Extracellular histone H3

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**Extracellular histone H3:
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Extracellular histone H3: biomarker and therapeutic target for the prevention of tissue damage

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Ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
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Chapter 1

General introduction

1

Histones in physiology

Histones are the most positively charged proteins found in biology [1]. These highly conserved, nuclear proteins are important mediators of gene regulation through their binding with negatively charged DNA. Histones comprise the basic protein unit of nuclear chromatin in all eukaryotic cells [1]. Chromatin consists of structural units referred to as nucleosomes, which are organized in a “beads-on-a-string”-like manner (Figure 1).

Nucleosomes are made up of so-called “core histones”, around which 146bp of DNA are wrapped, and are individually joined by the linker histone H1 [2]. The histone core consists of two histone H2A-H2B dimers and one H3-H4 tetramer (together forming an octamer), with N-terminal ends of the histones protruding from this structure.

Figure 1. Structural organization of histones inside the nucleus. Core histones are organized in nucleosomes, around which 146bp of DNA is wrapped (in orange). The core consists of 4 histone subtypes: H2A (in green) and H2B (in yellow), forming two dimers, and H3 (in magenta) and H4 (in cyan), forming one tetramer. N-terminal histone ends protrude from the nucleosomes and are prone to posttranslational modifications (PTMs), regulating the transcription of genes.

The N-terminal ends of histones are highly susceptible to posttranslational modification (PTM), which is an important way to regulate chromatin status, structure and gene expression [3,4]. These PTMs include amongst others acetylation, methylation, phosphorylation, citrullination and ubiquitination, and can act in combination with one another. All the possible PTMs together comprise the so-called “histone code”, which

regulates DNA accessibility to DNA-binding proteins [3]. Reflecting the importance of PTMs, aberrant histone modifications have been linked to human disease, including cancer, neurological disorders and inflammatory diseases [5,6].

Histones in disease

Release of extracellular histones

Besides their nuclear function and importance in epigenetics, histones can exert differential effects once released outside the cell. Various modes of histone release have been described, and these can be categorized into forms of passive release (e.g. cell death mechanisms) or active release (e.g. activated immune cell-mediated release) (Figure 2A) [7,8]. The passive release of histones via cell death can occur through both apoptosis and (forms of) necrosis. Apoptosis, which is considered to be a regulated form of cell death characterized by chromatin decondensation, DNA fragmentation and the formation of apoptotic bodies, is a process that avoids membrane disintegration [9]. However, it was described that histones can accumulate within or at the surface of apoptotic bodies and thereby leak from cells undergoing apoptosis [10,11]. Necrosis, on the other hand, is characterized by membrane disruption and is initiated by cellular injury and/or toxic stimuli [9]. The damaging of the cell membrane leads to a massive release of intracellular components, including histones and other nuclear material [12]. Active histone release was first described as part of a new anti-bacterial mechanism of the innate immune system. Neutrophils that were stimulated with pathogenic stimuli were found to release their granular and nuclear constituents as so-called neutrophil extracellular traps (NETs) by a process termed NETosis [13,14]. Citrullination of histones by peptidyl-arginine deaminase 4 (PAD4) is thought to be a key event in NETosis [15]. NETs are composed of DNA strands decorated with antimicrobial proteins such as granular enzymes and (citrullinated) histones [13,16,17], and are released to trap, disarm and kill invading pathogens. Later, the expulsion of these extracellular traps was also described for other types of immune cells such as mast cells, macrophages and eosinophils (termed ETosis) [18–20].

Within the cell, histones are organized together with DNA into nucleosomes. Extensive cell death should, in theory, release complexed histones into the circulation. However, the exact forms of extracellular histones and their fate in the blood stream are currently not well elucidated. This is in part due to technical limitations in distinguishing freely circulating histones from histones bound to DNA or as part of nucleosomes. The terms histones as well as nucleosomes are used interchangeably, but it is proposed that DNA, histones and intact nucleosomes can exert differential signaling events leading to immune cell activation [21]. Also, free histones were found to be cytotoxic whilst nucleosomes are

not [21,22]. In the remainder of this introduction, the primary focus will be on extracellular histones as such.

Pathological effects of extracellular histones

The initial hypothesis of the release of histones into the extracellular environment was that it served as part of the host defense repertoire. Already in the 1950s, histones were described to possess potent antibacterial activity [23]. In more recent years however, the release of intracellular material (including histones) into the extracellular milieu was associated with pathological consequences. Besides exogenous pathogens, also endogenous molecules were found to be potent triggers of the innate immune system. These host biomolecules are referred to as damage-associated molecular patterns (DAMPs) and include amongst others DNA, heat shock proteins and histones [24].

Identified as DAMPs, extracellular histones have been found to kill host cells, including endothelial cells [25,26] and cells from lung [27–29], liver [30,31], heart [32,33] and kidney [34,35]. The exact mechanisms of histone-mediated cytotoxicity are still being elucidated, but is thought to occur through both receptor-independent and receptor-dependent pathways (Figure 2B) [7,8]. Histones can directly interact with phospholipids, forming pores in cellular membranes and compromising membrane barrier function [36–38]. It is proposed that the charge as well as the tertiary structure of histones are involved in this process [36,39]. Core histones H3 and H4 are considered to be the most potent in terms of cytotoxicity [25]. Interestingly, histone H1 was specifically found to be released from damaged brain and is linked to neurotoxicity [40]. The exact underlying mechanisms contributing to this cell and tissue specificity remain to be investigated.

Extracellular histones have also been found to exert indirect effects by interacting with cell surface receptors such as toll-like receptors (TLR-) 2, 4 [30,31,34,41,42] and potentially 9 [31]. TLR signaling results in activation of the NLRP3 inflammasome [43], cytokine production and leukocyte recruitment [44,45], subsequently leading to inflammation, tissue injury and organ failure [46]. Targeting extracellular histones with an anti-histone antibody before LPS injection or directly after renal artery clamping resulted in reduction of kidney injury and rescued mice *in vivo* [34]. A similar beneficial effect was observed when TLR knockout mice were injected with a lethal dose of histones [34].

Elevated levels of circulating histones have been associated with severity of disease in for example autoimmune disorders [47], stroke [48], (transfusion-related) acute lung injury [27,29,31,49], ischemia/reperfusion injury (IRI) [31,34,50], kidney injury [34,51], liver injury [52,53], trauma [54] and sepsis [55,56] (Figure 2B). Histones are not only released as a consequence of cellular injury, they also actively contribute towards a self-sustaining cascade of ongoing cell death [57]. Therefore, extracellular histones have been proposed as both biomarkers and therapeutic targets in disease.

Figure 2. Modes of extracellular histone release and pathological effects *in vivo*. (A) Histones (represented as blue spheres) are released from cells undergoing a process of cell death (e.g. apoptosis or necrosis) or from stimulated immune cells (e.g. NETosis or ETosis). (B) Extracellular histones have pathological effects *in vivo*. Histones exert cellular effects by inducing direct pore formation leading to increased calcium (Ca^{2+}) influx and subsequent cell death, leading to additional histone release. Histones induce the production of pro-inflammatory cytokine via TLR2/4/9 activation and downstream signaling via e.g. MyD88 and NF κ B. Through TLR-signaling in platelets, circulating extracellular histones can also induce both platelet activation and aggregation, leading to thrombosis. Other mechanisms through which histones can increase thrombosis are the induction of phosphatidylserine (PS) on the platelet surface and the TLR2/4 dependent downregulation of thrombomodulin (TM) and upregulation of tissue factor (TF), all leading to enhanced thrombin (IIa) generation. Collectively, extracellular histones can lead to (multiple) organ failure and ultimately death. Images are made with the help of Servier Medical Art.

The coagulation system

Coagulation is the naturally occurring biological process of blood clot formation [58]. This process is tightly regulated in order to maintain a hemostatic balance, which prevents excessive clotting on the one hand (thrombosis) and bleeding on the other (hemorrhage).

The coagulation system can be initiated via two distinct pathways: the contact activation (or intrinsic) pathway and the tissue factor (TF) (or extrinsic) pathway [58]. Whilst the intrinsic pathway starts by the activation of coagulation factor XII (FXII) and the extrinsic pathway by TF exposure to the blood due to vessel injury, both pathways result in a sequential activation of coagulation factors. Ultimately, these pathways merge at the formation of activated coagulation factor X (FXa) to progress into the common coagulation pathway leading to the conversion of prothrombin into thrombin (IIa). As the key enzyme of the coagulation cascade, thrombin catalyzes the enzymatic cleavage of fibrinogen into fibrin and is therefore crucial in the formation of a stable blood clot. Thrombin further enhances coagulation via a positive feedback mechanism through activation of FXI, FVIII and FV, as well as platelet activation via protease-activated receptors (PARs) [59]. Activated platelets provide a phospholipid surface for the generation of FXa by FVIIIa and FIXa, and the generation of thrombin by FVa and FXa [60]. In addition, they can release procoagulant factors from their granules [61]. Besides their interaction with coagulation factors, platelets are involved in the initial formation of a blood clot by forming a platelet plug at the site of injury after which the coagulation factors come into play and form a stabilizing fibrin network [62,63].

Excessive clotting is prevented by endogenous anticoagulant systems which maintain the hemostatic balance by inhibiting thrombin formation. These include the activation of protein C (PC) via thrombin-thrombomodulin (TM), inactivation of FVIIIa and FVa by activated protein C (APC) and the inhibition of FX and thrombin by circulating serine protease inhibitors of which antithrombin (AT) is considered most relevant [58]. AT-mediated inhibition of thrombin and FXa is enhanced in the presence of heparin [64].

Histones in coagulation

Extracellular histones have been found to impair the PC-TM system [65]. Thrombin generation is enhanced dose-dependently by administration of exogenous histones *in vitro* and *in vivo* [65]. Also, histones were found to downregulate TM on vascular endothelial cells whilst upregulating TF, thereby enhancing thrombin formation [66] (Figure 2B). This upregulation of TF is regulated via TLR signaling and NF- κ B activation [67], and was also observed in human monocytes [68].

On the other hand, administration of recombinant TM (rTM) has been found to prevent histone-mediated lethal thrombosis in mice [69]. rTM has been approved for the treatment of patients with disseminated intravascular coagulation (DIC) in Japan, but it remains unclear how rTM exactly protects against histone toxicity. In DIC, microvascular thrombosis can lead to excessive consumption of coagulation factors and a subsequent increased risk of bleeding at other sites. Histone injection in mice can demonstrate these features by inducing thrombocytopenia, a prolonged prothrombin time, decreased fibrinogen levels, fibrin deposition in the microvasculature, and increased bleeding [70]. Extracellular histones also increase mechanical stability and fibrinolytic resistance of blood clots, delaying clot lysis [71]. In sepsis patients, in which DIC is frequently observed, histone H3 levels were found to negatively correlate with both AT and platelet counts [55], reflecting a hypocoagulable state after an initial activation of the coagulation system. Similar observations were made in trauma patients [72].

As well as their confirmed interaction with coagulation factors, histones can likewise affect platelet morphology. Extracellular histones induce both platelet aggregation and platelet activation [73,74] (Figure 2B). By binding to TLR2/-4, downstream signaling is triggered (e.g. ERK, p38, AKT and NF- κ B), as well as the influx of Ca^{2+} and the recruitment of fibrinogen. Histones also induce a procoagulant phenotype in human platelets by exposure of phosphatidylserine (PS) on the platelet surface, which results in accelerated blood clotting by generation of thrombin [75]. Notably, histone H4 is the most potent stimulator of platelet-dependent thrombin formation [73]. Direct injection of histones into mice resulted in an increase in plasma von Willebrand factor, contributing to platelet activation and development of deep venous thrombosis (DVT) [25,76,77]. Furthermore, platelet-deficient mice show a marked reduction in histone-induced cell death [70].

The microcirculation

The microcirculation comprises the small blood vessels of $<100\mu\text{m}$ in diameter (arterioles, capillaries and venules) and is involved in the exchange of oxygen and nutrients in tissues [78]. Important cell types of the microcirculation are the endothelial cells lining the vasculature, smooth muscle cells (predominantly in arterioles), red blood cells and leukocytes. These cells are involved in regulating vascular tone, blood flow, coagulation and immune function. Adequate microcirculatory function is therefore a prerequisite in maintaining proper organ function.

The glycocalyx has been described as another important regulator of the microcirculation. This layer covering the endothelial surface consists of glycosaminoglycans (GAGs) and associated proteoglycans, and provides a stabilizing barrier for the underlying

vasculature [79,80]. The glycocalyx attenuates blood cell-vessel wall interactions, possesses anticoagulant properties through the presence of e.g. TM and the endothelial protein receptor (EPCR), regulates signaling transduction to the endothelium and blocks leukocyte infiltration [80,81].

Dysregulation of the microcirculation, including a decreased microvascular density, oxygenation and glycocalyx thickness, is observed in pathological states such as sepsis [82]. Glycocalyx degradation is linked to increased oedema formation, enhanced leukocyte extravasation, and a decreased microcirculatory perfusion, leading to organ failure [83–85]. Observational studies in critically ill patients have shown an increase in circulating glycocalyx constituents (e.g. syndecan-1, hyaluronan), which correlate with disease severity and clinical outcome [86].

Histones and the glycocalyx

As histones are highly positively charged proteins and exert a variety of effects that involve the endothelium, the negatively charged glycocalyx is hypothesized to play an important role in the regulation of histone-mediated processes. In a pre-clinical rabbit model, a non-lethal dose of radiolabeled histones were injected and found to rapidly accumulate on heparan sulfate (HS) molecules in the lungs [87]. The authors demonstrated this charge-mediated interaction by testing several polycations competing with histones for binding to HS, and they showed that polyanions can form complexes with histones and neutralize them. This all resulted in diminished histone buildup in the lungs. The specificity of HS was studied by using heparinase III treatment, which specifically cleaves HS, and was found to degrade histone binding sites and abolish histone accumulation. It has been hypothesized that histones can displace AT from HS and disrupt the anticoagulant property of the glycocalyx, thereby inducing thrombosis. Interestingly, a study reported that mutant cells deficient in HS expression were more vulnerable for histone-induced cytotoxicity than cells expressing this GAG [88]. Circulating HS fragments were also found to inhibit histone-induced lung injury, which is partly due to direct binding to extracellular histones [89]. Other constituents of the glycocalyx, such as chondroitin sulfate [90] and high molecular weight hyaluronic acid [88], were found to protect against histone-induced cell death as well.

Several clinical studies have studied both histones and microcirculatory markers in clinical samples. In a study conducted in 39 sepsis patients, admission syndecan-1 levels were found to correlate with disease severity but not with histone H3 levels [91]. In another study performed in trauma patients, higher levels of syndecan-1 correlated with higher levels of histone-DNA complexes [92]. Additional studies should be performed to study

the potential association between histones and microcirculatory markers in various clinical settings. Also, the exact sequence of events involved in the interaction between the glycocalyx and histones and its possible implications warrants further investigation.

Models associated with tissue injury studied in this thesis

Machine preservation of ischemic donor kidneys

The shortage of donors and the limited supply of donor organs demand optimal use of the available donation pool. Besides living donation, organs from deceased donors are increasingly accepted for transplantation. Unfortunately, these organs inevitably suffer from ischemic damage due to increased time until organ procurement, potentially extended transportation to the recipients centre and thereby an overall prolonged transplantation procedure, leading to suboptimal organ transplants. The preferred method to preserve these donor kidneys is the use of hypothermic machine perfusion (HMP). It is considered to limit oxidative stress, ischemia and tissue damage, and improve graft survival and post-transplant graft function compared to cold storage [93–95]. HMP provides a continuous pulsatile flow through the kidney vasculature which can transport oxygen and nutrients to the tissue, and eliminate metabolic waste products. Especially vulnerable kidneys from old donors, donors with comorbidities and donors after circulatory death (DCD) can benefit from this preservation technique [96,97]. The increasing use of these marginal donor kidneys makes optimal organ preservation and selection even more important, underlining the need for efficient organ preservation methods and adequate pre-transplant viability tests.

Besides its benefits for transplant outcome, HMP at the same time provides an opportunity for testing kidney viability through the assessment of pump parameters and analysis of the circulating perfusate. Various studies have tested different metabolites in machine perfusate and found associations with the occurrence of delayed graft function (DGF) and primary non-function (PNF) [98]. We have tested the presence and role of histones in the prediction of short-term graft function and found that histone levels in the perfuse correlated positively with PNF, DGF and one-year graft survival [99], indicating that histones are potential biomarkers for kidney graft viability and transplantation outcome.

Sepsis

Sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection [100]. Sepsis is diagnosed if a patient has a suspected or proven clinical infection, accompanied by a Sequential Organ Failure Assessment (SOFA) score

of ≥ 2 . The SOFA score is based on different scores for the respiratory, cardiovascular, hepatic, coagulation and renal systems. The subcategory septic shock is characterized as sepsis accompanied by a lactate value of > 2 mmol/L and the use of vasopressors (e.g. norepinephrine) to maintain a mean arterial pressure of ≥ 65 mmHg. Current estimates indicate an incidence of over 30 million cases globally with accompanying mortality rates of up to 30% [101].

