comparison for GCS scores of 13–14 was a GCS score of 15. Baseline comparison for Observed LOC was "Unobserved LOC." Baseline comparison for PTA duration of between 1 and 24 h was PTA of less than 1 h. Baseline comparison for MRI findings was "No MRI Finding." Baseline comparison for presence of injuries to other parts of the body was "No Other Injuries." GCS, Glasgow Coma Score; LOC, loss of consciousness; MRI, magnetic resonance imaging; PTA, post-traumatic amnesia. Color image is available online.

consequences of prolonged inflammation in TBI. Here, we show that patients with mTBI experience a state of systemic low-grade chronic inflammation, for up to 1 year after the initial injury, which is much longer than reported previously and independent of trauma beside the mTBI.

Almost all cytokines, which were increased in patients with mTBI in this study, have been reported to be associated with TBI. MCP-1 has been associated with post-concussion syndrome in mTBI patients.[10] IL-8 (CXCL8) has been reported as a key mediator of neuroinflammation in severe TBI,[21] eotaxin (CCL11) has been associated with chronic traumatic encephalopathy,[29] and MIP-1β has been shown to be induced early in sports concussion.[30,31] These chemokines induce production of the pro-inflammatory cytokine IFN-γ, which was increased in this study. IFN-γ has been shown to increase cell recruitment to an injured brain and thus local inflammation.[32,33] Pro-inflammatory interleukins IL-17A[34] and IL-9,[35] as well as TNF[36] are suggested to be involved in neuroinflammation after experimental severe (IL-17A and IL-9) and clinical repeated mTBI (TNF), by stimulating and controlling recruitment of neutrophils.[37] To our knowledge, IL-9 has not been reported in clinical studies assessing TBI patients, yet. This may be due to pre-selection of known cytokines in previous studies. Given the large IL-9 elevations in patients with mTBI compared with controls in this study, further research into IL-9's relation to TBI is warranted. Growth factor FGF-basic has been shown to increase neurogenesis, preserve blood–brain barrier integrity, and enhance blood vessel proliferation, while suppressing autophagy.[38]

Notably, although each cytokine separately may be of limited pathophysiological importance for the clinical disease severity and outcome, and may differ substantially between individuals, the overall finding of sustained inflammation with an orchestra of many cytokines acting together and via different pathways might be of utmost importance. Crosstalk between the cytokines is frequently not detected when reductionistic systems such as isolated cells are used. We postulate that a holistic approach to understanding the neurobiological changes occurring in post-mTBI can be obtained only by using complex panels of assays in an *in vivo* situation, and that the possible implication to long-term neuroinflammation is a critical finding of our study that warrants further investigation.

Associations between increased cytokine plasma levels and injury-related and demographic variables

There were several findings from the best-subset regression analyses that point to more severe injury being associated with lower levels of cytokines in blood at admission. First, eotaxin, MIP-1β, IL-9, and IP-10 were all negatively associated with traumatic intracranial MRI findings, indicating that in all cases, visible traumatic lesions resulted in lower systemic cytokine levels. This finding was unsuspected. It may suggest that systemic low-grade inflammation in patients with mTBI reflects disease severity not obviously linked to neuroradiological findings, a fact reported in other diseases such as febrile status epilepticus and brain swelling in cerebral malaria.[39,40] Likewise, more severe injury indicated by lower GCS score was negatively associated with eotaxin and MIP-1β. Although GCS score has been shown to be an important predictor of morbidity in severe TBI,[41] it has shown relatively poor prognostic utility in mTBI,[42] and to our knowledge, its relation with inflammatory markers has not been investigated. Thus the significance of these findings remains to be explained.

On the other hand, LOC was positively associated with admission IFN-γ. IFN-γ is upregulated in the brain following TBI, but there is ongoing discussion about whether IFN-γ elevation is beneficial or detrimental.[43,44] A large positive association of other injuries with TNF was demonstrated. These results are unsurprising, as TNF release has been reported in response to bone fractures and virtually all forms of ischemia/reperfusion injury in the acute phase.[45,46] However, as TNF also was significantly increased in patients without other injuries at 3 and 12 months, TNF probably is associated with mTBI itself.

Age was associated with higher levels of the majority of cytokines: IL-8, eotaxin, MIP-1β, MCP-1, IL-17A, TNF, IL-1ra, IP-10, and PDGF. The impact of age on the immune system has been well characterized, and our results confirm that older individuals experience generally higher levels of inflammation.[47]

Lastly, sex showed different cytokine expression pattern in male compared with female patients. Although this phenomenon has been observed in several clinical and experimental studies in other diseases and is thought to be dependent on hormonal balances, our findings should be regarded as hypothesis generating.[48,49]

Limitations

Inclusion of non-hospitalized patients led to considerable variation in time (up to 72 h) of obtaining the first ''admission'' blood sample, as many patients first needed to be tracked down and invited to participate. Thus, initial, time-critical cytokine kinetics immediately following the injury might have been lost. However, as systemic cytokine levels remained elevated and even increased during the observation period in some cases (IL-17A and FGF-basic), the initial kinetics and the significance thereof might be of less relevance in comparison with the long follow-up period.

As can be seen from Figures 1 and 2, we did not obtain blood samples from individuals at all time-points. Loss to follow-up is an issue that poses problems for generalizing group differences between early and late time-points. However, using a linear mixed model circumvents the majority of these issues with missing data.

Previous studies in TBI patients have reported absolute plasma cytokine levels, which differ to levels reported in this study.[31,50] Methodologically, it is impossible to compare absolute cytokine levels when detection assays from different producers without standardized reference controls are used.[16] Hence, absolute cytokine levels in this study should only be compared between patients with mTBI and the matched healthy controls, whereas pattern of expression and relative group differences are comparable between studies.[10] In this study, only 11% of patients had MRI findings. Thus, regression analysis findings are based on relatively few patients and need to be verified in future studies.

Implications for diagnosis, follow-up and therapy

The distinct pattern of cytokine expression observed in this study indicates a previously undocumented pattern of persistent inflammation following mTBI for 1 year post-injury. Factors normally used to grade severity of a TBI, such as GCS score, LOC, MRI findings, and PTA, were not associated consistently with cytokine expression patterns in this study. A biomarker especially for triage of patients with mTBI to guide diagnostic procedures is warranted; however, previous studies have demonstrated low specificity for inflammatory markers for predicting, for example, CT findings.[51]

Thus, further studies are needed to investigate if the demonstrated inflammatory state is caused by neuroinflammation, and what are the clinical implications of prolonged inflammation with

outcome, such as post-concussion syndrome, and potential for manipulating it for improved outcome.

Conclusion

This prospective observational study including clinic outpatients indicates that low-level systemic inflammation persists during 1 year post mild TBI as a high number of cytokines with different functions and etiologies remain increased through the observation period.

Long-term cytokine increase was not explained by injury severity determined by MRI findings or other injuries beside mTBI, which may imply that inflammation post mTBI may not solely be caused by trauma per se or obvious pathology in the brain tissue.

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Author Disclosure Statement

No competing financial interests exist.

Supplementary Material

Supplementary Figure S1
Supplementary Table S1
Supplementary Table S2
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Supplementary Table S5
Supplementary Table S6

References

1. Maas, A.I.R., Menon, D.K., Adelson, P.D., Andelic, N., Bell, M.J., Belli, A., Bragge, P., Brazinova, A., Buki, A., Chesnut, R.M., Citerio, G., Coburn, M., Cooper, D.J., Crowder, A.T., Czeiter, E., Czosnyka, M., Diaz-Arrastia, R., Dreier, J.P., Duhaime, A.C., Ercole, A., van Essen, T.A., Feigin, V.L., Gao, G., Giacino, J., Gonzalez-Lara, L.E., Gruen, R.L., Gupta, D., Hartings, J.A., Hill, S., Jiang, J.Y., Ketharanathan, N., Kompanje, E.J.O., Lanyon, L., Laureys, S., Lecky, F., Levin, H., Lingsma, H.F., Maegele, M., Majdan, M., Manley, G., Marsteller, J., Mascia, L., McFadyen, C., Mondello, S., Newcombe, V., Palotie, A., Parizel, P.M., Peul, W., Piercy, J., Polinder, S., Puybasset, L., Rasmussen, T.E., Rossaint, R., Smielewski, P., Soderberg, J., Stanworth, S.J., Stein, M.B., von Steinbuchel, N., Stewart, W., Steyerberg, E.W., Stocchetti, N., Synnot, A., Te Ao, B., Tenovuo, O., Theadom, A., Tibboel, D., Videtta, W., Wang, K.K.W., Williams, W.H., Wilson, L., and Yaffe, K. (2017). Traumatic brain injury: integrated approaches to improve prevention, clinical care, and research. Lancet Neurol.16, 987–1048.
2. Bazarian, J.J., McClung, J., Shah, M.N., Cheng, Y.T., Flesher, W., and Kraus, J. (2005). Mild traumatic brain injury in the United States, 1998–2000. Brain Inj.19, 85–91.
3. McMahon, P., Hricik, A., Yue, J.K., Puccio, A.M., Inoue, T., Lingsma, H.F., Beers, S.R., Gordon, W.A., Valadka, A.B., Manley, G.T., and Okonkwo, D.O. (2014). Symptomatology and functional outcome in mild traumatic brain injury: results from the prospective TRACK-TBI study. J. Neurotrauma 31, 26–33.
4. McInnes, K., Friesen, C.L., MacKenzie, D.E., Westwood, D.A., and Boe, S.G. (2017). Mild traumatic brain injury (mTBI) and chronic cognitive impairment: a scoping review. PLoS One 12, e0174847.
5. Simon, D.W., McGeachy, M.J., Bayır, H., Clark, R.S.B., Loane, D.J., and Kochanek, P.M. (2017). The far-reaching scope of neuroinflammation after traumatic brain injury. Nat. Rev. Neurol. 13, 171.
6. Marcet, P., Santos, N., and Borlongan, C.V. (2017). When friend turns foe: central and peripheral neuroinflammation in central nervous system injury. Neuroimmunol. Neuroinflamm. 4, 82–92.
7. Johnson, V.E., Stewart, J.E., Begbie, F.D., Trojanowski, J.Q., Smith, D.H., and Stewart, W. (2013). Inflammation and white matter degeneration persist for years after a single traumatic brain injury. Brain 136, 28–42.
8. Fehily, B., and Fitzgerald, M. (2017). Repeated mild traumatic brain injury: potential mechanisms of damage. Cell Transplant. 26, 1131–1155.
9. Merchant-Borna, K., Lee, H., Wang, D., Bogner, V., van Griensven, M., Gill, J., and Bazarian, J.J. (2016). Genome-wide changes in peripheral gene expression following sports-related concussion. J. Neurotrauma 33, 1576–1585.
10. Sun, Y., Bai, L., Niu, X., Wang, Z., Yin, B., Bai, G., Zhang, D., Gan, S., Sun, C., Wang, S., Zhu, F., and Zhang, M. (2019). Elevated serum levels of inflammation-related cytokines in mild traumatic brain injury are associated with cognitive performance. Front. Neurol. 10, 1120.
11. Corrigan, J.D., Selassie, A.W., and Orman, J.A. (2010). The epidemiology of traumatic brain injury. J. Head Trauma Rehabil. 25, 72–80.
12. Skandsen, T., Einarsen, C.E., Normann, I., Bjoralt, S., Karlsen, R.H., McDonagh, D., Nilsen, T.L., Akslen, A.N., Haberg, A.K., and Vik, A. (2018). The epidemiology of mild traumatic brain injury: the Trondheim MTBI follow-up study. Scand. J. Trauma Resusc. Emerg. Med. 26, 34.
13. Einarsen, C.E., Moen, K.G., Haberg, A.K., Eikenes, L., Kvistad, K.A., Xu, J., Moe, H.K., Tollefsen, M.H., Vik, A., and Skandsen, T. (2019). Patients with mild traumatic brain injury recruited from both hospital and primary care settings: a controlled longitudinal magnetic resonance imaging study. J. Neurotrauma 36, 3172–3182.
14. Carroll, L.J., Cassidy, J.D., Peloso, P.M., Borg, J., von Holst, H., Holm, L., Paniak, C. and Pepin, M. (2004). Prognosis for mild traumatic brain injury: results of the WHO Collaborating Centre Task Force on Mild Traumatic Brain Injury. J. Rehabil. Med., 84–105.
15. Menon, D.K., Schwab, K., Wright, D.W., Maas, A.I., Demographics, Clinical Assessment Working Group of the International and Interagency Initiative toward Common Data Elements for Research on Traumatic Brain Injury and Psychological Health. (2010). Position statement: definition of traumatic brain injury. Arch. Phys. Med. Rehabil. 91, 1637–1640.
16. Henno, L.T., Storjord, E., Christiansen, D., Bergseth, G., Ludviksen, J.K., Fure, H., Barene, S., Nielsen, E.W., Mollnes, T.E., and Brekke, O.L. (2017). Effect of the anticoagulant, storage time and temperature of blood samples on the concentrations of 27 multiplex assayed cytokines: consequences for defining reference values in healthy humans. Cytokine 97, 86–95.
17. R Core Team. (2015). R: a language and environment for statistical computing. R Foundation for Statistical Computing: Vienna, Austria.
18. Pinheiro, J., Bates, D., Debroy, S., Sarkar, D., and R Core Team. (2015). nlme: linear and nonlinear mixed effects models. http://CRAN.R-project.org/package=nlme (Last accessed May 6, 2020).