Despite advances in understanding the molecular mechanisms and processes occurring during sepsis, there is still no adequate treatment modality besides supportive therapy. Major contributing factors for this lack of adherence are the absence of sensitive biomarkers and the heterogeneity of disease.

Many different biomarkers have been proposed, but are lacking sensitivity and specificity in the diagnosis of sepsis, the prediction of its prognosis or their use to guide therapy [102]. Recently, it was proposed that within sepsis different subphenotypes [103] or genomic blood endotypes [104] can be present, resulting in variations in disease progression and therapeutic susceptibility. Personalized medicine based on robust and reliable (bio)markers or patient signature could therefore provide a potentially more responsive treatment in sepsis.

Anti-histone treatment strategies discussed in this thesis

Activated protein C (APC)

APC is an important anticoagulant enzyme involved in the regulation of thrombin formation. APC is the activated form of its inactive precursor PC. PC is a vitamin K-dependent glycoprotein of 62 kDa that can be found in plasma at an average concentration of 70 nM [105]. PC can be proteolytically activated by thrombin by cleavage at arginine(Arg)169, resulting in the formation of an activation peptide and of APC, which circulates in plasma at 40 pM [105]. The generation of this serine protease is enhanced by TM and EPCR, both cofactors of thrombin, and is calcium(Ca^{2+})-dependent [106–108].

APC can regulate the formation of thrombin by cleavage of the activated coagulation factors V (FVa) and VIII (FVIIIa). For this inactivation to occur, cofactors protein S and FV are needed to facilitate the proteolysis of FVa at Arg306, Arg506 and Arg679, and FVIIIa at Arg336, Arg562 and Arg740 [109–113]. In this way, the formation of thrombin is down-regulated which makes the PC anticoagulant pathway crucial for the maintenance of the hemostatic balance.

Besides its initially described role as an important anticoagulant protein, research has shown that APC also possesses cytoprotective properties [114–120]. Activation of PAR-1 and EPCR by APC leads to endothelial barrier stabilization, and reduces inflammation

and apoptosis [119,121–127]. PAR-1 was found to be a prerequisite in the benefits of APC in sepsis and stroke [128,129]. Another cytoprotective mechanism of APC is the cleavage of histones both *in vitro* and *in vivo* [25,54,124]. This cleavage results in a decreased cytotoxic potency of histones. In addition, APC can reduce thrombin generation mediated by histone-DNA complexes, inhibit histone-related platelet activation [74] and inhibit NET formation [130]. Endogenous APC levels have been found to inversely correlate to extracellular histone levels in sepsis [56], but the association between increased histone levels was abolished when APC levels were elevated [54].

Despite these promising effects, APC has not yet been successfully implemented in the clinic. Human recombinant wildtype APC was first approved by the Food and Drug Administration for the treatment of sepsis, but was withdrawn from the market in 2011 due to lack of efficacy in reducing mortality [131]. Later, it was proposed that this APC was not effective as it was not tailored to its use as a cytoprotective molecule and too many sepsis patients, with a broad variety of underlying pathological mechanisms, were included in the clinical study. The recombinant form of APC still possessed its anticoagulant properties and was therefore not optimized for e.g. receptor activation or histone binding. Molecular engineered APC variants with altered anticoagulant activity have been tested in pre-clinical studies of stroke [129,132–135], IRI [136,137] and endotoxemia [138], and are described in more detail in an earlier review from our group [139]. Ongoing research investigates novel engineered APC variants not only devoid of its anticoagulant properties, but also optimized for binding and/or cleaving of cellular receptors or extracellular histones. Modifying APC to reduce its anticoagulant properties on the one hand, whilst enhancing its histone neutralizing on the other hand, could result in an optimized treatment strategy in histone-mediated diseases without affecting a patient's bleeding tendency.

Heparin

Heparin is a linear, highly sulphated and negatively charged polysaccharide. This polysaccharide belongs to the class of GAGs and is found in mast cells predominantly in the liver, lung and intestines [140,141]. Heparin preparations are usually obtained from calf lungs or porcine intestinal mucosa, and consist of a heterogeneous mixture of polysaccharides of varying chain length [142]. The average molecular weight of unfractionated heparin (UFH) varies between 17 and 20 kDa, whilst fractionated low-molecular weight preparations (LMWH) have an average molecular weight of 3.6 till 6.5 kDa [142]. Initially used as treatment for DVT [143], this potent anticoagulant has been used in the clinic for decades in various coagulation disorders [144]. Heparin binding to AT results in a conformational change and the formation of a bridging complex that greatly accelerates the inhibition of the serine proteases thrombin and FXa [145]. For this inhibition to occur, heparin molecules should have a specific pentasaccharide sequence

(3,6 di-O-sulfated 2-N-sulfated glucosamine) and be > 5kDa in length [142]. For this reason, LMWH is less anticoagulant than UFH.

Besides its anticoagulant functions, heparin has anti-inflammatory [146] and anti-apoptotic effects [147]. Heparin is also able to neutralize histones, thereby reducing their cytotoxicity [144,148,149] as well as histone-related platelet activation [65,70,74]. More on the nonanticoagulant functions of this versatile biomolecule will be discussed in chapter 2.

Rendering heparin devoid of its anticoagulant properties would greatly improve safety issues regarding a patient's bleeding tendency. In our group, we have employed this strategy by fractionating UFH using affinity chromatography with immobilized AT [148]. The heparin that does not bind to AT possesses greatly diminished anticoagulant activity whilst it is still able to reduce histone-induced cell death in various models of inflammation.

Aims and outline of this thesis

In this thesis we investigate the role of extracellular histones in several models of tissue injury, as well as strategies to facilitate the neutralization of these toxic molecules.

In **chapter 2** we describe the functions of heparin beyond coagulation. This pleiotropic molecule has been recognized as a potent anticoagulant after its initial discovery but has many other biological functions that could be employed in the clinic. Of special interest is the charge-mediated neutralization of histones, which could benefit different types of patients.

Extracellular histones are linked to inflammation and tissue injury and have been found to be increased in several clinical pathologies. Therefore, we study in **chapter 3** the presence of these cytotoxic molecules in another example of a clinically relevant model of tissue injury: ischemic transplant kidney preservation. We investigate the correlation between circulating histone levels in preserved ischemic kidneys with their function after transplantation, studying the potential of the quantitation of histone in preservation fluid to serve as a pre-transplantation viability marker.

In the next chapter, **chapter 4**, we investigate the role of extracellular histones in transplant kidneys in more detail. For this, we make use of an *ex vivo* porcine kidney perfusion model in which we study the appearance of histones over time, in kidneys with different extents of ischemic damage. As heparin is able to neutralize histones both *in vitro* and *in vivo*, we employ this anti-histone treatment in these kidneys in order to study histone kinetics and its effect on graft viability.

Similar to heparin, APC can be modified to improve its nonanticoagulant functions. In **chapter 5** we aimed to investigate parameters of glycocalyx degradation in septic

patients upon admission to the intensive care unit (ICU). The microcirculation is studied by both circulating plasma markers as well as a novel, automated and non-invasive imaging technique. These markers will be correlated to clinical outcome and tested for their predictive value in predicting hospital mortality.

Chapter 6 describes the prediction and use of different *in silico* designed molecular variants of APC, with the aim to specifically improve APC's activity towards extracellular histones and thereby its cytoprotective effects. We will test both the anticoagulant and proteolytic effects of the APC molecules to characterize these novel variants *in vitro*.

Chapter 7 critically discusses all the findings described in this thesis and places it in a general context, relating it to available literature.

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Chapter 2

The anticoagulant and nonanticoagulant properties of heparin

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Submitted

Abstract

Heparins represent one of the most frequently used pharmacotherapeutics. Discovered around 1926, routine clinical anticoagulant use of heparin was initiated only after the publication of several seminal papers in the early 1970's by the group of Kakkar. It was shown that heparin prevents venous thromboembolism (VTE) and mortality from pulmonary embolism in patients after surgery. With the subsequent development of low molecular weight heparins (LMWH) and synthetic heparin derivatives, a family of related drugs was created that continues to prove its clinical value in thromboprophylaxis and in prevention of clotting in extracorporeal devices.

Fundamental and applied research has revealed a complex pharmacodynamic profile of heparins that goes beyond its anticoagulant use. Recognition of the complex multifaceted beneficial effects of heparin, underscore its therapeutic potential in various clinical situations. In this review we focus on the nonanticoagulant activities of heparin and, where possible, discuss the underlying molecular mechanisms that explain the diversity of heparin's biological actions.

Heparin structure and biosynthesis

Heparin is a biomolecule that belongs to the class of glycosaminoglycans (GAGs). GAGs exist as large linear polysaccharide structures, composed of repeated two-dimensional structural motifs. Besides heparin, the family of GAGs includes heparan sulfate (HS), dermatan sulfate (DS), chondroitin sulfate (CS), keratan sulfate (KS) and hyaluronan. Each of these GAGs is composed of different repeating disaccharide units and, with the exception of hyaluronan, is sulfated at various positions. These sulfations introduce functionally important negatively charged groups to the GAGs. Both heparin and HS are complex heterogeneous mixtures of repeating uronic acid and D-glucosamine/N-acetyl-D-glucosamine units, with heparin being relatively larger and containing relatively more sulfated groups than HS [1,2]. The biological functions of heparin are for a large part dependent on electrostatic interactions and the influence of sulfation on these functions have been well-described since decades [3].

Heparin biosynthesis occurs in mammalian cells through a process that involves many enzymatic steps. After an initial core tetrasaccharide linker is synthesized, it is transferred to a cell-type specific core protein. This core protein is serglycin proteoglycan in case of heparin, where the tetrasaccharide linker is attached to the side chain oxygen atom of a serine residue and thereby forms an O-linked glycan. The linker then serves as the template onto which heparosan, the precursor molecule of heparin (and of HS), is synthesized by sequential enzymatic transfer of defined saccharide units. These saccharide units subsequently undergo further processing, including sulfations, and will finally result in the mature heparin molecules that are found exclusively in mast cell granules. This unique localization is in contrast to the ubiquitously expressed HS, which exists as part of proteoglycans in the extracellular matrix (ECM). Through the random cleavage at glucuronic acid residues by endo- β -D-glucuronidase, a heterogeneous mixture of heparin molecules ranging from 5-25 kDa in molar weight is generated. Of the fully processed heparin molecules, only one in three is able to bind to antithrombin (AT) [4]. The pharmaceutical-grade heparin that is extracted from animal tissues (mostly porcine mucosa) and that contains the mixture of heparin molecules with varying molecular weights is called unfractionated heparin (UFH).

The ubiquitously expressed heparan sulfate proteoglycans (HSPGs) are also heterogeneous with only less than 5% of HSPG able to bind AT [4]. Despite this low percentage, the contribution of HSPGs to anticoagulant homeostasis is considered important due to their presence in the endothelial glycocalyx.

The majority of heparins currently used have been purified from porcine intestines and bovine lungs that are collected in the slaughterhouse. These tissues are rich in mast cells that form a first line defence against invading pathogens. Potential contamination of pharmaceutical heparin together with the vulnerability of the supply chain and increasing

demand has led to the consideration of other animal sources and bio-engineered heparin [5]. However, none of these alternative strategies has yet led to a large scale production of pharmaceutical-grade heparin.

Heparin and its derivatives

The discovery of the separability of heparins' anticoagulant effects towards coagulation factor Xa (FXa) and thrombin (see next section on anticoagulant properties of heparin), led to the development of shorter heparin variants with reduced anticoagulant activity and bleeding risk as compared to UFH. These heparins are obtained by fragmentation of UFH by chemical or enzymological means and are commonly described as low molecular weight heparins (LMWHs). Where UFH molecules range in size from 5 to 25 kDa, those for LMWH vary between 1 and 10 kDa [6]. Many different types of LMWH exist (e.g. FDA approved dalteparin, enoxaparin, tinzaparin) with more recently the generation of second generation LMWH or ultralow molecular weight heparins (ULMWH) such as bemiparin and semuloparin (reviewed in [7]). All these LMWH preparations express different activities towards FXa and have different pharmacokinetic profiles. Consequently, they have different applications in clinical practice.

The use of LMWH offers several advantages over UFH as it has a higher bioavailability, allows better predictable dosing and is associated with less adverse reactions [8]. As a result, LMWH has become the heparin of choice in many standard clinical situations such as VTE, major surgery and acute coronary syndrome [7,9,10]. UFH, however, remains preferred in situations such as prevention of coagulation in extracorporeal devices and in patients with renal failure due to better hepatic clearance and improved reversibility with protamine [8].

Considering the drawbacks of heparin isolated from animal origin, a synthetic anticoagulant with a high affinity towards AT was developed [11]. The compound in question, fondaparinux, comprises a specific "pentasaccharide sequence" and will be discussed in the next section in more detail (see anticoagulant properties of heparin). Fondaparinux is approved for the prevention and therapy of VTE after major surgery, and has been successfully applied in for instance myocardial infarction [12]. Likewise selective modified forms of heparins such as (de)sulfated, deacetylated or glycol-split heparins are being developed as alternative anticoagulants and are currently tested in pre-clinical and clinical studies for their application in particular settings [13].

Anticoagulant properties of heparin

Of all its functions, the anticoagulant action of heparin has been described best. It is based on a proposed combined template-based/allosteric mechanism for the interaction between the plasma serine proteinase inhibitor AT, and either thrombin or FXa. Although the inhibition of factors VIIa, IXa, XIa and kallikrein by AT is also stimulated by heparin, these will not be discussed here as these are regarded of lesser importance than the inhibition of FXa and thrombin.

Heparin facilitates the interaction between AT and thrombin (or FXa) by binding tightly with a specific site to AT. This site is known as the “pentasaccharide sequence”, consisting of a specific sequence of 5 highly sulfated sugar units in the heparin polymer [14]. Figure 1A shows an experimentally derived structure for the ternary complex composed of heparin binding both thrombin and AT (adapted from [15]). Moreover, AT undergoes a conformational change upon binding to heparin (Figure 1B). This change in itself makes AT about twice as reactive to thrombin and up to 100 times more reactive to FXa [16,17]. The remaining 100 – 1000 fold increase of activity is due to the fact that thrombin binds loosely and aspecifically to the remainder of the heparin chain and is then guided rapidly to the bound AT by one-dimensional diffusion along the heparin molecule. FXa-inactivation is subject to the same mechanism but binds 10 times less strongly than thrombin and is inactivated 3 times less efficiently [18].

The interaction between the complex of heparin and AT with FXa or thrombin is thus essentially different. FXa can interact with the pentasaccharide-AT complex as such, whereas inactivation of thrombin requires a “slide” of 17 sugar units at the non-reducing end of the pentasaccharide [19]. Given this requirement in sugar length for the inactivation of thrombin by the heparin-AT complex, the “slide” can only occur with UFH and not with LMWH [8].

Figure 1. The interaction of heparin with antithrombin (AT) and thrombin. Three-dimensional structure of the ternary complex between thrombin and AT, bound to heparin. (A) Shown are the surface structures of thrombin (pale yellow) and AT (blue) that are bound by a heparin fragment (as ball presentation in red and green). This structure represents the 2.5Å crystal structure as is deposited in the protein data bank by accession number 1TB6.pdb [15]. (B) Conformational change induced by the binding of the pentasaccharide. Left: crystal structure of monomeric AT (1F1T.pdb, in blue) in the non-bound state, with the reactive centre loop in grey and P1 residue Arg393 in yellow. Note the hinge region (as indicated by the dashed arrow) in a folded conformation. Right: upon binding of a pentasaccharide (as indicated by the ring structures in green and red) the hinge region assumes a more elongated conformation, causing the reactive centre loop (in grey) and the P1 residue (in yellow) to be exposed (from PDB coordination file 2GD4.pdb).

Heparin as a direct and indirect modifier of coagulation pathways

The registration and application of heparin as an anticoagulant has spurred the research into mechanistic studies to explain the beneficial effects observed with its clinical use. Interestingly, not only antithrombotic functions were recorded but also a wide variety of nonanticoagulant properties of heparin were elucidated. These nonanticoagulant properties include heparin-mediated modulation of adhesion molecules [20,21], heparin-induced release of endothelial-bound tissue factor pathway inhibitor (TFPI) [22], modulation of fibrinolytic activators such as tissue plasminogen activator [23], the binding

of chemokines and cytokines [24,25], and more recently the neutralization of damage-associated molecular patterns (DAMPS) [26]. Hence, heparin expresses anticoagulant and nonanticoagulant activities (Figure 2) that collectively contribute to the therapeutic effects observed in the wide-range of clinical applications of heparin (Table 1).

Table 1. Physiological/pathological states and medical procedures for which heparin is being applied. Heparin is a multifunctional biomolecule which is used in many different types of medical applications and for various physiological and pathological states. Some examples of heparin's targets and applications, as extracted from references throughout this text, are given.