19. McLeod, A.I., and Xu, C. (2017). bestglm: best subset GLM. http://CRAN.R-project.org/package=bestglm (Last accessed May 6, 2020).
20. Thelin, E.P., Tajsic, T., Zeiler, F.A., Menon, D.K., Hutchinson, P.J.A., Carpenter, K.L.H., Morganti-Kossmann, M.C., and Helmy, A.J.F.N. (2017). Monitoring the Neuroinflammatory Response Following Acute Brain Injury. Front. Neurol. 8, 351.
21. Kushi, H., Saito, T., Makino, K., and Hayashi, N. (2003). IL-8 is a key mediator of neuroinflammation in severe traumatic brain injuries. Acta Neurochir. Suppl. 86, 347–350.
22. Helmy, A., Carpenter, K.L., Menon, D.K., Pickard, J.D., and Hutchinson, P.J. (2011). The cytokine response to human traumatic brain injury: temporal profiles and evidence for cerebral parenchymal production. J. Cereb. Blood Flow Metab. 31, 658–670.
23. Casault, C., Al Sultan, A.S., Banoei, M., Couillard, P., Kramer, A., and Winston, B.W. (2019). Cytokine responses in severe traumatic brain injury: where there is smoke, is there fire? Neurocrit. Care. 30, 22–32.
24. Di Battista, A.P., Rhind, S.G., Hutchison, M.G., Hassan, S., Shiu, M.Y., Inaba, K., Topolovec-Vranic, J., Neto, A.C., Rizoli, S.B., and Baker, A.J. (2016). Inflammatory cytokine and chemokine profiles are associated with patient outcome and the hyperadrenergic state following acute brain injury. J. Neuroinflammation 13, 40.
25. Ross, J., Sharma, S., Winston, J., Nunez, M., Bottini, G., Franceschi, M., Scarpini, E., Frigerio, E., Fiorentini, F., Fernandez, M., Sivilia, S., Giardino, L., Calza, L., Norris, D., Cicirello, H., Casula, D., and Imbimbo, B.P. (2013). CHF5074 reduces biomarkers of neuroinflammation in patients with mild cognitive impairment: a 12-week, double-blind, placebo-controlled study. Curr. Alzheimer Res. 10, 742–753.
26. Flachenecker, P., Bihler, I., Weber, F., Gottschalk, M., Toyka, K.V., and Rieckmann, P. (2004). Cytokine mRNA expression in patients with multiple sclerosis and fatigue. Mult. Scler. 10, 165–169.
27. Gill, J., Motamedi, V., Osier, N., Dell, K., Arcurio, L., Carr, W., Walker, P., Ahlers, S., Lopresti, M., and Yarnell, A. (2017). Moderate blast exposure results in increased IL-6 and TNFalpha in peripheral blood. Brain. Behav. Immun. 65, 90–94.
28. Parkin, G.M., Clarke, C., Takagi, M., Hearps, S., Babl, F.E., Davis, G.A., Anderson, V., and Ignjatovic, V. (2019). Plasma tumor necrosis factor alpha is a predictor of persisting symptoms post-concussion in children. J. Neurotrauma. 36, 1768–1775.
29. Cherry, J.D., Stein, T.D., Tripodis, Y., Alvarez, V.E., Huber, B.R., Au, R., Kiernan, P.T., Daneshvar, D.H., Mez, J., Solomon, T.M., Alosco, M.L., and McKee, A.C. (2017). CCL11 is increased in the CNS in chronic traumatic encephalopathy but not in Alzheimer's disease. PLoS One 12, e0185541.
30. Forstner, P., Rehman, R., Anastasiadou, S., Haffner-Luntzer, M., Sinske, D., Ignatius, A., Roselli, F., and Knoll, B. (2018). Neuroinflammation after traumatic brain injury is enhanced in activating transcription factor 3 mutant mice. J. Neurotrauma 35, 2317–2329.
31. Di Battista, A.P., Churchill, N., Rhind, S.G., Richards, D., and Hutchison, M.G. (2019). Evidence of a distinct peripheral inflammatory profile in sport-related concussion. J. Neuroinflammation 16, 17.
32. Zlotnik, A., and Yoshie, O. (2012). The chemokine superfamily revisited. Immunity 36, 705–716.
33. Kunis, G., Baruch, K., Rosenzweig, N., Kertser, A., Miller, O., Berkutzki, T., and Schwartz, M. (2013). IFN-γ-dependent activation of the brain's choroid plexus for CNS immune surveillance and repair. Brain 136, 3427–3440.
34. Sankar, S.B., Pybus, A.F., Liew, A., Sanders, B., Shah, K.J., Wood, L.B., and Buckley, E.M. (2019). Low cerebral blood flow is a noninvasive biomarker of neuroinflammation after repetitive mild traumatic brain injury. Neurobiol. Dis. 124, 544–554.
35. Niesman, I.R., Schilling, J.M., Shapiro, L.A., Kellerhals, S.E., Bonds, J.A., Kleschevnikov, A.M., Cui, W., Voong, A., Krajewski, S., Ali, S.S., Roth, D.M., Patel, H.H., Patel, P.M., and Head, B.P. (2014). Traumatic brain injury enhances neuroinflammation and lesion volume in caveolin deficient mice. J. Neuroinflammation 11, 39.
36. Juengst, S.B., Kumar, R.G., Arenth, P.M., and Wagner, A.K. (2014). Exploratory associations with tumor necrosis factor-α, disinhibition and suicidal endorsement after traumatic brain injury. Brain Behav. Immun. 41, 134–143.
37. Wojkowska, D.W., Szpakowski, P., and Glabinski, A. (2017). Interleukin 17A promotes lymphocytes adhesion and induces CCL2 and CXCL1 release from brain endothelial cells. Int. J. Mol. Sci. 18, 1000.
38. Chen, J., Hu, J., Liu, H., Xiong, Y., Zou, Y., Huang, W., Shao, M., Wu, J., Yu, L., Wang, X., Wang, X., and Lin, L. (2018). FGF21 protects the blood-brain barrier by upregulating PPARgamma via FGFR1/beta-klotho after traumatic brain injury. J. Neurotrauma 35, 2091–2103.
39. Gallentine, W.B., Shinnar, S., Hesdorffer, D.C., Epstein, L., Nordli, D.R., Jr., Lewis, D.V., Frank, L.M., Seinfeld, S., Shinnar, R.C., Cornett, K., Liu, B., Moshe, S.L., and Sun, S. (2017). Plasma cytokines associated with febrile status epilepticus in children: a potential biomarker for acute hippocampal injury. Epilepsia 58, 1102–1111.
40. Harawa, V., Njie, M., Kessler, A., Choko, A., Kumwenda, B., Kampondeni, S., Potchen, M., Kim, K., Jaworowski, A., Taylor, T., Mandala, W., Seydel, K., and Rogerson, S. (2018). Brain swelling is independent of peripheral plasma cytokine levels in Malawian children with cerebral malaria. Malar. J. 17, 435.
41. Perel, P., Arango, M., Clayton, T., Edwards, P., Komolafe, E., Poccock, S., Roberts, I., Shakur, H., Steyerberg, E., and Yutthakasemsunt, S. (2008). Predicting outcome after traumatic brain injury: practical prognostic models based on large cohort of international patients. BMJ 336, 425–429.
42. Tellier, A., Marshall, S.C., Wilson, K.G., Smith, A., Perugini, M., and Stiell, I.G. (2009). The heterogeneity of mild traumatic brain injury: where do we stand? Brain Inj. 23, 879–887.
43. Chandrasekar, A., Heuvel, F.O., Palmer, A., Linkus, B., Ludolph, A.C., Boeckers, T.M., Relja, B., Huber-Lang, M., and Roselli, F. (2017). Acute ethanol administration results in a protective cytokine and neuroinflammatory profile in traumatic brain injury. Int. Immunopharmacol. 51, 66–75.
44. Roselli, F., Chandrasekar, A., and Morganti-Kossmann, M.C. (2018). Interferons in traumatic brain and spinal cord injury: current evidence for translational application. Front. Neurol. 9, 458–458.
45. Durán, W.N. (2008). The double-edge sword of TNF-alpha in ischemia-reperfusion injury [Editorial]. Am. J. Physiol. Heart Circ. Physiol. 295, H2221–H2222.
46. Karnes, J.M., Daffner, S.D., and Watkins, C.M. (2015). Multiple roles of tumor necrosis factor-alpha in fracture healing. Bone 78, 87–93.
47. Gubbels Bupp, M.R. (2015). Sex, the aging immune system, and chronic disease. Cell. Immunol. 294, 102–110.
48. Stapleton, P.P., Strong, V.E., Freeman, T.A., Winter, J., Yan, Z., and Daly, J.M. (2004). Gender affects macrophage cytokine and prostaglandin E2 production and PGE2 receptor expression after trauma. J. Surg. Res. 122, 1–7.
49. Fan, H., Zhao, G., Ren, D., Liu, F., Dong, G., and Hou, Y. (2017). Gender differences of B cell signature related to estrogen-induced IFI44L/BAFF in systemic lupus erythematosus. Immunol. Lett. 181, 71–78.
50. Licastro, F., Hrelia, S., Porcellini, E., Malaguti, M., Di Stefano, C., Angeloni, C., Carbone, I., Simoncini, L., and Piperno, R. (2016). Peripheral inflammatory markers and antioxidant response during the post-acute and chronic phase after severe traumatic brain injury. Front. Neurol. 7, 189.
51. Lagerstedt, L., Egea-Guerrero, J.J., Rodriguez-Rodriguez, A., Bustamante, A., Montaner, J., El Rahal, A., Andereggen, E., Rinaldi, L., Sarrafzadeh, A., Schaller, K., and Sanchez, J.C. (2018). Early measurement of interleukin-10 predicts the absence of CT scan lesions in mild traumatic brain injury. PLoS One 13, e0193278.

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Article II

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Clinical paper

Complement activation is associated with poor outcome after out-of-hospital cardiac arrest

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Abstract

Background: Cardiopulmonary resuscitation after cardiac arrest initiates a whole-body ischemia-reperfusion injury, which may activate the innate immune system, including the complement system. We hypothesized that complement activation and subsequent release of soluble endothelial activation markers were associated with cerebral outcome including death.

Methods: Outcome was assessed at six months and defined by cerebral performance category scale (1–2; good outcome, 3–5; poor outcome including death) in 232 resuscitated out-of-hospital cardiac arrest patients. Plasma samples obtained at admission and day three were analysed for complement activation products C3bc, the soluble terminal complement complex (sC5b-9), and soluble CD14. Endothelial cell activation was measured by soluble markers syndecan-1, sE-selectin, thrombomodulin, and vascular cell adhesion molecule.

Results: Forty-nine percent of the patients had good outcome. C3bc and sC5b-9 were significantly higher at admission compared to day three ($p < 0.001$ for both) and in patients with poor compared to good outcome ($p = 0.03$ and $p < 0.001$, respectively). Unadjusted, higher sC5b-9 at admission was associated with poor outcome (odds ratio 1.08 (95% CI 1.01–1.14), $p = 0.024$). Adjusted, sC5b-9 was still associated with outcome, but the association became non-significant when time to return-of-spontaneous-circulation above 25 min was included as a covariate. Endothelial cell activation markers increased from admission to day three, but only sE-selectin and thrombomodulin were significantly higher in patients with poor versus good outcome ($p = 0.004$ and $p = 0.03$, respectively) and correlated to sCD14 and sC5b-9/C3bc, respectively.

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Conclusion: Complement system activation, reflected by sC5b-9 at admission, leading to subsequent endothelial cell activation, was associated with poor outcome in out-of-hospital cardiac arrest patients.