Targets and applications of heparin			
Acute myocardial infarction	Deep Vein Thrombosis	Ischemic stroke	Surgery
Alzheimer's Disease	Drug delivery	Pregnancy	Thrombotic stroke
Atrial Fibrillation	Infectious disease	Pulmonary Embolism	Transplantation
Cancer/Metastasis	Inflammatory Bowel Disease	Sepsis	Regenerative medicine

It should be mentioned that also procoagulant/antifibrinolytic properties of heparin have been observed. These include the stimulation of activated protein C (APC) inhibition by protein C (PC) inhibitor [27], the attenuation of fibrinolytic pathways [28], the altered inactivation of coagulation factor Va [29], and the stimulation of clotting of FXa initiated coagulation of plasma [4]. The latter two properties, however, have been tested in the absence of AT. Such condition may occur in individuals with a consumptive coagulopathy. Treating individuals with heparin may cause depletion of AT [30]. Further, of interest here are i) the observed acceleration of thrombin formation that occurs through inhibition of TFPI function by the nonanticoagulant proteoglycan AV513 [31], an effect which promotes the activation of coagulation factor V [4] and ii) the procoagulant properties of polyphosphate, a linear polymer of inorganic phosphate [32].

Figure 2. Heparin-ligand interactions in physiological and pathological states. Schematic illustration of the polypharmacological properties of heparin in cancer/metastasis (green), coagulation (red), cellular homeostasis (blue), the immune system (yellow) or properties able to affect multiple of these processes. Binding of heparin to a plethora of heparin binding biomolecules may result in alterations of important physiological processes and affect physiological states. (A)PC = (activated) protein C, AT=antithrombin, DAMPs = damage-associated molecular patterns, ECs = endothelial cells, GF = growth factor, PAR-1 = protease activated receptor-1, SMC = smooth muscle cell, TFPI = tissue factor pathway inhibitor.

Nonanticoagulant properties of heparin

Though heparin was discovered and developed as an anticoagulant, researchers realized that roughly 70% of the heparin molecules in UFH did not bind to AT [33–35], a fraction which was termed “inactive heparin” [33] or “low affinity material” [34,35]. Hence, over the years patients receiving UFH have been injected also with “inactive” heparin molecules. Given the low frequency and accepted minor side effects of UFH use, the “inactive” heparin molecules may have contributed positively to the overall therapeutic effects of UFH.

Indeed, it has been realized that the earlier termed “inactive” fraction of heparin contains activities that appear to be tissue protective. In those clinical situations where

use of heparin as an anticoagulant is indicated, tissue damage through ischemia and reperfusion is frequent. Therefore, in retrospect nonanticoagulant beneficial properties of heparin may well have contributed to the success of heparin.

Acknowledging the observed nonanticoagulant properties of heparin, both UFH and LMWH have been applied for several other applications besides their use as antithrombotic therapeutics [36,37]. These include applications such as surface coating of biomedical devices, treatment of haemodynamic disorders, modulation of growth factors, and serving as an adjunct to chemotherapeutic and anti-inflammatory drugs [38]. Currently there are nearly 250 different manually reviewed entries in the Swiss-Prot database for proteins that bind heparin and which biological properties can be modulated by heparin. Modulating effects of heparin not necessarily depend on its anticoagulant activity and an increasing number of described effects can be contributed to heparin's nonanticoagulant properties. The following sections provide an overview of the most studied nonanticoagulant properties of heparin, supported, if possible, by molecular mechanisms that explain the observed effects of heparin.

Growth factor modification

Binding of growth factors to heparins has already been described in the 1980's. In fact, the preparation of purified growth factors can proceed efficiently through the use of affinity chromatography using immobilized heparin [39].

Several observations may help to explain the effects that heparins have on growth factor function and availability. HSPG-bound fibroblast growth factor-2 (FGF-2) was shown to enter cells via non-clathrin mediated lipid raft dependent pathways, thus preventing the FGF-2 to be directed towards the lysosomes [40]. Notably, heparin needs charged molecules to be able to translocate to the cell nucleus and compete with DNA for binding to growth factors [41]. It is known that many growth factors (e.g. VEGF, FGF, PDGF) bind to GAGs present on the cell surface and in the ECM where they are deposited in the basement membrane [42]. As such, these growth factors are protected from proteolytic inactivation or physical denaturation [43,44]. At the same time, they can reside in the endothelial GAG layer and serve as a pool of ready-to-use growth factors that can for instance respond to vascular damage. When the GAG chains are broken down by physiological or pathological stimuli, the bound growth factors are released and may bind (likely in complex with the released GAG) to their respective cellular receptors [45].

Since heparin can interact directly with both growth factors and their respective receptors, it acts as a bridging molecule which can mediate the formation of high affinity growth factor-receptor complexes. Addition of exogenous heparin may thus compete with the cell-bound GAGs for binding to the immobilized growth factor pool and stabilize these growth factor-receptor complexes. This stabilization by heparin can

result in signaling events and has been observed for heparin sequences exceeding an octamer polysaccharide length [46,47]. Interestingly, for the promotion of VEGF activity, soluble heparin with a chain length of >22 saccharides is required, whilst a chain length of <18 sugar units results in the inhibition of VEGF binding to its receptor [48]. Therefore, LMWH appears to be a potent inhibitor of both angiogenesis and fibrosis [48,49].

In recent years, novel applications of heparin-growth factor modifications have been investigated in the field of nano- and regenerative medicine, see e.g. [50]. Heparin-tailored biomaterials/scaffolds such as hydrogels, nanoparticles, polymers and liposomes, have been studied in tissue engineering and drug delivery applications. Using high affinity delivery systems, heparin, growth factors and/or drugs can site-specifically be delivered to the tissue of interest and exert specific effects.

Anti-metastatic properties of heparin

Use of heparin as a potential chemotherapeutic adjunct has been under investigation for many years.

Interest in the anti-metastatic properties of heparin was sparked by a number of clinical observations indicating that cancer patients who were treated with heparin or heparin-derivatives for cancer-associated thromboembolic disease appeared to have prolonged survival [51,52]. Several pre-clinical studies investigating predominantly melanoma or mammary carcinoma tumors aimed to elaborate on the anti-cancer and anti-metastatic properties of heparin (reviewed in [53]).

Besides heparin's anti-FXa and anti-thrombin effects, an additional anticoagulant mechanism suggested to be involved in metastatic mechanisms is the induction of TFPI release from cells [22,54], a phenomenon that is observed in cancer patients receiving heparin [55]. TFPI exhibits antiangiogenic and antimetastatic effects *in vitro* and *in vivo* and its release is induced by both anticoagulant and nonanticoagulant LMWH [56]. Furthermore, heparin is thought to downregulate the expression and activity of tissue factor through modulation of growth factor receptor-mediated activation of nuclear factor-kappaB (NF- κ B) [57]. Another potential anti-metastatic mechanism of heparin is hypothesized to be the inhibition of fibrin deposition around tumour cells, which lifts the protection from cancer cells against immune cell attack [58].

Besides these anticoagulant mechanisms through which heparin is able to exert anti-cancer properties, nonanticoagulant mechanisms are assumed to be involved in these effects as well. One of the contributing nonanticoagulant activities was proposed to be the modulation of selectin activity (see section on anti-inflammatory properties). Heparin is known to block P- and L-selectin binding to natural and tumour cells ligands [59]. This implies that heparin is able to modify selectin-mediated events, thereby influencing cell-cell interactions that drive tissue growth and differentiation. Interestingly, there appears

to be specificity in the effects that glycans have on selectin binding, with e.g. heparin and HS showing differential interaction with selectins [60]. UFH and LMWH were shown to inhibit metastasis of carcinoma cells to an extent comparable to that of P-selectin deficient cells [53,61,62]. It is however difficult to describe this selectin-blocking heparin property as being specifically anti-metastatic since the exact mechanisms still remain under investigation [63,64].

Modulation of chemokine-chemokine receptor interactions constitutes another action of heparin affecting cell migration and metastasis, as was shown for the chemokine receptor CXCR4 and its ligand CXCL12 through *in vitro* studies [65]. Also the interaction of the integrin VLA-4 with its ligand VCAM-1 was found to be inhibited by heparin, reducing melanoma cell adhesion and experimental melanoma metastasis [66]. CXCR1/2 and CXCL8 are other potential targets for heparin [67]. Heparanase is an endoglycosidase that degrades HS in the ECM. Inhibition of heparanase activity by heparin was shown to inhibit tumour invasion and metastasis [68]. The inhibition is abolished by total desulfation of heparin [69]. Other ways through which heparin is thought to exert anti-cancer effects are the direct induction of apoptosis of carcinoma cells through cell cycle arrest [70] and the enhancement of tumor cell sensitivity to chemotherapy by increasing drug uptake and reversing the downregulation of tumour suppressor genes [71–73]. The Wnt signaling pathway is thought to be involved in this latter process [72].

In view of the close interplay between the haemostatic system and those mechanisms that underlie processes of cancer cell growth and metastasis, it is likely that observed therapeutic effects of heparin in cancer patients can be attributed to multiple effects of the drug. It appears that prescription of heparin (mainly LMWH or UFH) in cancer patients without associated thrombosis results in increased survival, in the absence of an increased bleeding risk, as was recently shown by means of a meta-analysis for particularly small cell lung cancer patients [74]. It should be noted however, that a recent multicentre randomized trial evaluating the prophylactic use of LMWH in >2000 lung cancer patients found no difference in overall or metastasis-free survival between heparin-treated and untreated groups, while there was an increase in clinically relevant non-major bleeding events [75]. Other trials indicated that patients with a “better prognosis” might benefit most from heparin treatment [76–78]. Of note, the aforementioned studies have used registered anticoagulant heparin formulations and it has been observed before that the anti-metastatic properties do not depend on the anticoagulant functions of heparin [79,80]. Potentially contributing to this is the activity of heparin cofactor II, a plasma serpin with anticoagulant activities, which is known to act through both pentasaccharide-containing heparin (anticoagulant heparin) as well as through nonanticoagulant heparin.

Via both *in vitro* study and animal studies it was shown that heparin cofactor II enhances metastasis in non-small cell lung cancer, which supported an observed correlation between

high pre-treatment plasma levels of heparin cofactor II and a reduced survival [81]. As such, nonanticoagulant heparins are being probed for their usefulness as adjuncts in preclinical antimetastatic models. Recent data suggest that such heparin derivatives could indeed provide the sought after anti-cancer effects, without affecting the coagulation system [80,82]. The fact that these heparin forms can be dosed to higher concentrations without risking bleeding in patients, may prove beneficial to their potential application.

Also, the effects of heparin on the inhibition of metastasis were not observed for fondaparinux, see e.g. [61], suggesting that the earlier listed anticoagulant properties are not per se the most significant contribution to the overall activity. Likewise, the mentioned inhibitory interaction of heparins with selectins is independent of the anticoagulant function of heparin and was described also for chemically modified forms of heparin *in vitro* [83]. As the haemostatic system is involved in cancer signaling, it remains to be proven what the true therapeutic value of inclusion of nonanticoagulant heparin in cancer treatment is and what the best treatment strategy is in current clinical practice. Hence, the possibility that different types of heparin have different therapeutic effects in various types of cancer should be considered. Therefore, clinical studies are needed that are designed to determine anti-cancer effects of a specified heparin type in a specified patient population.

Anti-inflammatory properties of heparin

Given the fact that endogenously produced heparin is stored in the granules of mast cells, it may not be surprising that pharmaceutical grade heparin has immunomodulatory properties. Mast cells perform a major role in inflammatory and allergic diseases as they contribute to increased vascular permeability and to allergic and anaphylactic reactions. It has been reported that heparin from mast-cells has pro-inflammatory properties via the stimulation of bradykinin [84]. However, endogenous heparin-like GAGs present on the endothelial cell surface and administered heparin appear to have quite the opposite effect. Patients with pathologies having a distinct immunological component seem to benefit from administration of heparin because of its anti-inflammatory properties, even though the evidence is not always equally convincing. These pathologies include conditions such as asthma [85], allergic rhinitis [86], inflammatory bowel disease [87], ocular disorders [88], cystic fibrosis [89] and burns [90]. Also in several cardiovascular conditions with a clear inflammatory component such as acute coronary syndrome [91], cardiopulmonary bypass [92] and thrombophlebitis [93], the use of heparin is beneficial. In organ preservation and transplantation, in which processes of both ischemia and reperfusion are apparent, heparin is used to reduce vascular thrombosis and ischemia-reperfusion injury [94,95]. However, the exact benefit or risk of heparin treatment during different stages of these processes is still a subject of debate.

Figure 3. The anti-inflammatory nature of heparin-ligand interactions. Illustration of the anti-inflammatory polypharmacology of heparin. Besides its use as anticoagulant, the anti-inflammatory properties of heparin are widely recognized. These properties depend on the overall effect of heparin affecting many different ligands. These ligands can be either found in plasma, are surface-bound or are present in the intracellular compartment.

The mechanisms by which heparin and its derivatives are able to express their anti-inflammatory properties reflect their multifaceted effects in biological processes. The anti-inflammatory properties can be roughly divided into 2 modes of action (Figure 3): (1) modulation through binding to soluble plasma ligands and (2) modulation through binding to cell surface bound receptors or macromolecules, with potential effects on downstream signaling pathways. In this way, heparin is able to interfere with several (if not all) stages of leukocyte transmigration and extravasation into the target tissue.

Modulation through binding to soluble plasma ligands

Heparin is known to interact with a wide variety of ligands, in particular proteins, and these interactions confer upon heparin its anti-inflammatory functions. Through binding of inflammatory mediators and enzymes, heparin can inhibit activation of inflammatory cells and subsequent propagation of the inflammatory response and tissue damage [96]. Examples of such ligands are complement proteins which have been described to express changed functional properties upon heparin binding [97]. Heparin interferes with both

the classical and alternative complement pathway by binding and inhibiting the formation of several complement factors (e.g. active C1 complex, C3 convertase) as well as the membrane attack complex (MAC), interfering with terminal cell lysis [98].

Likewise, pro-inflammatory molecules like chemokines and cytokines are able to bind to heparin, in which electrostatic forces and interaction sites seem to determine the relative affinity towards a particular molecule [99,100]. Binding of cytokines to cell surface GAGs and mast-cell derived heparins at the site of inflammation result in the protection of cytokines against proteolytic inactivation and rapid clearance from the circulation. Given that these cytokines and chemokines exert their pro-inflammatory functions through receptor-mediated events, the administration of exogenous heparin will result in competitive binding between these mediators and either the endogenous GAGs/heparins or the administered heparin. This can for instance be observed during an acute phase reaction, in which heparin binding proteins are elevated and can bind to administered heparin [101].

Modulation through binding to cell surface bound receptors or macromolecules

Several studies have shown that heparin is able to modify interactions between cells. Heparin binding to cell adhesion molecules and not heparin-induced altered expression of adhesion molecules appears to mediate these effects [59,60,109]. Particularly, the inhibition of interactions between endothelial cells and blood cells (e.g. leukocytes, platelets) by heparin results in a more anti-inflammatory state.

Heparin reduces tumor necrosis factor-alpha (TNF- α) induced leukocyte rolling *in vivo* [110]. This effect is likely caused by the binding of heparin to P-selectin that is present on the surface of activated endothelial cells and activated platelets [111]. Heparin also binds L-selectin on the surface of leukocytes and has anti-inflammatory activity *in vivo* [112]. HS that is present on activated endothelial cells is known to be of importance for the adherence and rolling of leukocytes to the endothelium, and exogenously added heparins can interfere with this interaction [113,114].

Interestingly, despite its structural similarity to P- and L-selectin, E-selectin does not bind heparin [60]. This difference relies on two specific amino acid residues in the EGF-like domain, meaning that E-selectin would be able to bind heparin if these are altered [115]. The reported downregulation of E-selectin and other adhesion molecules in heparin-treated endothelial cells is thought to occur (in part) through NF- κ B inhibition [116].

Intercellular adhesion molecule 1 (ICAM-1), a cell adhesion molecule of the immunoglobulin superfamily of proteins which binds strongly to the integrins CD11a/CD18 or CD11b/CD18, is present in the membranes of both endothelial cells and leukocytes. ICAM-1 interactions are important for the maintenance of the barrier function of endothelial tissue and for leukocyte endothelial transmigration. Heparin was shown to

attenuate ICAM-1 mediated interactions by binding to this adhesion molecule [117] and to suppress increased ICAM-1 expression during endothelial cell activation by reducing gene expression [118]. Platelet endothelial cell adhesion molecule or PECAM-1, expressed on the surface of platelets and immune cells, which is involved in transmigration of immune cells, is thought to bind to heparin in preferable mild acidosis conditions [119,120]. Also neuronal cell adhesion molecule (NCAM) [121] and the integrin macrophage-1 antigen (Mac-1) [122] have been shown to bind heparin. Mac-1 is present on the surface of several immune cells, including neutrophils, macrophages and NK cells [123]. This pattern recognition receptor consists of protein subunits CD11b/CD18 and binds to a number of ligands including the complement proteins iC3b and C4b and several bacterial surface epitopes. Heparin binding to Mac-1 was shown to inhibit the binding of Mac-1 ligands, resulting in modulation of inflammation and cell proliferation [124].