Keywords: Out-of-hospital cardiac arrest, Outcome, SC5b-9 protein complex, Cardiopulmonary resuscitation, Return of spontaneous circulation, Immune system, Endothelial cells

Introduction

Out-of-hospital cardiac arrest (OHCA) remains an important public health matter with a high mortality rate and is the third leading cause of death in Europe.[1] After successful resuscitation, the main reason for mortality and morbidity is the duration of hypoxia and a whole-body ischemia/reperfusion injury.[2] The brain is most susceptible to this reperfusion injury,[3] and two-thirds of hospital deaths are due to the neurological injury,[4]

The pathophysiology of ischemia/reperfusion injury is very complex with a plethora of players. Cardiopulmonary resuscitation (CPR) initiated reperfusion triggers the innate immune system by danger agents acting as ligands for pattern recognition molecules of the various branches of the innate immunity, including the complement system.[5,6] This induces a secondary and broad-acting systemic inflammatory response which, when over-activated or dysregulated, leads to tissue damage, organ failure and in worst case to death.[7]

Complement is present in plasma and thus immediately activated upon injury through the classical, lectin, or alternative pathway. All three pathways converge at the level of C3, which gets cleaved and forms a protease that cleaves C5 leading to the formation of the terminal complement complex. The terminal complement complex is either incorporated in cellular membranes, where it may result in lysis and inflammation, or it is released as a soluble molecule (sC5b-9) to the fluid phase.[8] All C3 and C5 derived complement activation products, including the anaphylatoxins C3a and C5a, can activate endothelial cells.

The aim of the present study was to investigate if complement activation with subsequent endothelial cell activation as part of the initial ischemia/reperfusion injury was associated with poor cerebral outcome and death in patients resuscitated after OHCA.

Material and methods

The present study is a planned sub-study of the prospective observational Norwegian Cardio-Respiratory Arrest Study (NORCAST, NCT01239420).[9] The aim of NORCAST was to assess the ability of currently recommended diagnostic tools to identify patients with a poor prognosis. Importantly, results of prognostic tests were blinded to clinicians to avoid hasty withdrawal decisions and self-fulfilling prophecies. The design and patient population in NORCAST has been previously described in detail.[9] Briefly, 259 comatose adult OHCA patients admitted to Oslo University Hospital Ullevål were included between October 1st, 2010 and January 30th, 2014. Post-resuscitation care was performed according to local standard procedures including targeted temperature management to 33 °C (TTM33) for 24 h and immediate coronary angiography with subsequent percutaneous coronary intervention, if a cardiac cause was suspected.[9] Hypoxic cause of cardiac arrest was defined as a non-cardiac cause of the arrest, where hypoxia of different reasons resulted in unresponsiveness, absence of breathing and loss of heart function. The Sequential Organ Failure Assessment (SOFA score) was performed to assess the extent of a patient's organ failure within the first 24 h of admission. Neurological outcome at 6 months was assessed by Neurology specialists who were blinded to all clinical and paraclinical scores during hospital admission and well acquainted with Cerebral Performance Category (CPC, 1–2 good outcome, 3–5 poor outcome with 5 including death) scoring. The examination on which CPC scoring was based consisted of structured neurological and cognitive examination as well as queries of functional level to patients and next-of-kin. For complement activation markers, the upper reference limit for each assay was set to the 95th percentile of a healthy reference population consisting of 20 female and 20 male blood donors without cardiovascular or autoimmune diseases.[10] Upper reference limit of sC5b-9 is >0.7 complement activation units (CAU)/mL and for C3bc >9 CAU/mL.[10] For endothelial markers such international reference limit do not exist and venous EDTA-blood obtained from twelve healthy volunteers with a close to similar age (median 59 years, interquartile range 51–68 years) and gender distribution (7 female: 5 male) compared to the study population was used as control.

Blood sampling protocol

At admission (before initiation of TTM33) and after 72 h (after rewarming), peripheral venous blood was obtained in ethylenediaminetetraacetic acid (EDTA) vacutainer tubes (BD, Plymouth, UK). Samples were immediately kept on crushed iced and centrifuged within 30 min at 2500 g for 20 min at 4 °C. Plasma was collected and stored at −80 °C until analyses.

Complement activation markers

Complement activation products, C3bc (common for classical, lectin and alternative pathways) and sC5b-9 (soluble terminal complement complex), were measured by in-house enzyme-linked immunosorbent assays (ELISA) which has previously been described[11,12] and later modified according to the protocol presented in Ref. 10.

Endothelial activation markers and sCD14

Soluble endothelial activation markers syndecan-1, sE-selectin (sCD62E), thrombomodulin (sTM), vascular cell adhesion molecule 1(sVCAM-1) and sCD14 were measured by DuoSet ELISA (R&D Systems, Minneapolis, MN) in EDTA plasma.

Ethics and data management

The Regional Committee for Medical Research Ethics of South-Eastern Norway approved NORCAST (Approval number REK S-O/A - 2010/1116a). Ethics and study management have been previously reported.[9] Written informed consent was obtained from close relatives or guardians within 24 h after hospitalization and from all patients regaining consciousness and decision-making capacity within six

months. Clinical, biochemical and outcome data were prospectively collected from medical records, questionnaires, and paramedic records as presented in the Utstein criteria.[13] Blood samples from healthy donors were collected into a biobank approved by the Regional Ethical Committee (REK S-04114).

Statistical analysis

Data were presented as medians, 25th–75th percentile, or frequencies and percentages, as appropriate. Patients with good and poor outcome were compared by Mann–Whitney *U* test for continuous and χ^2-test or Fisher's exact test for categorical data. The difference in complement or endothelial activation between CPC groups was assessed by Mann–Whitney *U* test. Kruskal–Wallis test was used for comparison of more than two groups. Bonferroni correction was applied to correct for multiple testing. The Wilcoxon signed-rank paired test was used to compare the complement and endothelial activation at admission and day three. Associations between cerebral outcome and SOFA score, time from cardiac arrest-to-return-of-spontaneous circulation (time-to-ROSC), cardiac arrest (unwitnessed vs. witnessed), initial rhythm (shockable vs. non-shockable), bystander CPR (yes vs. no), and cause of cardiac arrest were assessed by unadjusted and adjusted (multivariable) logistic regression model. To assess whether sC5b-9 and C3bc at admission was associated with the odds for poor outcome separately as well as combined, unadjusted logistic regression models were estimated. The models were further adjusted for SOFA score, time-to-ROSC, and cause of cardiac arrest. Interactions between sC5b-9 and time-to-ROSC, and between C3bc and time-to-ROSC were included into the adjusted model if significant. The results of logistic regression models were presented as odds ratios (ORs) with the corresponding 95% confidence intervals (CIs) and p-values. The regression coefficients and standard errors (SE) were presented for the variables included into the interaction term. For easier interpretation, the interactions were illustrated graphically. To assess how well the sC5b-9 and C3bc distinguishes between those with good and poor outcome, the area under the characteristic (ROC) curve with the corresponding 95% CI was calculated. The logistic regression models were estimated to assess the association between the CPC score and sC5b-9 at admission adjusted for endothelial markers Syndecan-1, E-selectin, Thrombomodulin, and VCAM, and CD14 measured at day three, one at a time. Also, the model combining all endothelial markers and CD14 was estimated. The models were adjusted for SOFA score, time-to-return-of-spontaneous circulation (time-to-ROSC), and cause of cardiac arrest. Area under the ROC curve calculated for the unadjusted and each adjusted model was compared. Correlation between complement and endothelial activation markers was assessed using Spearman's rank test. Results with *p*-values <0.05 were regarded as significant. Statistical analyses were performed in Statistical Package for the Social Sciences (SPSS) software (version 26, IBM, Armonk, NY), Stata (version 16.0, StataCorp, College Station, TX), SAS (version 9.4, SAS Institute, Cary, NC) and GraphPad Prism (version 8, GraphPad Software, San Diego, CA).

Results

Out of 259 successfully resuscitated patients after OHCA, 232 were included into the study (Fig. 1). Median age was 63 (54–69), 83% were male, and 82% had cardiac cause of arrest (Table 1). Outcome

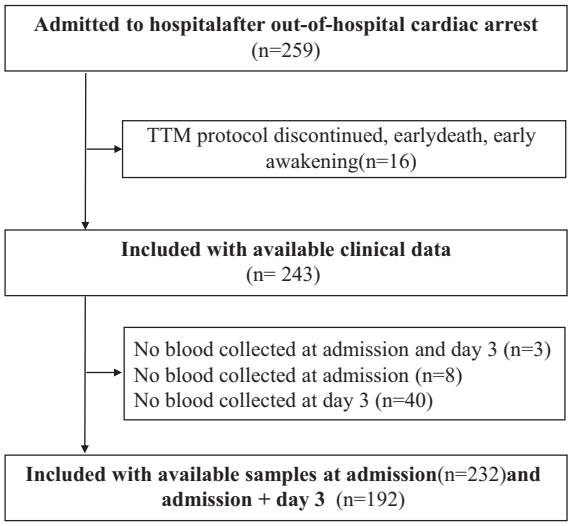

Fig. 1 – Flow chart of patient population in this study. Enrolment in study and exclusions, availability of clinical data and blood samples at each time point. TTM; target temperature management.

defined as best CPC score within six months after OHCA, was poor for 49% and good for 51% of patients with significant differences in time-to-ROSC and SOFA score at admission (Table 1). Clinical variables significantly associated with poor outcome in adjusted logistic regression analysis were non-shockable initial cardiac arrest rhythm, longer time-to-ROSC and higher overall organ dysfunction during the first 24 h after admission (Suppl. Table 1). Unwitnessed cardiac arrest was associated with outcome in unadjusted logistic regression model only, while performance of bystander CPR was not significantly associated with outcome (Suppl. Table 1).

Complement activation products

C3bc and sC5b-9 were markedly higher compared to upper reference limit in all patients (n = 232) both at admission and day three (Fig. 2A, B). C3bc and sC5b-9 were both significantly higher at admission compared to day three. Patients with poor outcome had significantly higher levels of C3bc and sC5b-9 at admission compared to patients with good cerebral outcome (Fig. 2A, B).

In unadjusted models, higher sC5b-9 at admission was associated with poor outcome (OR 1.08, 95% CI (1.01–1.14), $p = 0.02$, Suppl. Table 2), while C3bc was not (Suppl. Table 3). The area under the ROC curve (AUC) was 0.65 95% CI (0.57; 0.72) for sC5b-9 and 0.57 (0.49; 0.65) for C3bc. There was no additive effect on the ROC curve by combining sC5b-9 and C3bc (AUC 0.66 (0.58; 0.73)). Longer time-to-ROSC and higher SOFA scores at admission were also associated with poor outcome (OR 1.06 (1.05–1.09), $p < 0.001$ and OR 1.23 (1.06–1.43), $p = 0.006$, respectively, Suppl. Table 2). Interaction between sC5b-9 and time-to-ROSC was significant in an adjusted model ($p = 0.015$, Suppl. Table 2). Post hoc analysis of the interaction term showed that odds for poor outcome were increasing with increasing values of sC5b-9 when time-to-ROSC was below 30 min, while odds for poor outcome were dependent on time-to-ROSC alone at 30 min and longer (Suppl. Table 2, Fig. 3). There was no significant interaction between C3bc and time-to-ROSC in an unadjusted model,

Table 1 – Demographics and clinical characteristics at admission.

	Total (n = 232)	Good outcome (CPC 1–2) (n = 118)	Poor outcome (CPC 3–5) (n = 114)	*p*-value
Age (years)	63 (54–70)	62 (54–69)	64 (55–71)	*0.3*[a]
Sex				*0.5*[b]
Female	39 (17)	14 (36)	25 (64)	
Male	193 (83)	104 (54)	89 (46)	
Cause of cardiac arrest (CA)				
Acute myocardial infarction	89 (38)	48(54)	41 (46)	*0.5*[b]
Chronic cardiac disease	71 (31)	40 (56)	31 (44)	*0.3*[b]
Arrythmia (VF)	29 (13)	18 (62)	11 (38)	*0.2*[b]
Hypoxia induced CA	31 (13)	9 (29)	22 (71)	***0.01***[b]
Other causes	5 (2)	2 (40)	3(60)	*0.7*[c]
Unknown	7 (3)	1 (14)	6 (86)	*0.6*[c]
Survivors	123 (53)	114 (93)	9 (7)	<***0.001***[b]
Scores at admission				
SOFA score	11 (9.5–12)	10 (9–12)	11 (10–12)	<***0.001***[a]
Time-to-ROSC (min)	25 (16–33)	19 (12–28)	30 (24–40)	<***0.001***[a]

Data presented as median (25th–75th percentile) or n (%). Cerebral outcome is best CPC within 6 months after cardiac arrest. CPC; cerebral performance category, VF; ventricular fibrillation, CA; cardiac arrest, SOFA; Sequential Organ Failure Assessment, Time-to-ROSC; time to return of spontaneous circulation, min; minutes.

[a] Mann–Whitney *U* test.

[b] χ^2-test or

[c] Fisher's exact test.

and C3bc alone was not associated with outcome (Suppl. Table 3). Hypoxia as the cause of cardiac arrest increased the odds for poor outcome, with increased levels of sC5b-9 and C3bc at admission in comparison to acute myocardial infarction (OR 6.78 (1.90–24.16), $p = 0.003$, and OR 5.97 (1.75–20.36), $p = 0.004$, respectively, Suppl. Tables 2 and 3).

sCD14

sCD14, a co-receptor of TLR, was significantly higher in cardiac arrest patients (n = 192) compared to healthy controls and significantly higher at day three compared to admission (Suppl. Fig. 1). However, sCD14 was not associated with outcome alone or in adjusted models (Suppl. Table 4).

Markers of endothelial activation and damage

Glycocalyx damage marker sSyndecan-1 was higher in OHCA patients (n = 192) compared to healthy controls at admission and day three (Fig. 4A). It was significantly higher at admission compared to day three in OHCA patients (Fig. 4A). Markers of endothelial activation sE-selectin, sVCAM-1 and sTM were consistently higher in OHCA patients and concentrations at day three were significantly increased compared to admission (Fig. 4B–D). Among the four markers, only sE-selectin and sTM were significantly higher in patients with poor outcome compared to patients with good outcome (Fig. 5A, B). C3bc correlated significantly with sSyndecan-1 and sTM at admission ($R = 0.26$, $p < 0.001$ and R=0.17, $p = 0.02$, respectively) and day three ($R = 0.19$, $p = 0.007$ and R=0.2, $p = 0.007$, respectively, Suppl. Fig. 2). Likewise, sC5b-9 correlated significantly with sSyndecan-1 and sTM at admission ($R = 0.25$, $p < 0.001$ and R=0.23, $p = 0.001$, respectively) and at day three (R=0.16, $p = 0.03$ and R = 0.23, $p = 0.002$, Suppl. Fig. 2). sCD14 was significantly correlated with sE-selectin at day three ($R = 0.44$, $p < 0.001$). No other important correlations were observed (Suppl. Fig. 2). sE-selectin was the only endothelial parameter significantly associated with outcome in an unadjusted model (OR 1.02 [1.00–1.05], $p = 0.038$), but did not improve the discriminative ability of the model with only sC5b-9 (Suppl. Table 4). Adjustment did not improve the discriminative ability of sC5b-9 in combination with endothelial markers except for E-selectin with additive effect on the ROC curve when sC5b-9 was combined with sE-selectin (AUC 0.79 (0.72–0.85), $p = 0.047$, Suppl. Tables 4 and 5).

Discussion

A whole-body inflammatory reaction follows resuscitation after OHCA. In the present study, we show that initial strong activation of the complement system, in particular reflected by the terminal pathway activation product sC5b-9, was associated with poor long-term outcome. Following the initial rapid complement activation, endothelial activation occurred after admission. While the endothelial damage marker Syndecan-1 peaked at admission, all other measured markers of endothelial activation showed highest level at day three in contrast to complement activation, which then had markedly declined.

Complement activation

Complement activation at admission was associated with cerebral outcome. Complement has been shown to get activated immediately after initiation of resuscitation in OHCA patients with a swift decline after ROSC.[14] This might be due to release of damage associated molecular patterns (DAMPs) from injured cells followed by a chain of injurious events[15] leading to activation of complement signalling pathways, in particular the lectin pathway.[16] While the aforementioned study showed no relation of lectin pathway activation and outcome, this study confirms that the end-product of complement system activation sC5b-9 is associated with poor outcome. TTM33

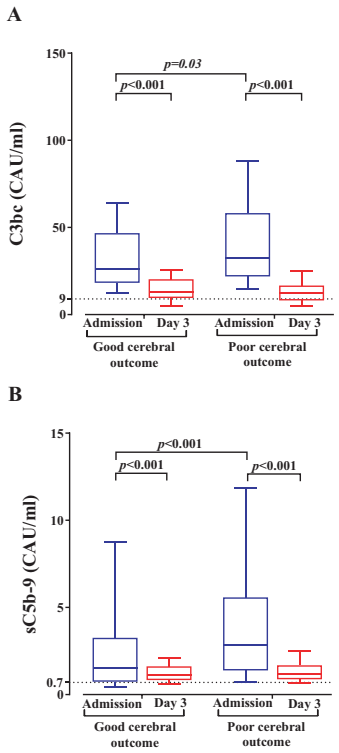

Fig. 2 – Patients (n = 232) with successful resuscitation after out-of-hospital-cardiac-arrest. Plasma levels of C3bc and sC5b-9 at admission (blue lines) were above upper reference limit (line) in most of the patients, significantly higher at admission compared to day three (red lines) and higher in patients with poor compared to good cerebral outcome (A, B).
Cerebral outcome was defined by cerebral performance category (CPC) as good; CPC 1–2 and poor; CPC 3–5. Data is shown as box plots with median as line and box indicating 25th–75th percentile and whiskers representing 10th–90th percentiles. Wilcoxon signed-rank paired test and non-paired Mann–Whitney *U* test.
CAU; complement arbitrary unit.

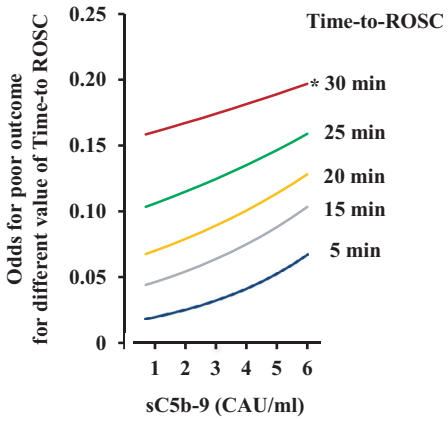

Fig. 3 – Interaction between sC5b-9 and time-to-ROSC is associated with poor outcome. Increasing values of sC5b-9 were associated with poor outcome when time-to-ROSC was ≤25 min but not when time-to-ROSC was 30 min or higher (asterix). Odd ratios adjusted for covariates SOFA, Time-to-ROSC and cause of cardiac arrest. CAU; complement arbitrary unit, Time-to-ROSC; time-to-return-of-spontaneous-circulation, SOFA; Sequential Organ Failure Assessment.

treatment of OHCA patients leads to suppression of complement activation with return to levels above reference limit after rewarming[17] while one study reports that TTM33 lead to reduction of the regulatory protein Map19 of the complement lectin pathway compared to TTM36.[18] All patients in this study were treated with TTM33 and it might thus be speculated that the initial complement activation is of special importance for outcome in OHCA patients. In line with this are findings that the degree of complement activation correlates with the severity of heart failure in patients developing cardiogenic shock after acute myocardial infarction.[19] Furthermore, blockade of C5 reduced infarct size and improved cardiac function in an experimental porcine model of severe myocardial infarction.[20]

An important factor influencing the reperfusion injury and degree of neurological injury is the duration of hypoxia i.e., the time-to-ROSC.[21] As in previous studies, we also verified an association between time-to-ROSC and poor cerebral outcome.[22] We add that for the same time-to-ROSC, odds for poor outcome increased with increasing levels of sC5b-9, suggesting that increased complement activation per se had an effect on outcome and might be an effect-modifier. The effect of association between sC5b-9 and outcome was absent in patients with time-to-ROSC 30 min, indicating that the impact of inflammation on cerebral outcome may be reduced when time-to-ROSC is long and ischemic brain damage becomes the only decisive factor. Despite the association between sC5b-9 and poor outcome, complement activation cannot be used as a decisive prognostic marker, given the large inter-patient variations of absolute values, which were in this study almost all above an internationally accepted upper reference limit.[10] However, complement inhibition in OHCA patients might be explored in further studies as inhibition of C5 has been shown to reduce ischemia/reperfusion injury in general, and our data on sC5b-9 support this hypothesis.

sCD14

sCD14 is a multifunctional molecule, which recognises and binds endogenous and exogenous danger signals.[23] It is also a co-factor for several TLRs which are mainly expressed by monocytes and macrophages. In the present study, sCD14 was increased upon admission and highest on day three, suggesting a maintained release, which might be due to shedding of membrane-bound CD14 protein or secretion via intracellular vesicles by inflammatory cells.[24] The prolonged release of sCD14 may indicate prolonged activation of the immune system which is in agreement with the pattern of complement and endothelial activation.

Endothelial activation

The endothelium is an active component of innate immunity. It gets injured during ischemia/reperfusion injury,[25] e.g. by complement. This again leads to retrograde complement activation resulting in a vicious circle with exaggerated inflammatory reaction causing tissue damage and organ failure.[26]

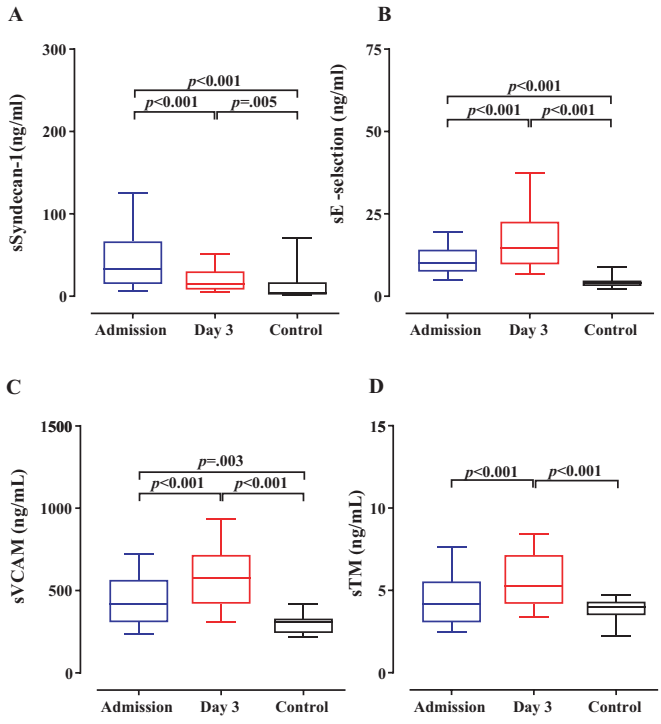

Fig. 4 – Patients (n = 192) with successful resuscitation after out-of-hospital-cardiac-arrest and blood samples both at admission and day three. Plasma levels of sSyndecan-1 (A), sE-selectin (B), and sVCAM (C) were significantly higher compared to healthy controls (black lines) at admission (blue lines) and day three (red lines), while sTM (D) was significantly higher at day three, only. sSyndecan was significantly higher at admission compared to day three, while sE-selectin, sVCAM and sTM were higher at day three compared to admission. Data is shown as box plots with median as line and box indicating 25th–75th percentile and whiskers representing 10th–90th percentiles. Wilcoxon signed-rank paired test and non-paired Mann–Whitney *U* test. sTM; sThrombomodulin.

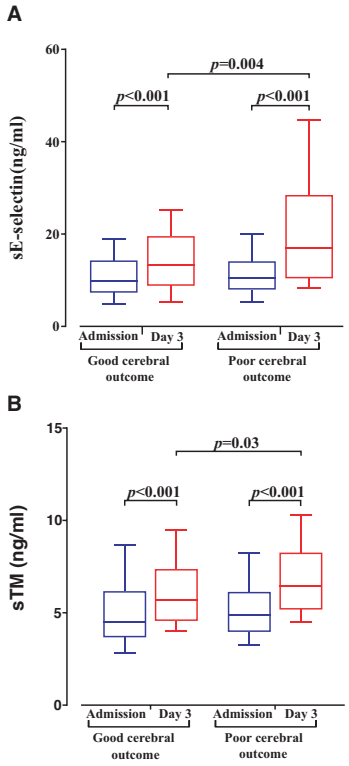

Fig. 5 – sE-Selectin (A) and sTM (B) in patients (n = 192) with successful resuscitation after out-of-hospital-cardiac-arrest. In patients with poor cerebral outcome, sE-selectin and sTM were significantly higher at day three (red lines) compared to admission (blue lines).
Cerebral outcome was defined by cerebral performance category (CPC) as good; CPC 1–2 and poor; CPC 3–5. Data is shown as box plots with median as line and box indicating 25th–75th percentile and whiskers representing 10th–90th percentiles. Wilcoxon signed-rank paired test and non-paired Mann–Whitney *U* test. sTM; sThrombomodulin.

Syndecan-1 was the only endothelial marker that peaked on the day of admission, which is in-line with previous findings describing shedding of glycocalyx as an initial event in OHCA patients, where Syndecan-1 is an integral part.[27] While sSyndecan-1 correlated significantly with complement activation, it was not associated with outcome. Thus, our findings confirm results of a previous study in 163 comatose patients after OHCA, where sSyndecan-1 was not associated with mortality,[22] which may imply that sSyndecan-1 is an endothelial damage marker, but not necessarily associated with prolonged inflammatory reperfusion injury.