Heparin was also shown to influence interaction of viruses with cells, as heparin can compete with several types of viruses for binding to HS on the cell surface. This attenuates the interaction between the virus and its target cells, which can result in inhibition of infection, see e.g. [125–127].

The above mentioned receptor-mediated anti-inflammatory effects of heparin can be considered as in direct contrast to the effects resulting from the heparin mediated inhibition of thrombin and FXa. Through the enhancement of inhibition of thrombin/FXa activity *in vivo*, heparin thus has a twofold indirect anti-inflammatory property since it is known to dampen the procoagulant responses of endothelial cells and platelets by limiting their activation by coagulation factors. In addition it was shown that both heparin and low anticoagulant heparin act in a PAR-1 dependent manner through a mechanism that does not depend on thrombin binding and can thus directly interfere with cell signaling [128].

In addition to endothelial cells heparin also targets vascular smooth muscle cells (VSMCs) and modulates their biology. Heparin induces switching from VSMC synthetic to contractile phenotype [129] and inhibits VSMC proliferation [130] by interfering with cell cycle progression [131]. This property of heparin can be employed in stent-technology to prepare coatings that control neointimal cell growth [132]. The modulation of VSMCs appears to depend neither on the anionic charge [133] nor on the anticoagulant properties of heparin [134].

Many experimental and clinical studies have illustrated in a more integrative approach the overall beneficial *in vivo* effects of heparin in inflammation. More recent studies confirmed that both UFH and LMWH reduce neutrophil sequestration and lung permeability with concomitant tissue protection in a rat model of LPS-induced acute lung injury [135,136]. The protective effect was likely independent of heparin's anticoagulant properties since heparins devoid of anticoagulant activity reduce neutrophil infiltration and protect lungs in a mouse model of LPS-induced sepsis [26].

Finally, heparin acts positively on vascular endothelium by restoring glycocalyx function after inflammation-induced glycocalyx shedding [137,138]. It is hypothesized that heparin can replenish the cell surface proteoglycan network by mobilising an intercellular pool of syndecan-1 [139] and that heparin can take over partly the functions of syndecan-1 [140].

As discussed above, heparin can influence intracellular signaling through modulating receptor-ligand interactions. Heparin was shown to inhibit p38 MAPK and NF- κ B activation in an *in vitro* LPS-induced inflammatory response on endothelial cells [141] as well as in an *in vivo* experimental model of LPS-induced inflammation [136]. Likewise, heparin was able to alleviate endothelial barrier dysfunction through regulation of p38 MAPK in an inflammatory model induced by the DAMP high mobility group box 1 (HMGB1) [142]. Whilst some report heparin does not affect the translocation of NF- κ B into the nucleus [143], others indicate it does and is hypothesized to compete with DNA for NF- κ B binding sites [141,144]. Another process in which heparin likely acts through NF- κ B is the decreased degranulation of mast cells and other immune cells after exogenous heparin administration. This decrease subsequently leads to a reduction in lysosomal enzyme activation and reactive oxygen species (ROS) generation [145]. As heparin was found to be an antioxidant, heparin is possibly exerting its effects through NF- κ B given the crosstalk between both ROS and NF- κ B [146].

An important indication of heparin's anti-inflammatory activities in the clinical setting is provided by recently published meta-analyses interrogating the effect of heparin use in patients with sepsis [147–149]. The general outcome of these analyses is that the use of heparin and LMWH results in reduced 28-day mortality in these critically ill patients, despite the recording of an increased number of bleeding events. Septic patients often have a compromised coagulation system. This awareness has prevented a more widespread clinical use of heparin as an anti-inflammatory agent in sepsis [150]. Since a significant part of heparin's anti-inflammatory actions does not depend on anticoagulant activity it is hypothesized that heparins with low-anticoagulant activity have beneficial therapeutic effects without increasing the risk of bleeding in patients with sepsis. Clinical trials are needed to address this hypothesis.

Conclusions and outlook

Discovered about a century ago as a water soluble anticoagulant obtained from animal tissues, heparin has ever since been used in the clinic because of its anticoagulant properties. Clinical use became widespread after Kakkar showed that systematic administration after surgical interventions dramatically decreased postoperative thrombosis with acceptable bleeding risk. Research over the past decades has unveiled diverse nonanticoagulant

properties of heparin that potentially have therapeutic value in a variety of diseases with inflammatory components. UFH is a mixture of polysaccharides with variable sugar compositions and molecular weights. So far it has not been demonstrated that all the polysaccharides in the mixture contribute to the nonanticoagulant properties. Further research is required to delineate the structure-function relationship of the various nonanticoagulant properties, in stark contrast to the structure-function knowledge available in the anticoagulant properties of heparin. Nevertheless, the anticoagulant property of heparin will impact its use in nonanticoagulant applications by limiting the dose due to bleeding risk. One strategy to exploit fully the nonanticoagulant properties therapeutically encompasses the formulation of heparins that have no or limited anticoagulant activity.

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Chapter 3

Presence of cytotoxic extracellular histones in machine perfusate of donation after circulatory death kidneys

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Abstract

Extracellular histones are cytotoxic molecules that are related to cell stress and death. They have been shown to play a crucial role in multiple pathophysiologic processes like sepsis, inflammation, vascular dysfunction and thrombosis. Their role in organ donation and graft function and survival is still unknown. The aim of this study was to assess whether an association exists between the presence of extracellular histones in machine perfusates and deceased donor kidney viability. Machine perfusates of 390 donation after circulatory death kidneys were analysed for histone concentration and corresponding graft function and survival were assessed.

Extracellular histone concentrations were significantly higher in perfusates of kidneys with post-transplant graft dysfunction (primary non-function (PNF) and delayed graft function (DGF)) and were an independent risk factor for DGF (OR=2.152 (1.199–3.863)) and one-year graft failure (HR=1.386 (1.037–1.853)), but not for PNF (OR=1.342 (0.900–2.002)). One-year graft survival was 12% higher in the group with low histone concentrations ($p=.008$) as compared to the group that contained higher histone concentrations.

This study warrants future studies to probe for a possible role of cytotoxic extracellular histones in organ viability and suggests that quantitation of extracellular histones might contribute to assessment of post-transplant graft function and survival.

Introduction

The use of hypothermic machine perfusion (HMP) to preserve deceased donor kidneys continues to increase [1–3], likely due to the general consideration that HMP limits the effects of ischemia to the tissue [4,5]. HMP performs superior to static cold storage in terms of graft survival and post-transplant graft function [6–8]. It is assumed that especially high-risk donor kidneys, i.e. kidneys from old donors, donors with increased morbidity and donors after circulatory death (DCD), may benefit from this preservation technique [1,3,9,10]. In addition to improved transplant outcome, HMP provides an opportunity to easily perform viability tests on donor kidneys by measurement of pump parameters or analysis of machine perfusate [11–13].

Machine perfusate metabolites and other components have been studied extensively over the last decades, but controversy exists regarding the value of such analyses to assess kidney quality. Several studies have shown the increased presence of a number of different metabolites in machine perfusates and associations with higher rates of delayed graft function (DGF) or primary non-function (PNF) were made [1,11–14]. However, none of these individual molecules at present provides conclusive predictive value for short-term graft function.

Recent insights into cellular mechanisms underlying microcirculatory stress and, consequently, organ failure point towards a critical role of (extracellular) histones [15,16]. Since extracellular histones H2B, H3 and H4 are cytotoxic and cause endothelial damage [17–23], we hypothesized that extracellular histones in the machine perfusate reflect cell stress and damage (Figure 1) and the activation of an amplifying cascade of histone release and endothelial damage which together ultimately contribute to transplant failure. The aim of this study was to assess whether an association exists between the presence of extracellular histones in machine perfusates and kidney viability. We measured histone H3 levels in a large group (n=390) of unselected machine perfused DCD kidneys and revealed that histone H3 levels correlated positively with DGF and one-year graft failure.

Material and Methods

Donor, graft and recipient data

Between January 1997 and March 2013, all DCD kidneys that were preserved using hypothermic pulsatile machine perfusion in our medical center, for at least four hours, were used for analysis. This included kidneys procured and transplanted in our Centre or elsewhere within the Eurotransplant region. Donor data were routinely registered and analysed for donor gender, age (years), most recent serum creatinine ($\mu\text{mol/L}$), cause of death (cerebrovascular, cardiac, respiratory, trauma, suicide, other) and DCD Maastricht category (1, 2, 3 or 4).

Figure 1. Top half: Histones H2A (orange) and H2B (yellow) form a dimer. Histones H3 (blue) and H4 (green) also form a dimer, which associates with another H3-H4 dimer to form a H3-H4 tetramer. All combined, a histone octamer is formed, which serves as the core structure of a nucleosome. DNA is wrapped around these histone cores in order to package the genome inside the nucleus. Lower half: A healthy renal cell contains a nucleus with nucleosomes. After ischemic injury the renal cell may die and release histones into the extracellular space.

The following graft characteristics were recorded: left or right kidney, type of machine perfusion (Gambro (Gambro, Lund, Sweden) or Lifeport (Lifeport Kidney Transporter, model no. LKT-100-P; Organ Recovery Systems, Des Plaines, IL), duration of machine perfusion time, WIT (defined as time of death, as witnessed by an absent circulation, until time of cold perfusion), cold ischemic time (CIT, defined as the period between start of cold perfusion until start of first anastomosis) and anastomosis time.

Recipient characteristics and follow-up data were recorded and retrieved: recipient gender, age (years), primary kidney disease (renovascular or other), dialysis type (no, hemo- or peritoneal dialysis), years of dialysis, number of previous transplants, total number of HLA mismatches (including HLA-A, HLA-B and HLA-DR; scoring 0-6) and immunosuppressive regimen. The immunosuppressive regimen changed during the study period, but mainly included use of a calcineurin inhibitor (cyclosporine or tacrolimus) and prednisolone. This was combined with sirolimus, mycophenolate mofetil or azathioprine, depending on the protocol at the time. Dacluzimab was given to patients with increased immunologic risk if needed.

The collection and storage of patient data was performed in agreement with the “Code of Conduct for Health Research” by the Dutch Federation of Biomedical Scientific Societies (<http://www.federa.org/>).

Kidney procurement, preservation and machine perfusion

All kidneys were procured from DCD donors who included all types of DCD donors according to the Maastricht criteria (DCD 1-4) [24]. Perfusion was either performed by using a double-balloon triple-lumen (DBTL) catheter or by direct cannulation of the aorta, as previously described [25]. After nephrectomy, kidneys were placed on pulsatile perfusion machines. Before May 2007 the Gambro PF-3B perfusion machines (Gambro, Lund, Sweden) were used with 500 mL UW-MPS (Belzer MPS; Trans-Med, Elk River, MN) as perfusion solution. This machine was set with a systolic pressure of 55 mmHg for the first hour, after which flow was kept constant. As of June 2007, kidneys were placed on the Lifeport Kidney Transporter (model no. LKT-100-P; Organ Recovery Systems, Des Plaines, IL) with 1 L of Kidney Perfusion Solution 1 (KPS-1; Organ Recovery Systems) and perfused at 30-40 mmHg pressure. All kidneys were perfused at a mean temperature of 4 degrees Celcius (°C) and pH was adjusted when lower than 7.10. Perfusion characteristics, i.e. temperature, pressure, flow, renal resistance were recorded during perfusion and any abnormalities were registered.

Sample collection and histone H3 analysis

During machine perfusion, perfusate samples were taken at 1, 2 and 4 hours after start of perfusion, centrifuged, cooled and stored at -80°C. To ensure a balanced state of perfusion, the samples of 4 hours of perfusion were used for biochemical analysis.

Histone H3 levels were determined semi-quantitatively as previously described [26]. In brief, kidney perfusate samples were centrifuged for 4 minutes at 3000xg, subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hemel Hempstead, UK) by semi-dry blotting. After blocking for one hour in triethanolamine-buffered saline with Tween + 5% (w/v) nonfat dry milk (Bio-Rad Laboratories, Hemel Hempstead, UK), membranes were incubated with a rabbit polyclonal H3 antibody overnight at 4°C (1:1000, sc-8654-R, Santa Cruz Biotechnology, Heidelberg, Germany), followed by incubation with a biotin-conjugated goat anti-rabbit IgG antibody for 30 minutes at room temperature (1:1000, Vector Laboratories Burlingame, CA, USA) and incubation with StreptABComplex/AP solution (1:3000, Dako Cytonation, Glostrup, Denmark) for 30 minutes at room temperature. Protein bands were detected by BCIP/NBT (Sigma-Aldrich, St. Louis, MO, USA) and band densities were quantified by ImageQuant TL software (GE Healthcare, Little Chalfont, UK). Purified H3 (Roche, Basel, Switzerland) served as a reference to

calculate unknown H3 concentrations ($\mu\text{g/mL}$) in the perfusate by comparing the densities of all H3 specific bands in these samples with the density of known concentrations of purified H3.

Study outcome parameters

Immediate graft function (IGF), delayed graft function (DGF) and primary non-function (PNF) were scored as outcome measures for short-term graft function. DGF was scored if dialysis was needed in the first week after transplantation. PNF was scored if the graft never showed adequate function and dialysis was needed all through the follow-up period of the patient. Serum creatinine levels were analysed for all recipients at three and twelve months and up to five years each year after transplantation if possible.

Data analysis

Numerical variables are presented as mean $\pm$ standard deviations if approximately normally distributed, and as median (interquartile range (IQR) otherwise. Categorical variables are presented as number (percentage). Analyses were performed on all perfusates collected, irrespective of any pre-analytical variable. A p-value of 0.05 or less was considered to represent statistical significance. To allow comparison of determined concentrations between the Gambro and Lifeport perfusion systems, the relative 2-fold concentration in the former was taken into account and corrected for.

Comparison of histone H3 concentrations between groups based on graft function (PNF vs. DGF vs. IGF) or based on type of donor (uncontrolled vs. controlled) was performed using the Mann-Whitney test. Univariable and multivariable binary logistic regression analysis was used for assessment of the risk of PNF (vs. DGF or IGF) or of DGF (vs. IGF), where the effects are presented as Odds Ratio (OR) (95% confidence interval (CI)). Multivariable analysis included clinically important risk factors (i.e. WIT, CIT, anastomosis time, donor age and type of donor (uncontrolled vs. controlled), the latter was excluded in subgroup analysis for uncontrolled and controlled DCD kidneys). The discriminative capacity of histone H3 concentrations on graft function was assessed by presenting our data as receiver operating curves (ROC) from which the area under the ROC (AURC) (95% CI) was calculated. For multivariable predictive analysis, predictive probabilities were calculated with and without histone H3 concentrations and the added value of extracellular histones to the model was assessed. Correlations between histone H3 concentrations and WIT, donor creatinine, donor age and post-transplant serum creatinine were tested with a t-test for Spearman's Rho (r_s). Categorical comparison between groups (one- and five-year graft survival, histone H3 concentrations below or above the median) was performed using Pearson Chi-square test. Graft survival was censored for death, for all patients who died with a functioning graft. A Kaplan-Meier analysis was used to analyse graft survival

and differences between curves were assessed using log-rank tests. A Cox proportional hazards model was applied to calculate the hazard ratio (HR) of histone H3 concentrations at one- and five-year graft failure. The multivariable Cox proportional hazard model included aforementioned clinical factors.

Results

Patients

From January 1997 until March 2013, 439 transplanted DCD kidneys were machine perfused by our centre. After careful assessment of machine perfusion records, 10 kidneys were excluded from analysis because of significant leakage, obstruction or documented malperfusion. Next, 10 kidneys were transplanted outside of the Eurotransplant region, and for six kidney transplantations, follow-up data could not be obtained; of another six, machine perfusion records were missing or incomplete. For 17 kidneys machine perfusate was not available, because samples were used in previous studies or sample collection was wrongly performed at the time. A remaining total of 390 perfusate samples were included.

Donor, graft and patient characteristics are shown in Table 1. Of the 390 kidneys entering analysis, a majority of 270 (69%) were procured from DCD-3 donors. Median donor age was 49 (IQR, 38-58) years. After death, the median WIT was 25 (IQR, 19-31) minutes, median CIT was 25 (IQR, 21-29) hours and median anastomosis time 38 (IQR, 31-47) minutes.

Graft function

A majority of transplantations resulted in DGF: 228 of the analyzed 390 (58.5%). Kidneys developed PNF in 76 (19.5%) cases and IGF in 86 (22.1%) cases after transplantation. Median overall machine perfusate extracellular histone concentration was 0.65 (IQR, 0.37-0.95) $\mu\text{g/mL}$. Extracellular histone concentration (in $\mu\text{g/mL}$) was significantly higher in kidneys which developed PNF (median 0.73 (IQR, 0.44-1.00), $p < .001$) and DGF (median 0.70 (IQR, 0.43-0.98), $p < .001$) compared to IGF (median 0.42 (IQR, 0.07-0.78)) (Figure 2). The difference between the histone H3 concentration in the PNF as compared to the DGF group, did not reach significance ($p = .437$).