Complement system and endothelial cell activation have been shown to correlate with systemic cytokine release and haemodynamic status early post-cardiac arrest. The magnitude of this response has been associated with the severity of this post-cardiac arrest syndrome,[5,8] independent on TTM.[28] Likewise, endothelial activation and damage markers have been shown to be independent of TTM, except for sE-Selectin, which is lower in TTM36 treated patients.[22] The present study confirms this previously observed pattern of complement and endothelial activation and that endothelial activation reflected by thrombomodulin is associated with outcome,[22] although individual correlations of single parameters are relatively weak. In a study on 163 patients with one-time blood sampling within approximately two hours after OHCA, Cox proportional-hazard analyses found sE-selectin to be a univariate predictor of mortality.[29] Our findings add that sE-selectin is associated with outcome, also when assessed three days after OHCA. In addition, we show that sE-selectin is correlated with sCD14 at day three after cardiac arrest and not with the complement system, highlighting the multifactorial pathogenesis of the post-cardiac arrest syndrome. Unfortunately, due to large inter-patient variations seen in our and the aforementioned studies, thrombomodulin and sE-selectin are not reliable prognostic markers. Especially sE-selectin levels are affected by body-mass index.[30] However, both sE-selectin and thrombomodulin could be used in future clinical intervention studies assessing effect of an intervention by reduction of these endothelial activation markers.

Dual inhibition of both the complement system and CD14 in post-OHCA patients may be a general approach to attenuate the innate immune system broadly, preventing downstream overreaction of the

immune system including activation of endothelial cells, cytokine storm, and adaptive immune system,[31,32] and ought to be investigated further.

Limitations

This was a post-hoc analysis of a single-center study, which limits generalizability. Despite a rigorous sampling routine, blood was not acquired from 40 patients at day three, decreasing power of especially endothelial markers, which are known to be time-sensitive and increase later than complement activation markers. All patients were treated with the same TTM33 protocol reducing generalization to internationally recommended post resuscitation care, which includes TTM36. However, by focusing on TTM33, confounders based on quality of care were minimized. Time-to-ROSC is a pure quantitative parameter, and the quality of CPR, which is a known predictor of outcome, was not assessed.[33] This could therefore impact on the data. As this is an observational study, no cause-effect conclusions can be made, the study should thus be regarded as hypothesis generating, and may facilitate design of future studies investigating interventions in the immediate post-cardiac arrest phase.

Conclusion

In comatose, resuscitated OHCA patients, activation of the complement system is present in the majority of patients at admission. In particular, increased sC5b-9 was associated with poor outcome. The whole-body inflammation included subsequent endothelial cell activation and sCD14 release three days after OHCA.

Authorship contribution statement

Authors have contributed as follows to (1) Conception and design of the study (all authors), or acquisition of data (Nakstad, Stær-Jensen, Seljeflot, Lundqvist, Sunde, Andersen), or analysis and interpretation of data (Chaban, Schjalm, Vaage, Benth, Mollnes, Pischke), (2) drafting the article or revising it critically for important intellectual content (all authors), (3) final approval of the version to be submitted (all authors).

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Data statement

All anonymised data is available upon request from the corresponding author.

Conflict of interests

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.resuscitation.2021.05.038.

REFERENCES

1. Gräsner JT, Wnent J, Herlitz J, et al. Survival after out-of-hospital cardiac arrest in Europe — results of the EuReCa TWO study. Resuscitation 2020;148:218–26.
2. Nolan JP, Neumar RW, Adrie C, et al. Post-cardiac arrest syndrome: epidemiology, pathophysiology, treatment, and prognostication. A Scientific Statement from the International Liaison Committee on Resuscitation; the American Heart Association Emergency Cardiovascular Care Committee; the Council on Cardiovascular Surgery and Anesthesia; the Council on Cardiopulmonary, Perioperative, and Critical Care; the Council on Clinical Cardiology; the Council on Stroke. Resuscitation 2008;79:350–79.
3. Kalogeris T, Baines CP, Krenz M, Korthuis RJ. Cell biology of ischemia/reperfusion injury. Int Rev Cell Mol Biol 2012;229–317 Elsevier.
4. Laver S, Farrow C, Turner D, Nolan J. Mode of death after admission to an intensive care unit following cardiac arrest. Intensive Care Med 2004;30:2126–8.
5. Bro-Jeppesen J, Johansson PI, Kjaergaard J, et al. Level of systemic inflammation and endothelial injury is associated with cardiovascular dysfunction and vasopressor support in post-cardiac arrest patients. Resuscitation 2017;121:179–86.
6. Sekhon MS, Ainslie PN, Griesdale DE. Clinical pathophysiology of hypoxic ischemic brain injury after cardiac arrest: a "two-hit" model. Crit Care 2017;21:90.
7. Mentzelopoulos SD, Zakynthinos SG. Post-cardiac arrest syndrome: pathological processes, biomarkers and vasopressor support, and potential therapeutic targets. Resuscitation 2017;121:A12-a4.
8. Bottiger BW, Motsch J, Braun V, Martin E, Kirschfink M. Marked activation of complement and leukocytes and an increase in the concentrations of soluble endothelial adhesion molecules during cardiopulmonary resuscitation and early reperfusion after cardiac arrest in humans. Crit Care Med 2002;30:2473–80.
9. Nakstad ER, Staer-Jensen H, Wimmer H, et al. Late awakening, prognostic factors and long-term outcome in out-of-hospital cardiac arrest — results of the prospective Norwegian Cardio-Respiratory Arrest Study (NORCAST). Resuscitation 2020;149:170–9.
10. Bergseth G, Ludviksen JK, Kirschfink M, Giclas PC, Nilsson B, Mollnes TE. An international serum standard for application in assays to detect human complement activation products. Mol Immunol 2013;56:232–9.
11. Garred P, Mollnes TE, Lea T. Quantification in enzyme-linked immunosorbent assay of a C3 neoepitope expressed on activated human complement factor C3. Scand J Immunol 1988;27:329–35.
12. Mollnes TE, Lea T, Harboe M, Tschopp J. Monoclonal antibodies recognizing a neoantigen of poly(C9) detect the human terminal complement complex in tissue and plasma. Scand J Immunol 1985;22:183–95.
13. Perkins GD, Jacobs IG, Nadkarni VM, et al. Cardiac arrest and cardiopulmonary resuscitation outcome reports: update of the Utstein

Resuscitation Registry Templates for Out-of-Hospital Cardiac Arrest: a statement for healthcare professionals from a task force of the International Liaison Committee on Resuscitation (American Heart Association, European Resuscitation Council, Australian and New Zealand Council on Resuscitation, Heart and Stroke Foundation of Canada, InterAmerican Heart Foundation, Resuscitation Council of Southern Africa, Resuscitation Council of Asia); and the American Heart Association Emergency Cardiovascular Care Committee and the Council on Cardiopulmonary, Critical Care, Perioperative and Resuscitation. Circulation 2015;132:1286–300.
14. Böttiger BW, Motsch J, Braun V, Martin E, Kirschfink M. Marked activation of complement and leukocytes and an increase in the concentrations of soluble endothelial adhesion molecules during cardiopulmonary resuscitation and early reperfusion after cardiac arrest in humans. Crit Care Med 2002;30:2473–80.
15. Ricklin D, Reis ES, Lambris JD. Complement in disease: a defence system turning offensive. Nat Rev Nephrol 2016;12:383–401.
16. Haugaard SF, Jeppesen AN, Troldborg A, Kirkegaard H, Thiel S, AM Hvas. The complement lectin pathway after cardiac arrest. Scand J Immunol 2018;88:e12680.
17. Bisschops LL, van der Hoeven JG, Mollnes TE, Hoedemaekers CW. Seventy-two hours of mild hypothermia after cardiac arrest is associated with a lowered inflammatory response during rewarming in a prospective observational study. Crit Care 2014;18:546.
18. Bro-Jeppesen J, Jeppesen AN, Haugaard S, et al. The complement lectin pathway protein MAp19 and out-of-hospital cardiac arrest: Insights from two randomized clinical trials. Eur Heart J Acute Cardiovasc Care 2020;9:S145–52.
19. Orrem HL, Nilsson PH, Pischke SE, et al. Acute heart failure following myocardial infarction: complement activation correlates with the severity of heart failure in patients developing cardiogenic shock. ESC Heart Fail 2018;5:292–301.
20. Pischke SE, Gustavsen A, Orrem HL, et al. Complement factor 5 blockade reduces porcine myocardial infarction size and improves immediate cardiac function. Basic Res Cardiol 2017;112:20.
21. Wu W, Chopra A, Ziegler C, McLeod SL, Lin S. Predictive value of hospital discharge neurological outcome scores for long-term neurological status following out-of-hospital cardiac arrest: a systematic review. Resuscitation 2020;151:139–44.
22. Bro-Jeppesen J, Johansson PI, Hassager C, et al. Endothelial activation/injury and associations with severity of post-cardiac arrest syndrome and mortality after out-of-hospital cardiac arrest. Resuscitation 2016;107:71–9.
23. Arroyo-Espliguero R, Avanzas P, Jeffery S, Kaski JC. CD14 and toll-like receptor 4: a link between infection and acute coronary events? Heart (British Cardiac Society) 2004;90:983–8.
24. Marcos V, Latzin P, Hector A, et al. Expression, regulation and clinical significance of soluble and membrane CD14 receptors in pediatric inflammatory lung diseases. Respir Res 2010;11:32.
25. Yang Q, He G-W, Underwood MJ, Yu C-M. Cellular and molecular mechanisms of endothelial ischemia/reperfusion injury: perspectives and implications for postischemic myocardial protection. Am J Transl Res 2016;8:765.
26. Karpman D, Ståhl A-l, Arvidsson I, et al. Complement interactions with blood cells, endothelial cells and microvesicles in thrombotic and inflammatory conditions. Immune Responses to Biosurfaces 2015;19–42 Springer.
27. Grundmann S, Fink K, Rabadzhieva L, et al. Perturbation of the endothelial glycocalyx in post cardiac arrest syndrome. Resuscitation 2012;83:715–20.
28. Bro-Jeppesen J, Kjaergaard J, Wanscher M, et al. The inflammatory response after out-of-hospital cardiac arrest is not modified by targeted temperature management at 33 °C or 36 °C. Resuscitation 2014;85:1480–7.
29. Johansson PI, Bro-Jeppesen J, Kjaergaard J, Wanscher M, Hassager C, Ostrowski SR. Sympathoadrenal activation and endothelial damage are inter correlated and predict increased mortality in patients resuscitated after out-of-hospital cardiac arrest. A post Hoc sub-study of patients from the TTM-trial. PLoS One 2015;10:e0120914.
30. Schumacher A, Seljeflot I, Sommervoll L, Christensen B, Otterstad JE, Arnesen H. Increased levels of markers of vascular inflammation in patients with coronary heart disease. Scand J Clin Lab Invest 2002;62:59–68.
31. Barratt-Due A, Pischke SE, Nilsson PH, Espevik T, Mollnes TE. Dual inhibition of complement and Toll-like receptors as a novel approach to treat inflammatory diseases-C3 or C5 emerge together with CD14 as promising targets. J Leukoc Biol 2017;101:193–204.
32. Barratt-Due A, Pischke SE, Brekke OL, et al. Bride and groom in systemic inflammation–the bells ring for complement and Toll in cooperation. Immunobiology 2012;217:1047–56.
33. Nolan JP, Berg RA, Callaway CW, et al. The present and future of cardiac arrest care: international experts reach out to caregivers and healthcare authorities. Intensive Care Med 2018;44:823–32.

Article III

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Complement ratios C3bc/C3 and sC5b-9/C5 do not increase the sensitivity of detecting acute complement activation systemically

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ABSTRACT

Background: Complement activation plays an important pathogenic role in numerous diseases. The ratio between an activation product and its parent protein is suggested to be more sensitive to detect complement activation than the activation product itself. In the present study we explored whether the ratio between the activation product and the parent protein for C3 (C3bc/C3) and for C5 (sC5b-9/C5) increased the sensitivity to detect complement activation in acute clinical settings compared to the activation product alone.

Materials and methods: Samples from patients with acute heart failure following ST-elevated myocardial infarction (STEMI) and from patients with out-of-hospital cardiac arrest (OHCA) were used. C3, C3bc and C5, sC5b-9 were analysed in 629 and 672 patient samples, respectively. Healthy controls (n = 20) served to determine reference cut-off values for activation products and ratios, defined as two SD above the mean.

Results: Increased C3bc/C3- and sC5b-9/C5 ratios were vastly dependent on C3bc and sC5b-9. Thus, 99.5 % and 98.1 % of the increased C3bc/C3- and sC5b-9/C5 ratios were solely dependent on increased C3bc and sC5b-9, respectively. Significantly decreased C3 and C5 caused increased ratios in only 3/600 (0.5 %) and 4/319 (1.3 %) samples, respectively. Strong correlations between C3bc and C3bc/C3-ratio and between sC5b-9 and sC5b-9/C5-ratio were found in the STEMI- ($r = 0.926$ and $r = 0.786$, respectively) and the OHCA-population ($r = 0.908$ and $r = 0.843$, respectively; $p < 0.0001$ for all). Importantly, sC5b-9 identified worse outcome groups better than sC5b-9/C5-ratio.

Conclusion: C3bc and sC5b-9 were sensitive markers of complement activation. The ratios of C3bc/C3 and sC5b-9/C5 did not improve detection of complement activation systemically.

Abbreviations: C3, Complement protein 3; C5, Complement protein 5; PRP, Pattern recognition protein; sC5b-9/TCC, Terminal complement complex.

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1. Introduction

The human complement system is a rapid and efficient immune surveillance system that protects the host and preserves homeostasis. Through pattern recognition proteins (PRP), the complement system provides an instant protection against exogenous threats, such as foreign intruders and organisms, or endogenous danger evoked by altered host cells and damaged self (Ricklin et al., 2010).

Complement is a cascade system built up of an elaborate network of soluble and cell-surface bound components, PRP, proteases, receptors and regulators (Bajic et al., 2015). Activation occurs via three routes, the classical, the lectin and the alternative pathway, all converging and leading to cleavage of the central complement component C3. The terminal pathway, on the other hand, leads to formation of the terminal C5b-9 complement complex (TCC), which either is formed in the soluble phase (sC5b-9) or inserted into a membrane as the membrane attach complex.

The cascade system is under strict control and a non-tuned balance between activation and regulation can trigger complement-driven pathological processes and diseases. Eculizumab, a monoclonal antibody targeting and preventing cleavage of C5, is FDA approved for only four rare conditions (Hillmen et al., 2006; Legendre et al., 2013; Paul, 2013; Dhillon, 2018). However, complement is suggested to play a key role in numerous other disease entities, where off-label use of eculizumab has been provided (Ricklin et al., 2017). This is an interesting development but also challenging, requiring solid complement-diagnostic tools and methods. Thus, a number of new complement inhibitors are under development to target different parts of the complement system, and some have already passed clinical trials and are and in clinical use. The development and use of complement inhibitors for several diseases have recently been extensively reviewed (Garred et al., 2021)

In the clinical setting, it may be difficult to ascertain the presence of complement activation. Low levels of C3 and C4 could be interpreted as an increased consumption and thus increased activation, but could also be explained by reduced synthesis due to liver failure, or dilution of the sample. Increased values can be seen during an acute phase reaction and in chronic inflammatory conditions (Ward, 2010; Jalal et al., 2018). The measurement of complement activation products is required for documenting *in vivo* complement activation. However, activation of complement occurs primarily locally, and a slightly increased formation of an activation product can easily be interpreted as insignificant. Thus, different reports have suggested that the ratio between an activation product and its parent protein (e.g. C3dg/C3, C3bc/C3 or C4bc/C4) is a more sensitive indicator of activation compared to the measurement of the activation product alone (Nurnberger and Bhakdi, 1984; Nielsen et al., 1996; Kim et al., 2019). This is an interesting approach that specifically takes into account borderline elevated samples. However, the evidence from the literature on this issue is limited.

The aim of the present study was to explore whether ratios between the activation products C3bc and sC5b-9 and their respective parent proteins C3 and C5 are more sensitive markers of complement activation compared to the activation products alone. Samples from two different prospective clinical studies including patients with acute heart failure following ST-elevation myocardial infarction (STEMI) (Husebye et al., 2014) and comatose out-of-hospital cardiac arrest (OHCA) patients were included (Nakstad et al., 2020).

2. Material and methods

2.1. Clinical trial samples

2.1.1. Levosimendan in Acute Heart Failure Following Myocardial Infarction (LEAF)

EDTA plasma samples from 60 patients were obtained from the previously published LEAF trial (Husebye et al., 2013). All patients had large STEMI, (the STEMI population), complicated by clinical symptoms of acute heart failure. The patients were further subdivided into patients with cardiogenic shock (shock-group), or heart failure without cardiogenic shock (non-shock group). Blood samples were drawn from inclusion to day 5. Complement activation products, including C3bc and sC5b-9 were measured and these results have already been published (Orrem et al., 2018). A total of 207 samples from 25 patients, previously analysed for C3bc, were available and analysed for C3 in the current study and enabled us to calculate the C3bc/C3 ratio. A total of 281 samples from 60 patients, previously analysed for sC5b-9, were available and analysed for C5 in the current study and enabled us to calculate the sC5b-9/C5 ratio.

2.1.2. Norwegian Cardio-Respiratory Arrest Study (NORCAST)

EDTA plasma samples were obtained from the Norwegian Cardio-Respiratory Arrest Study (NORCAST), a prospective study on blinded prognostication assessment in comatose patients with OHCA (the OHCA population) from both cardiac and non-cardiac causes (Nielsen et al., 1996). All patients were treated with standardized operating procedures (SOP) including targeted temperature management (TTM) to 33 °C for 24 h (Nielsen et al., 1996). Blood samples were obtained on admission and at day three and classified according to the patient's clinical outcome at six months post arrest defined as cerebral performance category (CPC) score 1–2 (good outcome) or CPC score 3–5 (poor outcome) (Mak et al., 2016). The complement activation products, including C3bc and sC5b-9, were measured and presented in a subsequent study (Chaban et al., 2021). In total, 422 samples from 246 patients, quantified for C3bc, and 391 samples from 230 patients, quantified for sC5b-9, were available for C3 and C5 quantification, enabling us to calculate the C3bc/C3- and sC5b-9/C5 ratio, respectively.

2.2. Controls

Twenty healthy controls (age 30–58 years) were analysed for C3bc, C3, sC5b-9 and C5 to define positive cut-off values of the ratios. A positive value was defined as two standard deviations above the mean and a normal value as equal or lower than the mean.

2.3. Detection of the complement components C3 and C5 and the complement activation products C3bc and sC5b-9

C3 was measured using nephelometry, BN II System from Siemens, (Erlangen, Gemany) at the Routine Immunological Laboratory at Oslo University Hospital. A commercially available enzyme-linked immunosorbent assay (Abcam, Cambridge, UK) was used to detect complement component C5. C3 and C5 were measured in both studied clinical cohorts, whereas C3bc and sC5b-9 already had been measured as mentioned above. The control samples were measured for C3 and C5 as described above and C3bc and sC5b-9 were quantified as described in detail previously (Bergseth et al., 2013)

2.4. Statistics

Graphpad prism version 8 (San Diego, CA) was used for statistical analyses. Data were analysed with one-way ANOVA followed by Bonferroni's multiple comparison test, and correlation analyses were measured in logarithmic scale using the non-parametric spearman correlation test. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Control samples, cut-off values and C3 and C5 concentrations

Cut-off values were based on measurement of the control samples and defined as two standard deviations below/above the mean (Fig. 1, grey bars). C3bc positive value was defined as > 4.9 AU/mL, C3bc/C3

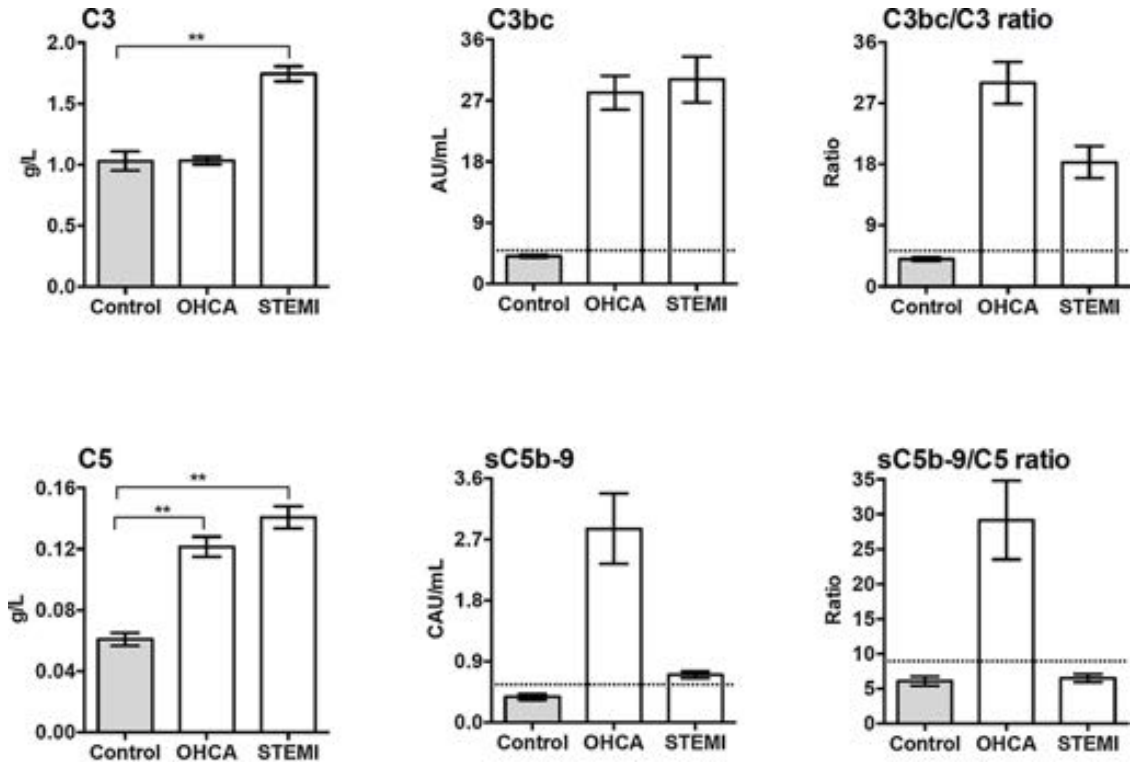

Fig. 1. C3, C3bc, C5 and sC5b-9 were analysed in samples from 20 healthy individuals (controls; grey bars) and in samples from two critically ill patients populations; a) patients with acute heart failure following ST-elevation myocardial infarction, the STEMI-population, b) patients with out-of hospital cardiac arrest, the OHCA-population (white bars). The ratios between the activation products and the native components were thereafter calculated. Positive cut-off values were defined as two standard deviations above the mean of the control, indicated by the dotted lines giving the following positive values; C3bc > 4.9 AU/mL, C3bc/C3 > 5.24 (upper panel), sC5b-9 > 0.56 AU/mL and sC5b-9/C5 ratio > 8.94 (lower panel). Statistical comparisons of C3- and C5-levels were performed between the controls and the MI- and CA-population. Data are presented as means ± 95 % CI. $^{**}p < 0.001$.

positive ratio as > 5.24, sC5b-9 positive value as > 0.56 AU/mL and sC5b-9/C5 positive ratio as > 8.94 (indicated by dotted lines in Fig. 1). For comparison, the mean values with 95 % CI for C3, C3bc, C5, sC5b9 and the calculated ratios C3bc/C3 and sC5b-9/C5 are shown for the STEMI and OHCA patients, respectively (Fig. 1). Notably, C3 concentration was significantly higher in the STEMI patients and C5 concentration was significantly higher in both STEMI and OHCA patients, compared to the control group ($p < 0.001$ for both) (Fig. 1, left panel.)

3.2. C3-activation as evaluated by levels of C3bc and the ratio of C3bc/C3

Increased C3bc/C3-ratio was vastly dependent on increased formation of C3bc. Altogether 597 out of 600 ratio-positive values (99.5 %) from both patient populations had increased levels of C3bc alone, whereas only three samples with increased ratios were caused by low or normal C3-values (Table 1). Notably, 15 out of 612 C3bc-positive samples (2.5 %) were not associated with increased ratios, underscoring the high sensitivity of C3bc alone. In the STEMI population, 100 % of the samples from the shock group and 91 % from the non-shock group were both C3bc and C3bc/C3 ratio positive (Table 1). In the OHCA population, altogether 96 % of the samples were both C3bc and C3bc/C3 ratio positive.

3.3. Terminal pathway activation evaluated by levels of sC5b-9 and the ratio sC5b-9/C5

Increased sC5b-9/C5-ratio was vastly dependent on increased formation of sC5b-9. From both patient populations, altogether 313 out of 319 ratio-positive samples (99.1 %) had increased levels of sC5b-9 alone, whereas only six samples with increased ratios were caused by normal or low sC5b-9-values (Table 2). Out of 510 sC5b-9 positive samples, 197 were not associated with increased ratios. In the STEMI population, 79 % (30/38) of the samples from the shock group were sC5b-9-positive, whereas only 42 % (16/38) of the samples were sC5b-9/C5-ratio positive. In the OHCA population, 92 % (149/162) of the samples from the poor outcome group were sC5b-9-positive, whereas only 71 % (115/162) of the samples were sC5b-9/C5-ratio positive.