The risk of PNF was not significantly increased by elevation of histone H3 levels as assessed by univariable and multivariable regression analyses (OR=1.417 (95% CI, 0.974-2.063) and 1.342 (95% CI, 0.900-2.002), respectively). Furthermore, the discriminative power of histone H3 concentrations on development of PNF was weak in univariable analysis (AUC=0.573 (95% CI, 0.502-0.645)). This significance increased however after correction of the data for confounding clinical factors in the multivariable model

Figure 2. Histone concentration are significantly higher in PNF (median 0.73 (IQR, 0.44–1.00) $\mu\text{g/mL}$, $p < 0.001$) and DGF (median 0.70 (IQR, 0.43–0.98) $\mu\text{g/mL}$, $p < 0.001$) groups, compared to IGF (median 0.42 (IQR, 0.07–0.78) $\mu\text{g/mL}$). Boxplots are presented as median and quartiles, and whiskers include full range of values. *Indicates significant differences between groups ($p < 0.05$).

(AURC=0.647 (95% CI, 0.577–0.717)), with a minor added value of extracellular histones to the predictive model (AURC without histones = 0.640 (95% CI, 0.571–0.708)) (Figure 3).

We observed that, for development of DGF, histone H3 concentration was an independent risk factor in both univariable (OR=3.060 (95% CI, 1.694–5.526)) and multivariable analysis (OR=2.152 (95% CI, 1.199–3.863)). However, the predictive value of extracellular histone concentrations in perfusates on DGF was poor (AURC=0.674 (95% CI, 0.604–0.743)), but increased to fair after correction for confounding clinical factors (AURC=0.710 (95% CI, 0.639–0.780)), in which histone H3 concentration added minor value to the predictive model (AURC without histones = 0.689 (95% CI, 0.621–0.757)) (Figure 3).

Extracellular histone concentrations correlated with serum creatinine at three months after kidney transplantation ($r_s=0.115$; $p=.044$). Thereafter, at one, two, three, four and five years follow-up, this correlation was no longer significant ($r_s=0.111$, $p=.059$; 0.119, $p=.053$; 0.110, $p=.086$; 0.091, $p=.190$; 0.103, $p=.180$, respectively) (Table 2).

Table 1. Donor, graft and recipient characteristics are shown as median (interquartile range) if the variable is numerical and as number (percentage) if categorical.

Characteristics		Univariable PNF OR (95% CI)	Univariable DGF OR (95% CI)
Donor gender			
Female/Male	143 (37%)/247 (63%)	0.756 (0.443-1.291)	0.500 (0.302-0.828) *
Donor age (years)			
	49 (38-58)	1.017 (0.999-1.035)	1.014 (0.998-1.031)
Donor creatinine (μmol/L)			
	85 (63-123)	1.004 (0.997-1.101)	1.014 (1.006-1.022) *
Donor cause of death			
Cerebrovascular	125 (32%)	1.612 (0.961-2.705)	1.586 (0.895-2.809)
Cardiac	93 (24%)	1.513 (0.867-2.640)	2.714 (1.318-5.589) *
Respiratory	6 (2%)		
Trauma	110 (28%)	0.462 (0.243-0.878) *	0.383 (0.228-0.644) *
Suicide	11 (3%)		
Other	45 (12%)		
Donor Maastricht category			
1	9 (2.3%)		
2	102 (26.2%)	1.509 (0.876-2.597)	2.459 (1.253-4.826) *
3	270 (69.2%)	0.616 (0.366-1.037)	0.431 (0.231-0.803) *
4	9 (2.3%)		
<u>Graft</u>			
Left/Right			
	199 (51%) / 191 (49%)	0.731 (0.442-1.209)	1.012 (0.616-1.663)
Perfusion machine (Gambro/Lifeport)			
	320 (82%) / 70 (18%)	1.075 (0.554-2.085)	2.754 (1.517-4.998) *
Machine perfusion time (hours)			
	19 (15-24)	1.010 (0.970-1.051)	1.044 (1.002-1.087) *
WIT (min)			
	25 (19-31)	1.028 (1.004-1.051) *	1.021 (0.995-1.048)
CIT (hours)			
	25 (21-29)	1.007 (0.969-1.046)	1.065 (1.022-1.109) *
Anastomosis time (min)			
	38 (31-47)	1.010 (0.995-1.025)	1.014 (0.997-1.033)
<u>Perfusate</u>			
Extracellular histones (μg/mL)			
	0.65 (0.37-0.95)	1.417 (0.974-2.063)	3.060 (1.694-5.526) *
<u>Recipient</u>			
Recipient gender			
Female/ Male	145 (37%) / 245 (63%)	1.209 (0.724-2.017)	0.675 (0.407-1.122)
Recipient Age (years)			
	55 (44-63)	1.013 (0.992-1.034)	1.007 (0.988-1.026)
Recipient primary disease			
Renovascular	97 (25%)	1.196 (0.679-2.104)	0.984 (0.552-1.754)
Other than renovascular	244 (63%)	0.963 (0.574-1.613)	1.142 (0.686-1.901)
Unknown primary disease	49 (13%)	0.783 (0.351-1.748)	0.786 (0.386-1.600)
Dialysis time (years)			
	3 (1-4)	1.133 (1.024-1.255) *	1.143 (0.999-1.309)

Table 1 (continued). Donor, graft and recipient characteristics are shown as median (interquartile range) if the variable is numerical and as number (percentage) if categorical. (Continued)

Characteristics		Univariable PNF OR (95% CI)	Univariable DGF OR (95% CI)
Dialysis type			
Hemodialysis	236 (61%)	1.072 (0.640-1.794)	1.463 (0.886-2.416)
Peritoneal dialysis	147 (38%)	0.891 (0.529-1.501)	0.810 (0.489-1.344)
No dialysis	6 (1.5%)		
Unknown	1 (0.3%)		
Re-transplantation, yes/no	50 (13%) / 340 (87%)	0.762 (0.342-1.698)	1.241 (0.582-2.648)
Total HLA (A, B, DR) mismatches	3 (2-3)	0.892 (0.711-1.118)	0.878 (0.694-1.112)
Cyclosporine/Tacrolimus/none			
Cyclosporine	26 %	0.907 (0.494-1.663)	0.724 (0.401-1.308)
Tacrolimus	71 %	0.594 (0.344-1.027)	1.172 (0.648-2.119)
None	4 %		
Antimetabolite, yes/no	64 %	0.652 (0.384-1.109)	0.525 (0.285-0.966) *
Induction therapy, yes/no	11 %	1.066 (0.467-2.432)	1.143 (0.470-2.778)

Univariable regression analysis of the risk of PNF and DGF is shown in the 2 columns on the right. For variables with multiple categories, odds ratios (ORs) are calculated for single variable versus all other variables (yes/no). Odds ratios are marked with an asterisk if statistically significant ($p < 0.05$).

Graft survival

Median follow-up was 6.1 (IQR, 3.8-8.7) years. Overall, one-year death-censored graft survival was 77% and at five years this was 71%. Figure 4 shows Kaplan-Meier curves with a significantly better survival of kidneys with histone H3 concentrations below the median of 0.65 µg/mL than those that have levels of extracellular histones that are above the median (log-rank, $p = .026$). Analysis excluding kidneys with PNF did not result in a significant difference (log-rank, $p = .443$). One-year graft survival was significantly better for the group with a histone concentration below the median concentration than above (graft survival = 83% vs. 71%; $p = .008$, respectively). This difference persisted up to five years (graft survival = 76% vs. 65%; $p = .014$, respectively).

The risk of graft failure within one year significantly increased with increasing histone concentration (HR=1.411 (95% CI, 1.071-1.858)) in a univariable Cox regression model. Multivariable analysis also resulted in a significantly increase (HR=1.386 (95% CI, 1.037-1.853)). The risk of five-year graft failure was significantly increased with increasing histone concentration in the univariable analysis (HR= 1.335 (95% CI, 1.026-1.738)), but not in the multivariable analysis (HR=1.270 (95% CI, 0.960-1.680)). Predictive value on graft failure within one and five years was weak after univariable analyses (AURC=0.547 (95% CI, 0.479-0.614) and AURC=0.537 (95% CI, 0.477-0.597), respectively). Although the predictive value increased after correction for confounding clinical factors (AURC=0.637 (95% CI, 0.573-0.702) and AURC=0.648 (95% CI, 0.590-0.707), respectively), there was no added value

of extracellular histones to the model for one and five year graft failure (AURC without histones = 0.641 (95% CI, 0.577-0.703) and AURC without histones=0.649 (95% CI, 0.591-0.707), respectively).

Factors associated with increased extracellular histone concentrations

Extracellular histone concentrations weakly correlated to WIT ($r_s=0.190$, $p<.001$) and donor creatinine ($r_s=0.167$, $p<.001$). Histone concentrations did not correlate with donor age. The median histone H3 concentration in uncontrolled donors was significantly higher than in controlled donors (median (IQR) = 0.73 (0.52-1.1) $\mu\text{g/mL}$ ($n=111$) vs. 0.59 (0.28-0.92) $\mu\text{g/mL}$ ($n=279$); $p<.001$, respectively) (Table 2).

Table 2. Correlation of histone concentration to donor, graft and outcome factors

Factors compared to histone concentration	Spearman correlation coefficient (r_s)	P-value
Warm ischemic time	0.190	<.001*
Donor serum creatinine	0.167	.001*
Donor age	-0.018	.725
Three-month serum creatinine	0.115	.044*
One- year serum creatinine	0.111	.059
Two-year serum creatinine	0.119	.053
Three-year serum creatinine	0.110	.086
Four-year serum creatinine	0.091	.190
Five-year serum creatinine	0.103	.180

*Indicates significant differences between groups ($p<0.05$).

Controlled vs. uncontrolled DCD

Because there is a difference in histone concentration between uncontrolled (Maastricht category 1 and 2) and controlled (Maastricht category 3 and 4) DCD donors, we performed subgroup analyses.

Perfusates from uncontrolled DCD donor kidneys ($n=111$) showed no significant difference in histone H3 concentrations between the groups with PNF, DGF or IGF (PNF vs. DGF, $p=.884$; PNF vs. IGF, $p=.988$; DGF vs. IGF, $p=.929$). Furthermore, histone H3 concentration was not associated with an increased risk of graft dysfunction (PNF or DGF). Only in multivariable predictive analyses, histone H3 concentration was predictive for PNF (AURC=0.664 (95% CI, 0.551-0.777)) and DGF (AURC = 0.690 (95% CI, 0.546-0.833)) (Figure 3).

Controlled DCD perfusates ($n=279$) contained a significantly higher median (IQR) concentration of histone H3 in kidney with PNF (0.74 (0.42-0.96) $\mu\text{g/mL}$, $p<.001$) and DGF

Figure 3. The predictive value of extracellular histones for PNF and DGF, for all DCD kidneys and after subgroup analysis for controlled and uncontrolled DCD kidneys. Receiving operator curves are shown and AUC (95% CI) calculated by multivariable analysis, including clinically important risk factors

(0.63 (0.39-0.97) $\mu\text{g/mL}$, $p<.001$) compared to IGF perfusates (0.32 (0.02-0.67) $\mu\text{g/mL}$). For controlled DCD, we found that histone H3 concentration is an independent risk factor for DGF (univariable OR=3.071 (95% CI, 1.588-5.941) and multivariable OR=2.433 (95% CI, 1.253-4.723)); for PNF however, such significance could not be shown. Furthermore, the discriminative power for development of PNF and DGF of extracellular histone

levels in this subgroup is comparable to the entire group of perfusates (PNF univariable AURC=0.597 (95% CI, 0.512-0.683); PNF multivariable AURC=0.643 (95% CI, 0.559-0.726); DGF univariable AURC=0.683 (95% CI, 0.607-0.759) and DGF multivariable AURC=0.710 (95% CI, 0.631-0.788) (Figure 3).

One-year death-censored graft survival was 78% and at five years this was 72%. Comparing the groups with histone H3 concentrations above and below the median, Kaplan Meier analysis showed a significant (log rank, $p=.012$) difference in graft survival in favour of the lower concentrations. Notably, one- and five-year graft survival were significantly better in the group with lower concentrations (at one year 84% vs. 72% ($p=.019$) and at five years 79% vs. 66% ($p=.015$).

Figure 4. Kaplan-Meier graph of graft survival after transplantation. After one year, the group with histone H3 concentrations below the median had a 12% higher graft survival compared to group with histones H3 concentrations above the median ($p=.008$). Data was censored at the time of death for patients who died with a functioning graft.

Discussion

Many studies have sought to identify possible relations between measurable biomolecules and organ injury, post-transplant graft function or survival. It has been shown that kidneys release a plethora of biomolecules over time during machine perfusion. The release of these molecules appears to reflect the pathophysiologic processes that occur during organ donation.

Interest in extracellular histones has grown rapidly during the last decade. Studies have demonstrated a crucial role of extracellular histones multiple pathophysiologic processes like sepsis, inflammation, vascular dysfunction and thrombosis [15,16,18,22,27–29]. It has been shown that extracellular histones are in fact cytotoxic molecules that can kill various cell types, including endothelial cells, and contribute to organ failure [15,18,21,22,26,29,30]. Hence, histones once released into the circulation may initiate an amplifying cascade of cell death and histone release culminating in microcirculatory damage and consequently organ failure. Extracellular histones have not yet been studied in the pathophysiologic process of kidney injury and organ viability in a clinical setting of organ donation and transplantation. In this study we addressed the relevance of measuring extracellular histones in the machine perfusate of hypothermic machine perfused DCD kidneys before transplantation.

The incidence of PNF in this group of DCD kidneys is high. Donor kidneys in this group were procured in the period that the waiting lists exceeded five years for kidney transplantation and we were able to extend the donor pool threefold by transplanting marginal donor kidneys. These kidneys were further characterised by a very long cold ischemia time due to allocation issues and were mainly transplanted in fragile recipients in whom a prolonged waiting time for transplantation was considered not desirable. Despite the relatively good graft survival of functioning grafts and although transplantation of marginal kidneys with a high incidence of PNF may provide a survival benefit to recipients who alternatively would wait for a standard criteria donor kidneys, we considered this percentage as too high and adapted our acceptance criteria for marginal donor kidneys [31].

Essentially, every nucleated cell contains core histones, which include the histones H2A, H2B, H3 and H4 in an equimolar ratio (Figure 1). Considering the equimolar presence of the cytotoxic histones H2B, H3 and H4, measurement of histone H3 is considered a proxy-measurement of H2B and H4 as well. Recently, we were able to show a correlation between disease severity and mortality in sepsis and extracellular histone H3 levels in patients in the intensive care unit [27]. Therefore, we decided to measure the levels of histone H3 in our collection of machine perfusates.

To verify that differences in perfusion protocols and time of storage did not influence extracellular histone H3 measurements we compared mean histone H3 concentrations for IGF, DGF and PNF between the different perfusion protocols used here, and analysed

a possible influence of storage time. We neither observed differences between perfusion protocols nor did we observe an influence of storage time. This notion is supported by several studies that show that long-term storage only results in minor decreases of nucleosome concentrations and repeated freeze-thaw cycles do not affect measured concentrations of nucleosomal DNA fragments [32–34]. We also verified the latter observation by measuring histones repeatedly in our set-up (mean average coefficient of variation = 7.24 ± 0.08 % for 3 independent experiments; *data not shown*).

The average extracellular histones concentration is strikingly higher in kidneys which showed post-transplant dysfunction. The apparent prognostic benefit that correlates with lower concentrations of extracellular histones in perfusate was especially seen in graft survival analysis, where we found a 12% higher one-year survival for the lower concentration half (Figure 4). The survival of functioning grafts appears to be equal. Moreover, in contrast to three recent studies [12,35,36], which did not find a significant association of any molecule measured in machine perfusate to one-year graft failure, extracellular histones in this study were associated with an increased risk of one-year graft failure. Also, we found that extracellular histones are an independent predictor of DGF when adjusted for relevant confounding factors. Subsequent subgroups analyses yielded similar results for controlled DCD kidney transplantation. Analyses on uncontrolled DCD only showed a significant association to graft function in the multivariable prediction model for DGF and PNF, likely due to a small group size of uncontrolled DCD and the lower amount of IGF (n=12) in this group. We have shown that extracellular histone concentration is associated with graft function and graft survival, however its value to predict the outcome after transplantation is very limited and equivalent to the predictive value of most other machine perfusate biomolecules [12–14,35,36]. Therefore, the currently available data on perfusate histones concentration does not aid in the decision to either accept or refuse a kidney for transplantation.

Here, we have shown for the first time that extracellular histones are present in the perfusate of hypothermically machine perfused DCD kidneys. We observe that extracellular histones are likely to represent an independent risk factor for DGF and one-year graft failure and that there is an association to PNF. DCD machine perfused kidneys with low histone concentrations have a significantly better survival after transplantation. Future studies are necessary to assess if graft survival can be improved if the extracellular histones concentration is reduced either by better organ preservation or by therapies that may filter or neutralize these potentially cytotoxic agents.