Table 1
C3-activation measured by increased formation of the activation product C3bc, versus increased formation of the C3bc/C3 ratio in patients with acute heart failure following ST-elevation myocardial infarction (**STEMI**) and patients with out-of-hospital cardiac arrest (**OHCA**).[a]

Patient population	C3bc: **positive** C3bc/C3: normal	C3bc: normal C3bc/C3: **positive**	C3bc: **positive** C3bc/C3: **positive**	C3bc: normal C3bc/C3: normal	Number of samples
STEMI	9 (4)[b]	0	192 (93)	6 (3)	207
Shock	0	0	37 (100)	0	37
Non-shock	9 (5)	0	155 (91)	6 (4)	170
OHCA[c]	6 (1.5)	3 (0.5)	405 (96)	8 (2)	422
Poor outcome	1 (0.5)	1 (0.5)	163 (97)	3 (1.5)	168
Good outcome	4 (1.5)	2 (1.0)	198 (96)	3 (1.5)	207

[a] C3bc positive defined as >4.9 AU/mL and normal as ≤ 4.9 AU/mL. C3bc/C3 ratio positive defined as >5.24 and normal as ≤ 5.24.
[b] Number of samples with percentages in parenthesis.
[c] In the cardiac arrest study clinical data were available for 375 out of 422 samples.

Table 2
C5-activation measured by increased formation of sC5b-9 (TCC) versus increased formation of the sC5b-9/C5 ratio in patients with acute heart failure following ST-elevation myocardial infarction (**STEMI**) and patients with out-of-hospital cardiac arrest (**OHCA**).[a]

Patient population	sC5b-9: **positive** sC5b-9/C5: normal	sC5b-9: normal sC5b-9/C5: **positive**	sC5b-9: **positive** sC5b-9/C5: **positive**	sC5b-9: normal sC5b-9/C5: normal	Number of samples
STEMI	109 (39)[b]	4 (1)	44 (16)	124 (44)	281
Shock	15 (39)	1 (3)	15 (39)	7 (18)	38
Non-shock	94 (39)	3 (1)	29 (12)	117 (48)	243
OHCA[c]	88 (22.5)	2 (0.5)	269 (69)	32 (8)	391
Poor outcome	35 (21)	1 (1)	114 (71)	12 (7)	162
Good outcome	46 (23)	1 (0.5)	135 (67.5)	18 (10)	200

[a] sC5b-9 positive defined as >0.56 AU/mL and normal as ≤ 0.56 AU/mL. sC5b-9/C5 ratio positive defined as >8.94 and normal as ≤ 8.94.
[b] Number of samples with percentages in parenthesis.
[c] In the cardiac arrest study clinical data were available for 362 out of 391 samples.

3.4. Correlations between the activation product C3bc, C3, and the ratio of C3bc/C3

Correlation between C3bc and the ratio of C3bc/C3 measured in both the STEMI- and OHCA populations were highly significant ($p < 0.0001$), with high correlation coefficients ($r = 0.926$ and $r = 0.908$, respectively, Fig. 2, right panel). In contrast, only weak correlations were found between C3bc and C3 with $r = 0.197$ ($p = 0.0043$) and $r = 0.117$ ($p = 0.016$), respectively, (Fig. 2, left panel). In controls, the correlation between C3bc and C3 was moderate ($r = 0.474$, $p = 0.034$), whereas no correlation was found between C3bc and C3bc/C3-ratio ($r = 0.035$, $p = 0.88$) (data not shown).

3.5. Correlations between the activation product sC5b-9, C5 and the ratio of sC5b-9/C5

The correlation between sC5b-9 and the ratio of sC5b-9/C5 in the STEMI and OHCA populations were highly significant ($p < 0.0001$) with high correlation coefficients, $r = 0.786$ and $r = 0.843$, respectively (Fig. 3, right panel). No correlation between sC5b-9 and C5 were found, $r = 0.048$ (p = 0.42) and $r = 0.027$ ($p = 0.59$), respectively (Fig. 3, left panel). In controls, we did not find any correlation between sC5b-9 and

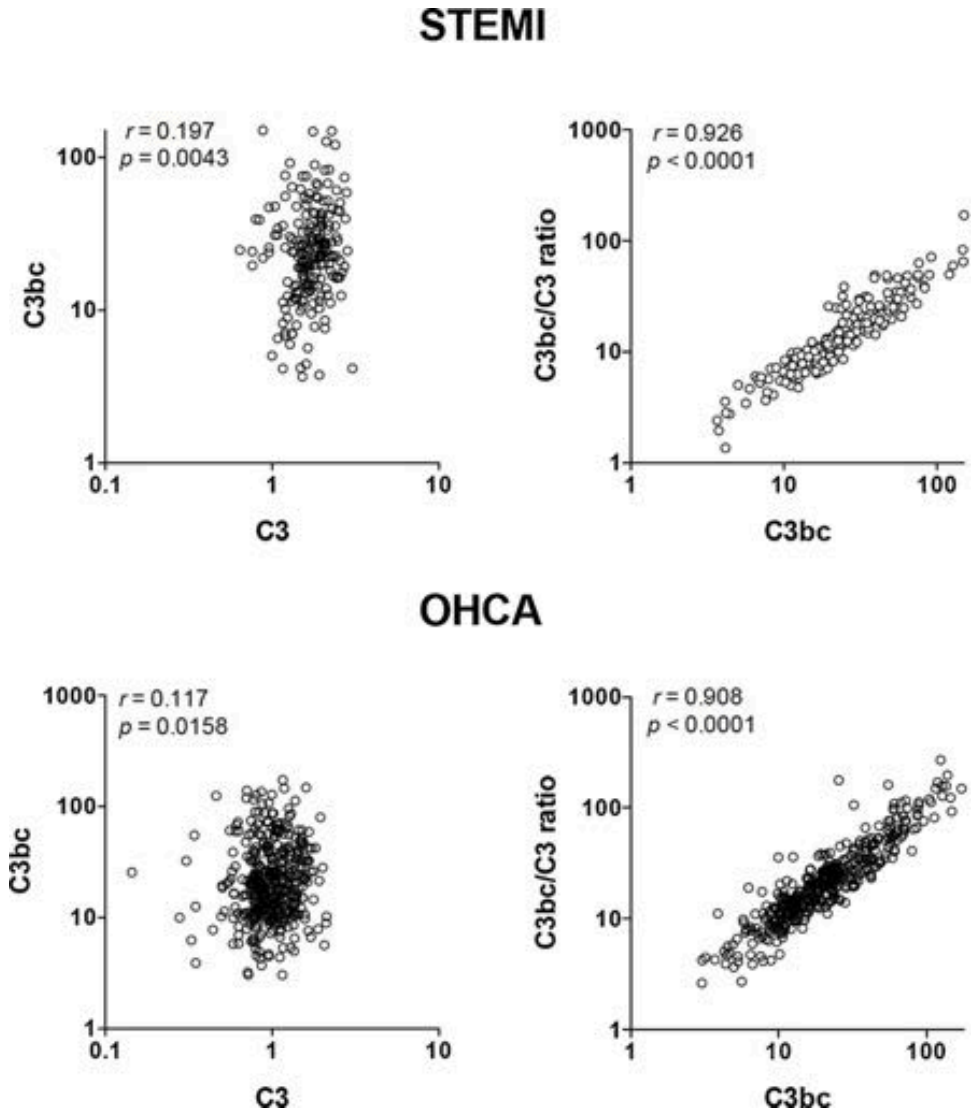

Fig. 2. The correlations between C3bc and C3 and between C3bc and the C3bc/C3 ratio in the STEMI (upper panel) and OHCA (lower panel) population were performed in logarithmic scale using the non-parametric spearman correlation. A p value < 0.05 was considered statistically significant.

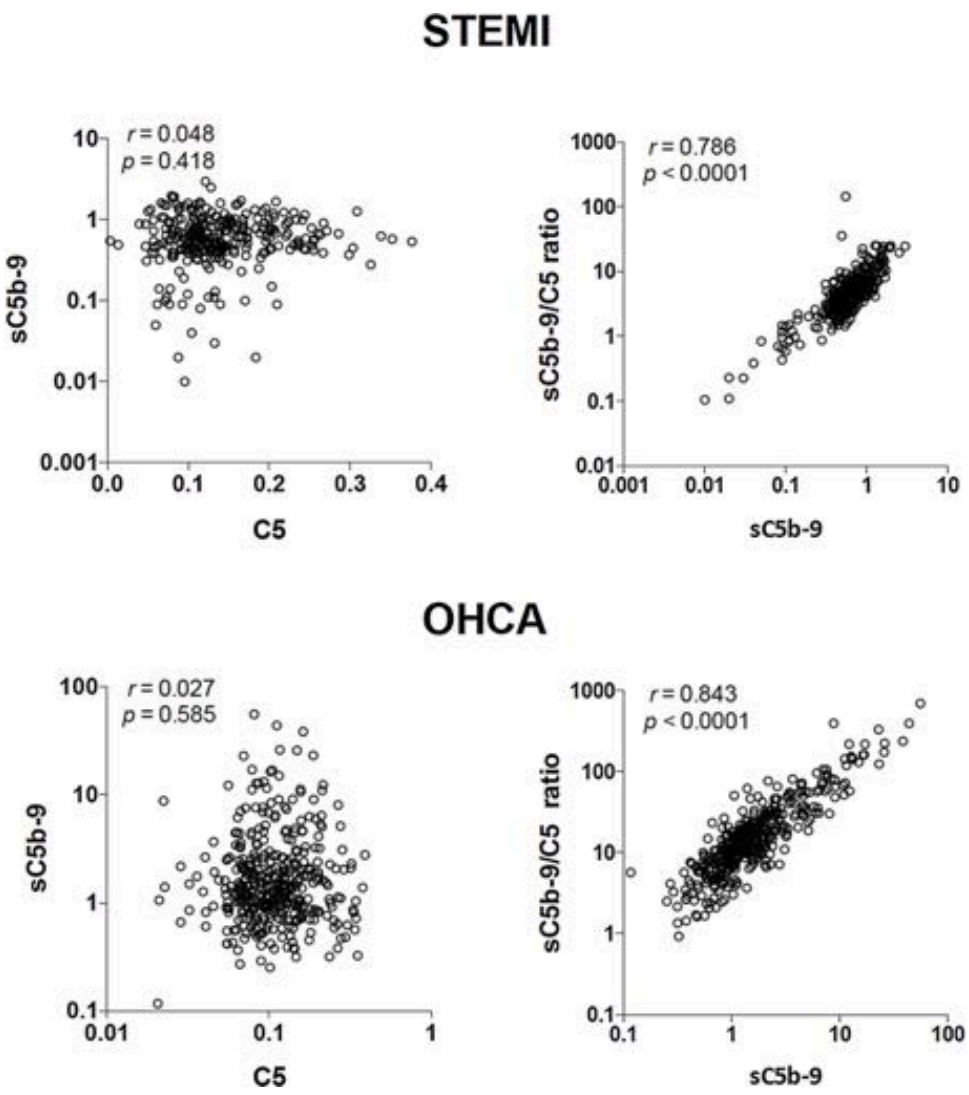

Fig. 3. The correlations between sC5b-9 and C5 and between sC5b-9 and the sC5b-9/C5 ratio in the STEMI (upper panel) and OHCA (lower panel) population were performed in logarithmic scale using the non-parametric Spearman correlation test. A p value < 0.05 was considered statistically significant.

C5 ($r = 0.05$, $p = 0.84$), however, the correlation between sC5b-9 and the ratio of sC5b-9/C5 was strong ($r = 0.743$, $p = 0.0002$) (data not shown).

4. Discussion

The present study demonstrates strong correlations between the complement activation products C3bc and sC5b-9, and the ratio to their native components, C3 and C5, in two clinical cohorts comprising patients with STEMI complicated with acute heart failure and comatose OHCA patients. Importantly, the study documents that the ratio of the activation products, C3bc/C3 and sC5b-9/C5, were not more sensitive in detecting complement activation systemically than the activation products itself. In fact, the increased ratios of C3bc/C3 and sC5b-9/C5 were almost exclusively dependent on increased levels of C3bc and sC5b-9. Of particular interest was that normal or low C3 or C5 concentrations caused increased ratios in only 3/600 (0.5 %) and 6/319 (1.8 %) of the samples, respectively. No correlations were found between C3bc and C3 or sC5b-9 and C5. In STEMI-patients developing cardiogenic shock, and in OHCA-patients with poor outcome, sC5b-9 was indeed a more sensitive marker of complement activation compared to the sC5b-9/C5 ratio.

The complement activation products are normally present in traces, but may increase log-folds when activation occurs, not least due to the alternative pathway amplification loop, responsible for 80 % of the magnitude of the activation irrespective of the activation pathway (Harboe et al., 2004, 2006). In particular, sC5b-9 is a stable activation product, highly resistant to thawing and freezing and demonstrate low intra- and inter-assay variability (Bergseth et al., 2013). Both C3bc and sC5b-9 are excellent markers to detect complement activation systemically. Thus, a slightly increased concentration of the activation product imbalances the ratio, reflected by the convincingly strong correlation between the activation product and the ratio. This is in fact not surprising given that approximately 1 % of the components are activated under physiological condition, a 10-fold increase in the activation product will only give a reduction by 10 % of the parent molecule, and hardly influence the ratio. If the corresponding C3bc concentration is increased from 5 to 50 AU/mL, it will considerably increase the ratio by 10 times, consistent with the finding in this study.

Thus, positive ratios were almost solely dependent on increased activation products and rarely on low concentration of the native components. The samples included in the present study were from patients with acute, critical illness, OHCA or acute heart failure after STEMI with or without cardiogenic shock, both incidents likely to cause regional or global ischemia/reperfusion injury and complement activation (Banz and Rieben, 2012). However, the study cohorts comprise a

wide range of complement activation patterns, from no activation to excessive activation, thus elucidating the value of complement-activation ratios over a broad range of complement activation in a comprehensive context.

Previous studies have suggested that ratios may be useful and more sensitive than activation products in measuring ongoing complement activation (Nurnberger and Bhakdi, 1984; Kim et al., 2019). These reports refer to systemic lupus erythematosus (SLE), a chronic condition where the disease activity is related to complement consumption and decreased concentration of C3 and C4. Recently, it was demonstrated that the relative changes in iC3b/C3-ratio discriminated between active and inactive form of SLE (Kim et al., 2019). Thus, the usefulness of a ratio might be greater in chronic diseases characterized by complement consumption, but in acute disease like in the present study, the ratio does not appear to be better or a more useful marker, even with very low levels of native components.

The control levels of C3 and C5 used in this study correspond to the levels referred to in the literature (Bergseth et al., 2013). However, the C5 level was significantly higher in both patient populations compared to the control group, whereas the level of C3 was significantly higher in the STEMI population only. Previous reports document that an increased level of C3 is associated with increased arterial stiffness, cardiovascular disease and obesity (Engstrom et al., 2005; Muhammad et al., 2017; Kirschfink and Mollnes, 2003; Nilsson et al., 2014) which may explain the elevated level of C3 observed in the STEMI population. The observation of increased levels of C5 in both patient cohorts, whereas C3 was increased only in the STEMI population is of interest and may have several explanations. The different levels in C3 and C5 may reflect different mechanisms of low-grade chronic inflammation among these patients, or alternatively a rather specific C3 increase as an acute response to the insult. Mechanistically, this could be explained by a differential efficacy and stability of the C3- and the C5 convertases. Such a phenomenon is known from other conditions like certain nephritic factors (autoantibodies to the convertases) where some stabilize only the C3 convertase and C3 is activated without a consecutive activation of C5 (Mollnes et al., 1986). We recently showed the same phenomenon by activation complement by air bubbles, leading to a huge activation of C3 and the alternative pathway, with a limited activation of the terminal pathway (Storm et al., 2021). This interesting differential activation of the convertases could under some conditions be explain by the density of C3b molecules generated by the C3 convertase (Mannes et al., 2021). Finally, it was recently shown that properdin binding to the convertase to a great extent explained the shift towards C5 activation after alternative pathway activation (Michels et al., 2021). In any case, the increased levels of the native components, C3 and C5, reduce the likelihood for a positive ratio as the denominator increases, thus making the ratio a less sensitive marker of complement activation.

sC5b-9 is a robust marker of terminal complement pathway activation due to its detection of complement activation to the final stage and the relatively long half-life compared to other complement activation products (Deppisch et al., 1990; Mollnes, 1985). The sC5b-9/C5-ratio has previously not been investigated, and in the present study, increased sC5b-9/C5-ratio was vastly dependent on increased formation of sC5b-9. Interestingly, when looking at subgroups, increased formation of the activation product sC5b-9 occurred more frequently than the ratio, both among STEMI patients with cardiogenic shock and OHCA patients with poor outcome. It is therefore tempting to suggest that the activation product sC5b-9 might be a more robust marker than the ratio itself, at least in critically ill patients.

In conclusion, the present study demonstrates a strong correlation between the complement activation products and their ratios to native components in critically ill patients with complement activation detected systemically. Compared to the activation products, the ratios were less sensitive in detecting complement activation systemically than the activation products alone. Both C3bc and sC5b-9 are sensitive markers of complement activation and sufficient for screening of clinically significant complement activation.

Data availability

The present study is based on clinical data and plasma samples provided from two clinical trials, the NORCAST (Nakstad et al., 2020) and Leaf trial (Nielsen et al., 1996). The samples were taken snap-frozen, stored properly, and analyzed in our lab.

Data will be made available on request.

Declaration of Competing Interest

The authors report no declarations of interest.

References

Bajic, G., Degn, S.E., Thiel, S., Andersen, G.R., 2015. Complement activation, regulation, and molecular basis for complement-related diseases. EMBO J. 34 (22), 2735–2757.

Banz, Y., Rieben, R., 2012. Role of complement and perspectives for intervention in ischemia-reperfusion damage. Ann. Med. 44 (3), 205–217.

Bergseth, G., Ludviksen, J.K., Kirschfink, M., Giclas, P.C., Nilsson, B., Mollnes, T.E., 2013. An international serum standard for application in assays to detect human complement activation products. Mol. Immunol. 56 (3), 232–239.

Chaban, V., Nakstad, E.R., Stær-Jensen, H., Schjalm, C., Seljeflot, I., Vaage, J., et al., 2021. Complement activation is associated with poor outcome after out-of-hospital cardiac arrest. Resuscitation 166, 129–136.

Deppisch, R., Schmitt, V., Bommer, J., Hansch, G.M., Ritz, E., Rauterberg, E.W., 1990. Fluid phase generation of terminal complement complex as a novel index of bioincompatibility. Kidney Int. 37 (2), 696–706.

Dhillon, S., 2018. Eculizumab: a review in generalized myasthenia gravis. Drugs 78 (3), 367–376.

Engstrom, G., Hedblad, B., Eriksson, K.F., Janzon, L., Lindgarde, F., 2005. Complement C3 is a risk factor for the development of diabetes: a population-based cohort study. Diabetes 54 (2), 570–575.

Garred, P., Tenner, A.J., Mollnes, T.E., 2021. Therapeutic targeting of the complement system: from rare diseases to pandemics. Pharmacol. Rev. 73 (2), 792–827.

Harboe, M., Ulvund, G., Vien, L., Fung, M., Mollnes, T.E., 2004. The quantitative role of alternative pathway amplification in classical pathway induced terminal complement activation. Clin. Exp. Immunol. 138 (3), 439–446.

Harboe, M., Garred, P., Borgen, M.S., Stahl, G.L., Roos, A., Mollnes, T.E., 2006. Design of a complement mannose-binding lectin pathway-specific activation system applicable at low serum dilutions. Clin. Exp. Immunol. 144 (3), 512–520.

Hillmen, P., Young, N.S., Schubert, J., Brodsky, R.A., Socie, G., Muus, P., et al., 2006. The complement inhibitor eculizumab in paroxysmal nocturnal hemoglobinuria. N. Engl. J. Med. 355 (12), 1233–1243.

Husebye, T., Eritsland, J., Muller, C., Sandvik, L., Arnesen, H., Seljeflot, I., et al., 2013. Levosimendan in acute heart failure following primary percutaneous coronary intervention-treated acute ST-elevation myocardial infarction. Results from the LEAF trial: a randomized, placebo-controlled study. Eur. J. Heart Fail. 15 (5), 565–572.

Husebye, T., Eritsland, J., Arnesen, H., Bjørnerheim, R., Mangschau, A., Seljeflot, I., et al., 2014. Association of interleukin 8 and myocardial recovery in patients with ST-elevation myocardial infarction complicated by acute heart failure. PLoS One 9 (11), e112359.

Jalal, D., Renner, B., Laskowski, J., Stites, E., Cooper, J., Valente, K., et al., 2018. Endothelial microparticles and systemic complement activation in patients with chronic kidney disease. J. Am. Heart Assoc. 7 (14).

Kim, A.H.J., Strand, V., Sen, D.P., Fu, Q., Mathis, N.L., Schmidt, M.J., et al., 2019. Association of blood concentrations of complement split product iC3b and serum C3 with systemic lupus erythematosus disease activity. Arthritis Rheumatol. (Hoboken, NJ) 71 (3), 420–430.

Kirschfink, M., Mollnes, T.E., 2003. Modern complement analysis. Clin. Diagn. Lab. Immunol. 10 (6), 982–989.

Legendre, C.M., Licht, C., Muus, P., Greenbaum, L.A., Babu, S., Bedrosian, C., et al., 2013. Terminal complement inhibitor eculizumab in atypical hemolytic-uremic syndrome. N. Engl. J. Med. 368 (23), 2169–2181.

Mak, M., Moulaert, V.R., Pijls, R.W., Verbunt, J.A., 2016. Measuring outcome after cardiac arrest: construct validity of Cerebral Performance Category. Resuscitation 100, 6–10.

Mannes, M., Dopler, A., Zolk, O., Lang, S.J., Halbgebauer, R., Höchsmann, B., et al., 2021. Complement inhibition at the level of C3 or C5: mechanistic reasons for ongoing terminal pathway activity. Blood 137 (4), 443–455.

Michels, M., Maas, R.J.F., van der Velden, T., van de Kar, N., van den Heuvel, L., Volokhina, E.B., 2021. The role of properdin in C5 convertase activity and C5b-9 formation in the complement alternative pathway. J. Immunol. (Baltimore, Md : 1950) 15 (10), 2465–2472.

Mollnes, T.E., 1985. Early- and late-phase activation of complement evaluated by plasma levels of C3d,g and the terminal complement complex. Complement (Basel, Switzerland) 2 (2–3), 156–164.

Mollnes, T.E., Ng, Y.C., Peters, D.K., Lea, T., Tschopp, J., Harboe, M., 1986. Effect of nephritic factor on C3 and on the terminal pathway of complement in vivo and in vitro. Clin. Exp. Immunol. 65 (1), 73–79.

Muhammad, I.F., Borne, Y., Ostling, G., Kennback, C., Gottsater, M., Persson, M., et al., 2017. Acute phase proteins as prospective risk markers for arterial stiffness: the Malmo Diet and Cancer cohort. PLoS One 12 (7), e0181718.
Nakstad, E.R., Staer-Jensen, H., Wimmer, H., Henriksen, J., Alteheld, L., Reichenbach, A., et al., 2020. Late awakening, prognostic factors and long-term outcome in out-of-hospital cardiac arrest - results of the prospective Norwegian Cardio-respiratory Arrest Study (NORCAST). Resuscitation 149, 170–179.
Nielsen, E.W., Johansen, H.T., Hogasen, K., Wuillemin, W., Hack, C.E., Mollnes, T.E., 1996. Activation of the complement, coagulation, fibrinolytic and kallikrein-kinin systems during attacks of hereditary angioedema. Scand. J. Immunol. 44 (2), 185–192.
Nilsson, B., Hamad, O.A., Ahlstrom, H., Kullberg, J., Johansson, L., Lindhagen, L., et al., 2014. C3 and C4 are strongly related to adipose tissue variables and cardiovascular risk factors. Eur. J. Clin. Invest. 44 (6), 587–596.
Nurnberger, W., Bhakdi, S., 1984. Plasma C3d/C3 quotient as a parameter for in vivo complement activation. J. Immunol. Methods 74 (1), 87–91.
Orrem, H.L., Nilsson, P.H., Pischke, S.E., Grindheim, G., Garred, P., Seljeflot, I., et al., 2018. Acute heart failure following myocardial infarction: complement activation correlates with the severity of heart failure in patients developing cardiogenic shock. ESC Heart Fail. 5 (3), 292–301.
Paul, F., 2013. Hope for a rare disease: eculizumab in neuromyelitis optica. Lancet Neurol. 12 (6), 529–531.
Ricklin, D., Hajishengallis, G., Yang, K., Lambris, J.D., 2010. Complement: a key system for immune surveillance and homeostasis. Nat. Immunol. 11 (9), 785–797.
Ricklin, D., Barratt-Due, A., Mollnes, T.E., 2017. Complement in clinical medicine: clinical trials, case reports and therapy monitoring. Mol. Immunol. Sep;89, 10–21.
Storm, B.S., Christiansen, D., Fure, H., Ludviksen, J.K., Lau, C., Lambris, J.D., et al., 2021. Air bubbles activate complement and trigger hemostasis and C3-Dependent cytokine release ex vivo in human whole blood. J. Immunol. (Baltimore, Md : 1950) 207, 2828–2840.
Ward, P.A., 2010. The harmful role of c5a on innate immunity in sepsis. J. Innate Immun. 2 (5), 439–445.

Article IV