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Chapter 4

Release of extracellular histones from porcine kidney grafts depends on the level of ischemic injury

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Abstract

Deceased donor kidneys are increasingly preserved via machine perfusion. Viability is affected by ischemia and the subsequent release of toxic molecules. A recently identified molecule is the cytotoxic extracellular histone H3, which can be released from activated immune cells and dying host cells, contributing to cellular damage. Details on this release and its implications for kidney preservation are lacking. Therefore, we studied the appearance of extracellular histones in perfused porcine kidneys with variable ischemic injury. A strategy to neutralize histones was tested on its effect on histone release and graft viability.

First, we deprived 7 porcine kidney pairs from intravascular content by extensive flushing. Of each pair, one kidney was put on hypothermic (4°C) machine perfusion (HMP) and the other on subnormothermic (28°C) machine perfusion (SNMP) for 4 hours. Second, 7 kidney pairs were put on SNMP for up to 8 hours with one kidney receiving perfusion liquid with nonanticoagulant heparin at the start of perfusion. Perfusate was analysed for histone H3 and ischemic markers, whilst post machine perfusion biopsies were studied for cellular injury.

Kidneys on SNMP showed significantly higher histone H3 levels than kidneys on HMP (median (IQR) = 0.48 (0.20-0.83) µg/mL vs. 0.02 (0.00-0.06) µg/mL; $p=0.045$, respectively). Perfusate histone H3 concentrations increased over time in the SNMP group only (Friedman test = $\chi^2=44.559$; $p<0.001$ vs. $\chi^2=11.613$; $p=0.393$ for HMP). Tissue analysis revealed significantly more cell death in the SNMP group. Heparin treatment did not affect graft viability or total histone H3 release, but resulted in a faster and more pronounced appearance of a lower molecular weight variant compared to untreated kidneys.

Our study shows that kidneys are able to release extracellular histones and that this release is associated with the amount of ischemic injury. Heparin did not impair total histone release, but did result in the appearance of histone fragments which appeared to represent histones of lower molecular weight. The implications of these findings should be investigated in more detail.

Introduction

Kidney transplantation is the preferred treatment for patients with end-stage renal disease, with improved quality of life and life expectancy as compared to dialysis [1,2]. Due to a persisting shortage of donor organs, the donation pool has over the years been expanded with (marginal) organs from deceased donors [3], categorized into donation after brain death (DBD) and donation after circulatory death (DCD). Kidney procurement from these donors is logistically and medically more challenging than in living donations and results for DCD kidneys in inevitable periods of ischemia, contributing to a higher rate of post-transplant graft dysfunction [3–5]. Nevertheless, graft survival of functioning DCD kidneys is similar to DBD kidneys, and shows a survival benefit for dialysis patients [6].

Early preservation of DCD kidneys was done by static cold storage (CS). Later, machine perfusion was being explored as a novel method of organ preservation [7,8]. In a large randomized clinical trial, preservation of DCD kidneys via hypothermic machine perfusion (HMP) was found to be superior over CS in terms of one-year graft function and survival after transplantation [9]. Renal resistance is found to decrease during machine perfusion, whilst vasodilatation may improve perfusion, removal of vasoactive and inflammatory mediators, and delivery of oxygen and nutrients after transplantation [10,11]. These benefits are especially apparent in the first hour(s) of perfusion [12].

Besides its superiority over CS in terms of post-transplant graft function, an important benefit of HMP is that kidneys can be monitored during preservation by assessing renovascular and perfusate parameters [7,12]. Several circulating perfusate markers have been correlated to clinical parameters of both the transplant and the recipient with variable specificity and sensitivity [13,14]. An earlier study by our group added extracellular histones to this list of potential biomarkers, as higher levels of extracellular histone H3 were associated with a higher rates of graft dysfunction after transplantation [15].

Originally located in the nucleus, where core histones H2A, H2B, H3 and H4 organize DNA into chromatin and regulate gene expression [16,17], release of extracellular histones is known to have pathological consequences as these proteins are cytotoxic towards host tissues [18–21] and serve as damage-associated molecular patterns (DAMPs) [22]. Histone release can occur through (un)regulated cell death [23,24] or active immune cell-mediated expulsion [25,26].

Extracellular histones have also been linked to ischemia/reperfusion injury (IRI) and kidney injury [21,27,28]. In IRI, an ischemic insult is followed by reperfusion with activated leukocytes, implying that both forms of release contribute to circulating histones. Extracellular histones function as DAMPs through TLR2/-4 in post-ischemic and septic acute kidney injury [28]. Direct injection of histones into murine kidneys results in increased levels of neutrophil recruitment, tubular injury and renal failure, but are abrogated when administering anti-histone antibodies [28].

All in all, extracellular histones are potent cytotoxic molecules that can trigger a damaging positive feedback cascade leading to additional cell death and histone release. Their potential deleterious role in organ preservation and its implications for the utility of (marginal) donor kidneys, prompted use to investigate histone release in ischemically injured kidney grafts in more detail. We used an *ex vivo* machine perfusion model using porcine kidneys, with a minimal role for blood components and immune cells, to test the association of extracellular histones with ischemic damage. Using this model, the development of this process was studied over time and was also tested with the use of an anti-histone intervention to study its potential benefit in kidney preservation.

Material and Methods

Study design

First perfusion model: extent of ischemic injury and histone release

For this first part of the study, we used a model in which we aimed to compare histone release between two groups of kidneys with a different extent of ischemic injury. In each experiment a comparison was made between kidneys that had been obtained from the same animal. A total of 8 pigs were used for this part of the study, of which 7 kidney pairs were used until the end of machine perfusion (T8½, Figure 1). One pair of kidneys served as a negative control and was not put on the final 4 hour machine perfusion period. A graphic presentation of the porcine kidney perfusion model system is presented in Figure 1.

All kidneys were retrieved from local slaughterhouse pigs, with a mean age of 6-7 months and weight of 105 kg. After bilateral procurement, each pair of kidneys was inspected for mechanical injury. Kidneys with intact renal capsule, parenchyma and vasculature were accepted for further study. After 25 minutes of warm ischemia (WIT), vasculature of both kidneys was flushed simultaneously with ice cold Histidine-Tryptophane-Ketoglutarate (HTK, **Essential Pharmaceuticals, Ewing, USA**) for 20 minutes, while kidneys were being topically cooled with ice cold 0.9% NaCl. Venous outflow was discarded during the flush out. Thereafter, kidneys were packed in 0.9% NaCl solution at 4°C and kept on cold storage for 3 hours of cold ischemia. The start of perfusion was recorded as the end of the warm ischemia and start of cold ischemia time (CIT). After cold storage, kidneys were weighed and simultaneously hypothermically perfused at 4°C using two Lifeport Kidney Transporters (model no. LKT-100-P; Organ Recovery Systems, Des Plaines, USA) for 1 hour with 0,5L of Kidney Perfusion Solution 1 (KPS-1; Organ Recovery Systems) per kidney at a pressure of 40 mmHg. Thereafter, kidneys were flushed for another 15 minutes with ice cold HTK and venous outflow was again discarded.

Figure 1. Paired ischemic porcine kidney perfusion model. Porcine kidneys are retrieved from the slaughterhouse and flushed 25min after sacrifice (warm ischemia time or WIT). Kidneys are then put on cold storage for 3h until the first period of hypothermic machine perfusion (HMP) at 4°C. Subsequently, kidneys are flushed one more time before being assigned to another 4h period of HMP or subnormothermic machine perfusion (SNMP) at 28°C. Regular perfusate sampling is done during this final perfusion period and at the end, biopsies are taken for further analysis.

After this first period of machine perfusion and two former flush-outs, each kidney was assigned to one of the following groups: hypothermic or subnormothermic machine perfusion (HMP and SNMP, respectively). HMP was performed similar to the initial perfusion period, but extended to 4 hours and performed at a pressure of 30 mmHg. SNMP comprised of kidney perfusion for 4 hours with KPS-1 at a mean temperature of 28°C. To eliminate cellular contamination, new perfusate and different perfusion packs were used for this machine perfusion period. Machine perfusate samples were taken from the Lifeport sampling port in the last 4 hour perfusion period and were immediately centrifuged at room temperature (RT) at 3000g for 3 minutes, aliquoted, and stored at -80°C until further analysis.

Second perfusion model: nonanticoagulant heparin and histone release

In the second part of our study, we altered our perfusion setup (see Figure S1). In this model, we aimed to not only determine histone H3 kinetics after an extended machine perfusion period, but also study how these kinetics are affected when using an anti-histone based treatment strategy. Nonanticoagulant heparin has been shown to neutralize extracellular histones *in vitro* as well as *in vivo*, thereby limiting histone-induced cell death and organ damage [29]. In short, this heparin is devoid of anticoagulant properties by removal of all heparin molecules that are able to bind to antithrombin. Therefore, we were interested in the effect of heparin on histone release and toxicity in our DCD kidneys.

For this new setup, both kidneys were procured and handled in a similar manner as described above up to the final machine perfusion period (T4½, Figure 1). Then, both kidneys from each pair were assigned to SNMP for 4 hours (n=3 pairs) or 8 hours (n= 4 pairs). One kidney from each pair was perfused for 4 hours liquid containing 0.31mg/mL or 54 units/mL of nonanticoagulant heparin. For 4 out of 7 kidney pairs, the perfusion period was extended to 8 hours of SNMP to study histone H3 levels over a longer time period.

Perfusate analysis

To validate the presumed ischemic differences between the two groups in our first perfusion setup, we investigated whether the SNMP kidneys were metabolically more active and would therefore sustain more ischemic injury than their HMP counterparts. Lactate (mmol/L), glucose (mmol/L), pH, pO₂ (kPa) and pCO₂ (kPa) were analysed with a blood gas analyser (GEM Premier 4000, Instrumentation Laboratory, Lexington, USA) using standardized laboratory methods. Lactate dehydrogenase (LDH, U/L) and total glutathione-S-transferase (GST, U/L) were measured using a Roche Cobas 8000 system (Roche Diagnostic International Ltd, Rotkreuz, Switzerland). Samples taken at t=10, 60, 120, 180 and 240 minutes of machine perfusion were measured and used to validate the porcine perfusion model.

Anti-histone H3 Western blotting

Perfusate taken at different time points was analysed for the presence of histone H3 using semi-quantitative Western blotting as previously described [15]. Briefly, kidney perfusate samples were subjected to SDS-PAGE gel electrophoresis and transferred to PVDF membranes (Bio-Rad Laboratories, Hemel Hempstead, UK) using semi-dry blotting. Membranes were blocked and incubated with primary anti-histone H3 antibody (1:1000 o/n at 4°C, sc-8654-R, Santa Cruz Biotechnology, Heidelberg, Germany), followed by a secondary biotin-conjugated IgG antibody (1:10000 for 30 minutes at RT, ab97083, Abcam, Cambridge, UK) and a streptavidin-biotin/alkaline phosphatase complex (Vectastain ABC-Alkaline Phosphatase for 1:500 for 30 minutes at RT, Vector Laboratories, Burlingame, USA). Histone H3 bands were stained by BCIP/NBT (Sigma-Aldrich, St. Louis, USA) and band densities were quantified by ImageQuant TL software (GE Healthcare, Little Chalfont, UK), as compared to known concentrations of purified calf thymus H3 (Roche, Basel, Switzerland).

Tissue Sampling

Wedge biopsies were taken of renal parenchyma and in most cases medulla at the end of machine perfusion (T8½ or T12½), except for the two kidneys for control analysis, which were biopsied at an earlier stage (T4½). Tissues were formalin-fixed and paraffin-embedded (FFPE) before sectioning at 4 µm thickness for immunohistochemistry.

Immunohistochemistry

Histopathologic examination of all kidneys was performed in order to study the extent of tissue damage in our perfusion setups. Morphology and extent of ischemic injury were assessed on 4 µm FFPE tissue sections using conventional hematoxylin and eosin (H&E) and Periodic acid–Schiff (PAS) staining. Additional immunohistochemical analysis was performed using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) for DNA fragmentation as an end stage of apoptosis according to the manufacturer's instructions (Roche). A counterstain was performed with hematoxylin. Representative images were taken from the outer, middle and inner region of the cortex.

Histological sections stained with H&E and PAS were scored blindly and independently by two experienced nephropathologists. Biopsies were scored for ischemic injury and cell death based on the following parameters: brush border loss, necrosis, intraluminal casts and debris, and tubular dilation. Scores were assigned to 10 cortical fields at 400x magnification on a scale of 0 to 3 (0 involves <25% cortex; 1 involves 25-50% cortex; 2 involves 50-75% cortex; and 3 involves >75% cortex). TUNEL staining was scored blindly according to overall staining positivity on a scale from 1 to 5 (1 involves 0-20% staining positivity; 2 involves 20-40% staining positivity; 3 involves 40-60% staining positivity;

4 involves 60-80% positivity; 5 involves 80-100% staining positivity) by two independent researchers.

Anti-histone H3 immunogold electron microscopy (EM)

Kidney biopsies were chemically fixed at 4°C with a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in PHEM buffer. After washing with PHEM containing 50 mM glycine, tissues were embedded in 12% gelatine and infused in 2.3 M sucrose. Mounted gelatine blocks were frozen in liquid nitrogen after which thin sections were prepared in an ultracryomicrotome (Leica EM Ultracut UC6/FC6, Vienna, Austria). Ultrathin cryosections were collected with 2% methylcellulose in 2.3 M sucrose. Then, cryosections were incubated at RT on drops of 2% gelatin/PBS for 20 minutes at 37°C, followed by 50 mM glycine/PBS for 15 minutes, 10% BSA/PBS for 10 minutes and 5% BSA/PBS for 5 minutes. Then they were incubated with anti-histone H3 antibody (1:50 in 5% BSA/PBS for 30 minutes at RT, ab1791, Abcam). After three washes of 10 minutes with drops of PBS, sections were incubated using protein A coupled to 10 nm diameter colloidal gold particles (1:50 in 1% BSA/PBS for 20 minutes at RT, AURION, Wageningen, the Netherlands). This was followed by another three PBS washes of 10 minutes and two washes of 10 minutes with distilled water. As a control for non-specific binding of the colloidal gold-conjugated antibody, the primary antibody was omitted. Observations were done in an Electron Microscope Tecnai G2 Spirit equipped with an Eagle 4kx4k CCD camera (FEI Company, The Netherlands).

Cell culture

The permanent human endothelial cell line EA.hy926 (CRL-2922, ATCC) and porcine kidney epithelial cell line PK-15 (CCL-33, ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (all from Lonza, Walkersville, USA). In addition, EA.hy926 cells were supplemented with hypoxanthine (5 mM), aminopterin (20 µM) and thymidine (0.8 mM) (Lonza).

In vitro cytotoxicity assay

The EA.hy926 and PK-15 cell lines were grown to confluence. Cells were washed twice with PBS before incubation with 5-40 µg/mL purified calf histone H3, 10 µg/mL purified calf histone H1 (both Roche), 10 µg/mL human recombinant histones H2A, H2B and H4 (all New England Biolabs, Ipswich, USA) or 50 µg/mL histone mix (Roche) for 1 hour in serum-free DMEM. Next, cells were collected and cell viability was assessed by flow cytometry employing a BD Accuri C6 flow cytometer using annexin A5-FITC (PharmaTarget

BV, Maastricht, The Netherlands) and propidium iodide (PI) staining (Invitrogen Life Technologies, Carlsbad, USA) as previously described [29].

Immunofluorescent staining

EA.hy926 cells were seeded and grown to confluence. Cells were equilibrated to 4°C, 22°C or 37°C before incubation with 10 µg/mL histone H3 in serum-free DMEM for 1 hour. Cells were then washed, stained with 2,5 µg/mL PI and 0,5 µg/mL Hoechst 33342 (ThermoFischer, Mannheim, Germany) for 10 minutes and imaged using a EVOS FL inverted digital microscope (ThermoFischer). Six random images were taken at 20x magnification per tested condition. The percentage of PI positive cells was quantified for each image using ImageJ software (x86, Wayne Rasband, National Institute of Health, USA) and then averaged to generate a mean PI positivity for each condition.

Data analysis

All data were analysed using IBM SPSS statistics Version 22.0 (IBM Corp., Armonk, USA) or GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, USA). Numerical variables are presented as mean $\pm$ standard deviation (SD) if approximately normally distributed or as median (interquartile range (IQR)) if stated. Categorical variables are presented as numbers (percentage).

For statistical analysis of immunohistochemical, flow cytometry and immunofluorescence data, one-way ANOVA was performed, while applying a Bonferroni correction when comparing all groups and a Dunnett test when comparing groups to control. For analysis of histone concentrations in the perfusate, the non-parametric Friedman test was used to test if concentration changes recorded over time for machine perfusion groups (SNMP and HMP) were significant and presented as Chi-square and p-value per group. Secondly, to describe the difference of concentration changes over time between both groups, we used a linear mixed model. This model was applied, assuming a linear regression model, in which both types of machine perfusion (SNMP and HMP) and machine perfusion sample time were used as fixed effect and a random intercept was included. For determination of the difference between groups, we used the interaction term of these two parameters (type of machine perfusion * machine perfusion sample time) and presented the p-value. Extracellular histone release over time was investigated for each type of perfusion group.

To compare concentrations of lactate, LDH, GST and glucose between machine perfusion groups (SNMP vs. HMP), the Mann-Whitney U test was used per point of machine perfusion sample time (t=10, 60, 120, 180 and 240 minutes). The Friedman test was used to test if concentrations changed over time per aforementioned marker in both machine perfusion groups. Association of these 4 parameters to histone concentration was tested with a Student's t-test for Spearman's Rho (rs) on these time points. In the

intervention perfusion model, histone concentrations per time point were compared by performing an independent t-test. Significant differences were indicated by a *p-value of <0.05, **p-value of <0.01 or ***p-value of <0.001.

Results

Validation of ischemic injury within the machine perfusion model

We determined several ischemic markers in machine perfusate including LDH, lactate and glucose, to validate our perfusion model (Figure 2). LDH is widely used as marker of tissue damage and also proposed as kidney viability marker during machine perfusion of deceased donor kidneys [13]. Kidneys increasingly released LDH into the extracellular compartment in both the SNMP and HMP group (Friedman test: SNMP Chi-square 23.886, $p < .001$ and HMP Chi-square 16.914; $p = .002$; Figure 2A, Table S1). After 30 minutes there was a significant difference between both groups in LDH concentration within the perfusate, with higher levels in SNMP kidneys (Figure 2A, Table S1).

Figure 2. Validation of the ischemic kidney perfusion model. Changes of biomarkers (A) LDH, (B) glucose and (C) lactate concentrations over time in machine perfusate per group (HMP vs SNMP), as measured on 5 time points (10, 60, 120, 180, 240 min). Data are presented as median and whiskers include interquartile range. Concentration difference between groups was measured per time point with the Mann-Whitney U test *Indicates significant differences between groups ($p < 0.05$).

We used perfusate glucose deprivation as marker of metabolic activity and perfusate lactate increase as end product of metabolic activity of the kidney [32]. Figures 2B and 2C show changes in glucose and lactate concentrations over time during SNMP and HMP. There was uptake of glucose by kidneys in both SNMP and HMP groups over time (Friedman test: SNMP Chi-square 16.000, $p=.003$ and HMP Chi-square 18.000; $p=.001$; Table S1). For all time points measured, perfusate glucose concentration was significantly higher in the HMP group compared to the SNMP group (Figure 2B, Table S1).

Kidneys in both groups produced lactate during machine perfusion (Friedman test: SNMP Chi-square 28.000, $p<.001$ and HMP Chi-square 24.361; $p<.001$). The release of lactate was significantly higher for SNMP kidneys as compared to the HMP kidneys (Figure 2C, Table S1). All other tested markers are depicted in Table S1.

Immunohistochemical analysis of ischemic kidneys

Overall morphology and tubular injury of control, HMP and SNMP kidneys were individually scored by two blinded, experienced nephropathologists. H&E staining showed a non-significant trend with control<HMP<SNMP towards increased scores with respect to tubular dilation, cast deposition, brush border loss and necrosis as assessed by morphological scoring (Figure 3). Brush border integrity, as assessed by PAS staining, was not significantly different between both groups (Figure 3).

As histones and ischemia are both associated with cell death, we hypothesized that SNMP kidneys would have a higher level of cell death than HMP kidneys. This was investigated by performing a TUNEL staining on post machine perfusion kidney biopsies. Scoring by two blinded, independent researchers showed that SNMP kidneys had a higher TUNEL positivity than HMP kidneys (Figure 4; $p<0.01$ for SNMP vs HMP), and thus have a decreased cellular viability. No differences were observed between control kidneys and kidneys on HMP.

Figure 3. H&E and PAS staining of ischemic porcine kidneys in our perfusion model. A total of n=7 kidneys from both HMP and SNMP, and 2 control kidneys were studied for differences in overall morphology and brush border integrity by an independent pathologist. Representative images from each group are shown. Upper panel: Top row represents H&E staining and the bottom row represents PAS staining. Lower panel: Scoring of H&E images for all groups. Scale bar represents 50 μ m.

Figure 4. TUNEL staining of ischemic porcine kidneys in our perfusion model. A total of $n=8$ kidneys from each group were examined for extent of cell death using TUNEL staining labeling DNA breaks. Upper panel: representative images from each group are shown: (A) positive staining control, (B) control, (C) HMP and (D) SNMP kidney. Quantification of the TUNEL staining is shown in the lower panel (Data are means $\pm$ SD. ** $p<0.01$). Scale bar represents $50\mu\text{m}$.

Extracellular histone release and ischemic injury

We measured histone concentrations within the machine perfusate to investigate ischemia dependent histone release into the extracellular space. During the initial stages (e.g. procurement, storage and initial flushing and perfusion stages) we could not detect extracellular histones in the perfusate (data not shown). At the final stage of the machine perfusion, extracellular histones were detected (Figure 5). Histone H3 concentrations were significantly higher after 4 hours of SNMP compared to HMP (median concentration = 0.48 (0.20-0.83) $\mu\text{g/mL}$ vs. 0.02 (0.00-0.06) $\mu\text{g/mL}$; $p=0.045$, respectively; Figure 5).

Furthermore, there was a significant change in histone concentration over time in the SNMP group, while this was not the case in the HMP group (Friedman test = $\chi^2=44.559$; $p<0.001$ vs. $\chi^2=11.613$; $p=0.393$, respectively). Additionally, analysis using a mixed linear model showed that histone concentration in the SNMP group increases over time and differs significantly to the histone concentration changes of the HMP group (p -value for the estimate of fixed effect for the interaction term of machine perfusion group * time of machine perfusion = 0.001). Figure 5 demonstrates the increase of histone concentration over time during SNMP, with the concentration of histones during HMP remaining low.

Figure 5. Release of extracellular histones is dependent on the amount of ischemic injury. Extracellular histone H3 was determined semi-quantitatively using Western blotting. Perfusate levels of total histone H3 increased over time in the SNMP group, whilst remaining constant in the HMP group. Data are presented as median and whiskers include interquartile range.*Indicates significant differences between groups ($p<0.05$).

Cytotoxic effects of extracellular histones

We tested two cell types most likely involved in not only ischemia itself, but also possible histone-induced cell damage in our perfusion model: endothelial cells and epithelial cells.

Upon incubation of both cell lines with histone H3, cell viability decreases in a concentration-dependent manner (Figure 6A). Histone-mediated cytotoxicity is not specific for one of these cell types as cell viability response to histone addition did not differ between both cell lines. There are indications that in our model both endothelial cells and tubular cells are involved in extracellular histone release, as we observed cytoplasmic localization of histone H3 and extracellular localization within peritubular capillaries and tubuli (Figure S2). Considering the likely presence of the full spectrum of extracellular histone isoforms in the circulating perfusate, also histones H1, H2A, H2B and H4 were tested. Endothelial cells incubated with different types of histone revealed a variable effect on cell viability for the different histone isoforms (Figure 6B). For these cells, histone-mediated cytotoxicity appears to be isotype specific, with the linker histone H1 being the least cytotoxic, followed by histone H2A, H2B, H3 and H4. The mixture of histones (consisting of all histone subtypes at equal molar ratio) induces the most cell death.

Figure 6. Cytotoxicity of extracellular histones to endothelial and kidney epithelial cells. (A) Endothelial cells (EA.hy926) and porcine kidney epithelial cells (PK-15) were incubated with 5-40 µg/mL histone H3 in serum-free DMEM for 1h at 37°C. (B) PK-15 cells were incubated with 10 µg/mL of individual histones H1, H2A, H2B, H3 and H4, or a mixture of these histones (Hmix) under the same conditions as in (A). Cell viability was assessed using flow cytometry with PI and annexin-A5 FITC. Data is shown as the mean + SD. *p<0.05 and ***p<0.001 as compared to control.

Considering our model compares different extents of ischemia by perfusion, we tested whether the cytotoxicity of extracellular histones is dependent on temperature. For this experiment, EA.hy926 cells were incubated with a fixed concentration of histone H3 for 1h at 4°C, 22°C or 37°C. Then the DNA-binding fluorescent probe Hoechst 33342 was added to quantify dead cells. Immunofluorescent images show that the number of PI positive nuclei, as a measure of histone-induced cell death, increases with increasing temperature (Figure 7). Incubation of cells with histone H3 at 4°C degrees for 60 minutes

Figure 7. Temperature-dependent cytotoxicity of extracellular histone H3. Endothelial cells (EA.hy926) were incubated with 10 $\mu\text{g}/\text{mL}$ histone H3 in serum-free DMEM for 1h at 4°C, 22°C or 37°C. Left panel: representative images from histone treated cells. Nuclei were stained using Hoechst 33342 (in blue) and necrotic cells were stained using PI (in green). Right panel: The number of PI positive cells were quantified in 6 random image fields using ImageJ software. Data is expressed as the mean $\pm$ SD of the average percentage of PI positive cells per condition (n=5).

hardly results in cell death (6.6%) whilst incubation at 37°C results in significantly more cell death (27.6%, $p < 0.01$). Incubation at an intermediate temperature of 22°C induced a moderate level of cell death (16.6%) even though this is not significantly different from the levels of induced cell death for the other temperatures tested.

Nonanticoagulant heparin and histone release

As we observed substantial histone release in SNMP kidneys (Figure 5), we used this setup to test our heparin intervention and investigate its effect on the kinetics of extracellular histone appearance and on organ viability. Western blot analysis of machine perfusate did not reveal significant differences in the total amount of extracellular histone H3 between heparin treated or untreated SNMP kidneys at any time point up to 8h of perfusion (Figure 8A). Interestingly, in both groups a histone H3 band of an apparent lower molecular weight of about ± 14 -15 kDa (LMW-H3; estimated size ± 12.4 -13.4 kDa) gradually appeared over the course of perfusion besides the full-length protein band with an apparent MW of ± 17 kDa (UniProt F1RVA0 determined MW of 15.4 kDa) (Figure 8C).

Figure 8. Time-dependent release of histone H3 in heparin treated and untreated SNMP kidneys. Extracellular histone H3 was determined in SNMP kidneys with or without circulating nonanticoagulant heparin. SNMP kidneys treated with heparin showed a similar release in total histone H3 (A) but a more rapid and pronounced increase in levels of a lower molecular weight histone H3 (LMW-H3) variant (B) compared to untreated counter kidneys. (C) A representative Western blot image of full-length histone H3 (17 kDa apparent MW) and LMW-H3 (15 kDa apparent MW) in perfusate of untreated SNMP kidney at t=5, 6, 7 and 8 hours of perfusion. *Indicates significant differences between groups ($p < 0.05$).

Despite the total histone release being similar in both groups, this LMW-H3 was released faster and more pronounced in kidneys with circulating heparin as compared to their counter parts (Figure 8B). The increase in LMW-H3 became apparent for the heparin kidneys after 2 hours of perfusion whilst this only started to occur after 6 hours of perfusion for the untreated counterparts. For both SNMP groups, this increase was exponential up to at least 8h of perfusion. Significant differences in LMW-H3 levels between both groups were observed for all time points between 2 and 8 hours of perfusion.

Discussion

More insight into the various processes occurring during different stages of organ transplantation would provide a basis for novel interventions that can expand the use of marginal kidneys. As extracellular histones present in human donor kidneys during machine perfusion are associated with graft dysfunction after transplantation [15], we aimed to investigate their release in more detail in an *ex vivo* machine perfusion model using kidneys of porcine origin. One kidney of a pair was subjected to a low level of ischemic damage (perfusion at 4°C or HMP), and the other to a high level of ischemic damage (perfusion at 28°C or SNMP).

Ischemic differences were validated as shown by established markers, but we observed that such differences cannot be determined by study of the overall morphology and brush border integrity after 4 hours of perfusion. This could be due to a relative insensitivity in assessing acute kidney damage via the qualitative methods. Nevertheless, the TUNEL staining (indicative of apoptosis) did indicate a difference in the extent of cell death between both groups of kidneys. It has been shown that cold storage predominantly induces necrosis, with subsequent warm reperfusion inducing apoptosis [34–36]. Minimizing the extent of ischemic damage could therefore be beneficial in reducing cell death, as well as potential histone release.

The appearance of extracellular histone H3 in the preservation fluid showed a significant difference between both HMP and SNMP in terms of concentrations detected. Extracellular histones were released exponentially over time in kidneys with substantial ischemic damage, whilst this release was not seen in less ischemic kidneys. As LDH induces histone release and vice versa in cultured cells *in vitro* [37] and in glomeruli *in vivo* [38], our findings are in line with the observation that in ischemic damage histones can be both indicators of ongoing tissue damage as well as active contributors. While in our study we specifically looked at histone H3, we regard this isotype as a proxy for other isotypes as they complexed in nucleosomes and are expected to be released as such.

Our *in vitro* experiments have shown that extracellular histones exert cytotoxic effects depending on concentration, histone isotype and temperature, with endothelial and epithelial cells being equally sensitive. In our setup, cytotoxicity appears to be regulated primarily through active processes, as most cell death was observed at 37°C and only minimal cell death at 4°C. These processes could be regulated by toll-like receptors, as histones can bind them [28] and they are upregulated in DCD kidneys [39]. We have indications that both endothelial cells and tubular cells are involved in the release of histones, because of the extranuclear localization of histone H3 within the cytoplasm and the extracellular localization within peritubular capillaries and tubuli. Also, these

cells were found to be positive in the TUNEL staining, suggesting that death of these cells results in histone release.

In our study we performed SNMP up to 8 hours as adequate perfusion is then still maintained. In a clinical setting, the period of machine perfusion may even be up to 24 hours or longer (own cohort data) [9,15]. The time-dependent exponential release of circulating histones is expected to continue during prolonged machine perfusion as these proteins can stimulate additional histone release, which would favour shorter SNMP or HMP (in which no histones were detected up to 4h). However, it is still debated whether extended machine perfusion would be more beneficial than shorter perfusion in terms of graft function and transplant outcome [40,41].

The increasing appearance of cytotoxic proteins in the preservation fluid led us to test charge-mediated neutralization of these toxic molecules with nonanticoagulant heparin, which has been shown to reduce histone-induced cell death in inflammation [29]. Administration of this heparin at the start of SNMP did not result in changes in perfusion parameters, LDH levels or tissue damage as compared to kidneys that did not receive heparin (data not shown). Unexpectedly, the total amount of circulating extracellular histone H3 did also not differ between both groups. Despite a similar extent of injury induced by SNMP, local concentrations of extracellular histones and other perfusate components could be different in both groups. Histones are known to bind cell surface proteoglycans [42] and locate at the cell surface of apoptotic cells [43]. Heparin could possibly lead to an increase in the circulating histone pool as it can serve as a carrier molecule able to liberate cell-bound histones and retain them in the circulating perfusate. Conventional anti-histone H3 immunohistochemistry and EM could not accurately assess possible differences in extracellular histone H3 staining (data not shown).

Interestingly, we did observe the progressive appearance of a lower molecular weight histone H3 variant (LMW-H3) in SNMP kidneys, with heparin accelerating this phenomenon. The presence of this variant is hypothesized to be the result of histone proteolysis and/or modification. Binding of histones to heparin could lead to an increased affinity of (circulating) protease(s) for histone substrate and initiate proteolysis faster than in untreated kidneys. After searching the literature, several examples of histone-cleaving proteases were found: neutrophil elastase (NE) [44], granzyme A [45], plasma hyaluronan-binding protein [46], cathepsins [47], trypsin(-like) enzymes [48], mast cell tryptase [49], plasmin [50] and activated protein C (APC) (own observations and [51]). In addition, many other proteins can react with histones in plasma, including proteinase inhibitors which might be blocked by heparin binding [52].

Based on their known interactions with heparin [53,54], and the ability of immune cells to release DNA strands decorated with histones and elastase [25], we analysed the cleavage of histone H3 by APC and elastase *in vitro*. Cleavage by elastase, and less likely

by APC, resulted in the appearance of a similarly sized histone band on Western blot (Figure S3A). However, the addition of heparin in this setup did not accelerate histone cleavage by elastase (Figure S3B). Instead, it led to an enhanced detection of full-length histone H3, most likely by preventing protein adsorption to the surface of the reaction tube. Earlier evidence has showed that heparin is able to inhibit elastase activity [55,56], with a minimum required chain length of 12-14 saccharides [57]. In antithrombin inactivation, however, heparin-antithrombin complexes serve as substrates for elastase and are prerequisites for the enzymatic reaction [53]. Even if this observed discrepancy between the effects of heparin on enzyme function is due to differences in substrates or reaction conditions, we could not fully confirm or reject neither elastase nor APC as the responsible determinant for the observed LMW-H3 in our perfusion model.

Therefore, we attempted to deduce the exact cleavage site within the histone protein by determination of the LMW-H3 sequence via mass spectrometry. Although we were not able to identify the exact histone mass and sequence using this approach, we did detect some histone H3 fragments in both the native histone H3 and LMW-H3 (Figure S4). The analysis seems to indicate that the cleavage site is located in the N-terminus, as fragments were detected in all other regions except the first 28 residues of the N-terminus. Furthermore, this histone region is prone to proteolysis [58] which could result in an estimated 2-3 kDa loss in protein size for the cleaved product. More sophisticated perfusate analysis should therefore be conducted to determine the exact mass and origin of LMW-H3 as well as identify other molecules of interest, with recent advances in mass spectrometry [59], proteomics [60,61] and NMR [62–64] serving great potential.

As epigenetics is increasingly studied in the context of transplantation [65], we also tested our samples for histone modifications, since these may contribute to the gel electrophoretic mobility behaviour of histones. In our perfusion samples, we did not detect phosphorylation or citrullination of histone H3 (data not shown). Other modifications should however still be considered, as heparin is known to inhibit for instance histone acetyl transferases (HATs) [66] and transforming growth factor (TGF) [67] which is involved in histone methylation [68].

A limitation of this study is that our model did not allow the accurate measurement of functional markers such as creatinine, blood urea nitrogen, urinary markers and urine output [69,70], as kidneys are deprived from blood and do not function as *in vivo*. Novel preservation strategies such as oxygenated normothermic perfusion with perfusion fluid or autologous blood should be considered in order to better assess graft function [71] and plasma-related heparin effects [72–74]. Furthermore, this study specifically looked at a pre-transplantation setting. A more direct translational approach would be the use of a porcine transplantation model, which could be highly relevant since histones were associated with post-transplantation outcome in humans [15] and addition of the

direct thrombin inhibitor Melagatran in HMP resulted in a lower serum creatinine after transplantation [75]. Finally, as we did not label our heparin, we could not investigate in more detail the localization of this polysaccharide in the machine perfused kidney. Sedigh et al. has shown that a biotinylated heparin conjugate bound to the inner surface of the vessel wall during machine perfusion of porcine kidneys and could restore the endothelial glycocalyx [76].

In summary, we have shown that extracellular histones are released from ischemically damaged kidneys in a time- and ischemia-dependent manner. Addition of nonanticoagulant heparin did not result in different histone H3 levels, but did result in the earlier release of a lower molecular weight histone H3 variant into the circulating perfusate. The further implications of these findings in organ preservation should be investigated, as optimal graft preservation is paramount.

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Supplemental Methods

Histone H3 cleavage experiments

5 µg/mL of purified calf thymus histone H3 was incubated in 25 mM Hepes (pH 7.5), 175 mM NaCl (HN) buffer with 10 nM activated protein C (APC) for 10 minutes or with 0.2 µg/mL elastase from porcine pancreas (Sigma-Aldrich) for 60 minutes at 37°C. In a separate experiment, 5 µg/mL histone H3 was incubated with 0.2 µg/mL elastase in the presence of heparin (0-50 µg/mL) in HN for 30 minutes at 37°C. Histone H3 cleavage was assessed using Western blot analysis as earlier described.

In-gel digestion and LC-MS/MS analysis for identification of histone H3 bands

A perfusate sample taken at 4 hours of SNMP was subjected to SDS-PAGE gel electrophoresis and Coomassie Brilliant Blue staining (Bio-Rad). Both histone H3 protein bands were excised from the gel and processed on a MassPREP digestion robot (Waters, Manchester, UK) for peptide extraction, according to an earlier described protocol [30]. LC-MS/MS analysis was then used for peptide separation and protein identification as previously described [31]. The MS data were searched using Sequest HT Proteome Discoverer 2.2 search engine (Thermo Scientific, Rockford, USA) against the UniProt database according to optimized settings [31], with proteins and peptides requiring a minimum length of six amino acids. The sequences of the identified peptides were matched to the full-length porcine histone H3 sequence determined at 15.4 kDa (UniProt F1RVA0).

Supplemental Material

Table S1: Friedman and Mann-Whitney tests for differences between type of perfusion group per time point per biomarker

		H3 (5 time points)	Lactate	Glucose	LDH	GST	pH	pO2	pCO2
Friedman HMP	Chi-square	6.333	24.361	18.000	16.914	1.172	11.075	9.257	7.387
	p-value	0.176	<.001*	.001*	.002*	.883	.026*	.055	.117
Friedman SNMP	Chi-square	18.719	28.000	16.000	23.886	5.600	14.071	8.547	1.640
	p-value	.001*	<.001*	.003*	<.001*	.231	.007*	.073	.802
10 min	HMP median (IQR)	0.00 (0.00-0.00)	0.6 (0.3-0.7)	9.9 (9.8-10.3)	5 (4-11)	7.9 (3.5-9.9)	6.99 (6.96-7.08)	26.0 (24.6-27.6)	2.9 (2.3-3.8)
	SNMP median (IQR)	0.00 (0.00-0.00)	1.0 (0.4-1.0)	9.3 (9.1-9.9)	11 (8-23)	7.9 (6.4-10.2)	6.84 (6.82-6.87)	25.6 (24.4-26.1)	5.6 (4.9-5.6)
	Mann-Whitney p-value	1.000	0.259	.035*	.165	.818	.001*	.383	.017*
60 min	HMP median (IQR)	0.00 (0.00-0.00)	0.6 (0.6-0.8)	8.7 (8.4-9.2)	21 (17-25)	6.5 (4.1-11.7)	6.89 (6.81-6.97)	23.5 (21.7-24.9)	4.0 (2.8-6.9)
	SNMP median (IQR)	0.00 (0.00-0.10)	2.2 (1.5-2.7)	8.1 (7.7-8.3)	40 (32-47)	10.3 (7.3-12.2)	6.80 (6.80-6.84)	23.5 (20.9-25.3)	5.4 (4.6-9.9)
	Mann-Whitney p-value	.805	.001*	.015*	.001*	.366	.038*	.902	.128
120 min	HMP median (IQR)	0.00 (0.00-0.05)	0.8 (0.7-1.1)	8.7 (8.1-8.8)	36 (34-48)	10.0 (5.0-10.9)	6.89 (6.80-6.90)	24.8 (24.2-25.7)	4.3 (3.5-6.4)
	SNMP median (IQR)	0.15 (0.00-0.22)	4.0 (2.6-4.4)	7.5 (7.0-7.7)	60 (58-85)	11.4 (6.3-13.7)	6.80 (6.80-6.80)	23.7 (22.8-24.6)	7.9 (4.5-9.3)
	Mann-Whitney p-value	.097	.001*	.001*	.038*	.318	.038*	.128	.073
180 min	HMP median (IQR)	0.00 (0.00-0.00)	1.0 (0.7-1.0)	8.2 (7.6-8.6)	48 (35-51)	8.5 (8.4-10.2)	6.86 (6.80-7.00)	24.0 (22.6-26.0)	4.9 (2.9-7.0)
	SNMP median (IQR)	0.11 (0.02-0.26)	5.7 (3.6-6.4)	6.9 (6.2-7.2)	95 (76-117)	10.9 (6.6-12.8)	6.80 (6.80-6.80)	22.9 (21.1-24.9)	7.1 (4.8-7.7)
	Mann-Whitney p-value	.011*	.001*	.008*	.004*	.383	.026*	.456	.209
240 min	HMP median (IQR)	0.02 (0.00-0.06)	1.0 (0.9-1.1)	8.2 (8.1-8.5)	56 (41-62)	11.3 (5.5-12.6)	6.90 (6.80-6.94)	22.8 (22.0-24.7)	4.3 (2.8-8.6)
	SNMP median (IQR)	0.48 (0.20-0.83)	7.4 (5.2-9.0)	6.0 (5.7-7.1)	118 (61-130)	10.4 (7.5-13.2)	6.80 (6.80-6.81)	24.1 (23.1-25.8)	7.9 (5.6-8.7)
	Mann-Whitney p-value	.004*	.001*	.001*	.038*	.836	.165	.456	.165

Figure S1. Paired ischemic porcine kidney perfusion model with an anti-histone intervention. A similar setup was used as before, except both kidneys were assigned to SNMP for the final period of machine perfusion. One kidney from each pair received non-anticoagulant heparin (0.31 mg/mL or 54 U/mL), whilst the other did not receive treatment. A total of 7 kidney pairs were used, from which 3 pairs were perfused up to 4h and the other 4 pairs were perfused up to 8h of SNMP. Regular perfusate sampling was done and tissue was collected at the end of SNMP.

Figure S2. EM images of anti-histone H3 immunolabeling after 4h of SNMP. Histone H3 (small black dots indicated with black arrows) was detected by immuno-EM using antibody labeling with 10 nm gold particles. Top row: representative images are shown for histone H3 localization inside the nucleus (left) and outside the nucleus/in the cytoplasm (right). Bottom row: extracellular histone H3 is released from both endothelial cells (left image) and tubular cells (right image). Scale bar represents 200 nm or 500 nm as indicated.

Figure S3. Western blot analysis of H3 cleavage by APC and elastase. (A) 5 $\mu\text{g/mL}$ histone H3 was incubated with 10 nM APC (H3+APC) for 10 minutes at 37°C or with 0.2 $\mu\text{g/mL}$ elastase (H3+E) for 1 hour before analysis with SDS-PAGE and anti-histone H3 Western blotting. Cleavage of H3 by these enzymes was compared to a perfusate sample from a heparin-treated SNMP kidney ($t=4$ h). (B) 5 $\mu\text{g/mL}$ histone H3 was incubated with 0.2 $\mu\text{g/mL}$ elastase in the absence or presence of increasing amount of heparin (0-50 $\mu\text{g/mL}$) for 30 minutes at 37°C and analysed for cleaved histone H3 as performed in (A). Blots are representative of three independent experiments.

Figure S4. Mass spectrometry analysis of histone H3 bands from perfusate samples. (A) + (B) A representative perfusate sample of a SNMP kidney at $t=4$ hours was subjected to SDS-PAGE gel electrophoresis and Coomassie staining. Both histone H3 bands (as earlier identified with Western blot analysis) were excised from the gel, digested with trypsin and processed with LC-MS/MS analysis for peptide identification according to the described methods. Identified peptides were then matched to the porcine histone H3 sequence (UniProt F1RVA0). (A) The highlighted residues in green indicate the identified peptides belonging to the full-length histone H3 band (apparent MW of ± 17 kDa; UniProt mass 15.4 kDa), whilst (B) shows the identified peptides for the LMW-H3 band (apparent MW of ± 14 -15 kDa; estimated ± 12.4 -13.4 kDa). The red square indicates the potential cleavage region within the N-terminus that could lead to the generation of LMW-H3.

Chapter 5

Decreased endothelial glycocalyx thickness is an early predictor of mortality in sepsis

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Abstract

Microcirculatory alterations play an important role in the early phase of sepsis. Shedding of the endothelial glycocalyx is regarded a central pathophysiological mechanism causing microvascular dysfunction, contributing to multiple organ failure and death in sepsis. The objective of this study was to investigate whether endothelial glycocalyx thickness at an early stage in septic patients relates to their clinical outcome.

We measured the perfused boundary region (PBR), which is inversely proportional to glycocalyx thickness, of sublingual microvessels (5-25 μm) using sidestream dark field imaging. The PBR in 21 sepsis patients was measured within 24 hours of admission to the intensive care unit (ICU). In addition, we determined plasma markers of microcirculatory dysfunction and studied their correlation with PBR and mortality.

Endothelial glycocalyx thickness in sepsis was significantly lower for non-survivors as compared to survivors, as indicated by a higher PBR of 1.97 [1.85, 2.19] μm compared to 1.76 [1.59, 1.97] μm , $p = 0.03$. Admission PBR was associated with hospital mortality with an area under the curve of 0.778 based on the receiver operating characteristic curve. Furthermore, PBR correlated positively with angiopoietin-2 ($\rho=0.532$, $p=0.03$), indicative of impaired barrier function. PBR did not correlate with APACHE IV, SOFA score, lactate, syndecan-1, angiopoietin-1 and heparin-binding protein.

An increased PBR within the first 24 h after ICU admission is associated with mortality in sepsis. Further research should be aimed at the pathophysiological importance of glycocalyx shedding in the development of multi organ failure and at therapies attempting to preserve glycocalyx integrity.

Introduction

The microcirculation has been increasingly acknowledged to play a key role in the pathophysiology of various disease states [1,2]. Microcirculatory derangements may include decreased microvascular density, perfusion and oxygen exchange, along with increased flow heterogeneity in septic shock patients [3,4]. The glycocalyx, a gel-like layer covering the luminal side of the vascular endothelium, is an important regulator of the microcirculation. It primarily consists of proteoglycans with covalently linked glycosaminoglycans, glycoproteins and glycolipids [5,6]. Under physiological conditions the endothelial glycocalyx provides an important stabilizing barrier as it regulates activation of coagulation factors such as protein C and tissue factor, prevents leakage of plasma components, inhibits platelet activation and blocks leukocyte adhesion [7,8].

Glycocalyx degradation has been linked to increased edema formation, enhanced leukocyte extravasation, and a decreased microcirculatory perfusion, leading to organ failure [9–11]. A surrogate marker of glycocalyx shedding is the transmembrane heparan sulfate proteoglycan syndecan-1, which was found to correlate with sepsis severity and mortality [12–15]. Interestingly, syndecan-1 levels in non-survivors remained elevated over the course of disease signifying ongoing glycocalyx shedding, while they decreased in sepsis survivors [12]. Whereas there appears to be a clear relationship between biomarkers of glycocalyx degradation and mortality in sepsis, no studies are available that link direct visualization of the glycocalyx with mortality in sepsis.

Over the last decade, the development of non-invasive imaging techniques such as sidestream dark field (SDF) imaging has resulted in a better understanding of the role of the microcirculation in disease [16–19]. SDF imaging makes use of the easily accessible sublingual microcirculation to investigate the condition of microvessels in more detail. Several studies have indicated that the sublingual vasculature is representative for the rather inaccessible intestinal or cerebral microvascular beds, because it is affected in a similar manner [20–22]. SDF imaging has been used in many clinical studies to investigate the microvasculature in pathological states such as stroke [23], chronic kidney disease (CKD) [24–26] and sepsis [3,4,19,27–30]. One of the measured parameters is the perfused boundary region (PBR), an inverse parameter of glycocalyx thickness on endothelial cells of sublingual microvessels [31]. Thus, an increased PBR indicates a decreased glycocalyx thickness. The PBR was indeed shown to be significantly increased in critically ill patients as compared to healthy controls [28]. However, it remains unclear whether the PBR itself is a prognostic marker able to identify sepsis patients at high risk for organ failure and death.

In our study, we aimed to investigate the association between glycocalyx degradation and mortality in septic patients. The glycocalyx was assessed within 24h after intensive

care unit (ICU) admission by SDF imaging and results were related to biomarkers of glycocalyx degradation (syndecan-1 and heparin-binding protein (HBP)) and vascular permeability (angiopoietin (Ang)-1 and Ang-2)).

Material and Methods

Patients

Twenty-one septic patients admitted to the ICU of the Maastricht University Medical Center (MUMC) in Maastricht, the Netherlands, were included between May 2016 and September 2017. Inclusion criteria were: age > 18 years, absence of oral bleeding, wounds and infections, absence of variations in mean arterial pressure (MAP) > 10 mmHg in the 10 minutes prior to measurements, absence of variations in medication and fluid administration in the 10 minutes prior to measurements and a diagnosis of sepsis according to the Sepsis-3 criteria [32]. Exclusion criteria were: noninvasive ventilation and administration of a high nasal flow cannula. Additionally the SDF camera had to be available and a researcher experienced with performing SDF measurements had to be present. Demographic variables, routine laboratory data and physiological parameters were collected during the first 24 hours after ICU admission. A subgroup of patients with septic shock was identified according to the Sepsis-3 criteria, defined as sepsis accompanied by a lactate value of > 2mmol/L and the use of vasopressors (norepinephrine) to maintain a mean arterial pressure (MAP) of ≥ 65 mmHg [32]. All procedures complied with the guidelines of the Declaration of Helsinki and were approved by MUMC Institution's Medical Ethical Committee (project number 154269). Informed consent was obtained from all patients or their representatives.

Sublingual glycocalyx measurement

The sublingual endothelial glycocalyx thickness was studied by non-invasive SDF imaging. Image acquisition (CapiScope HVCS, KK Technology, Honiton, UK) and analysis was performed by GlycoCheck™ software (Microvascular Health Solutions Inc., Salt Lake City, UT, USA) [33]. In brief, images of approximately 14 mm² are recorded when image quality is deemed sufficient by the software (this is based on focus, motion and intensity). All microvessels between 5 and 25 μ m in diameter are then automatically identified. These microvessels are divided in vascular segments of 10 μ m along the length of the detected vasculature. The lateral movement of red blood cells (RBC) into the glycocalyx at these points can be detected, which is expressed as the PBR. A disturbed glycocalyx leads to an increased lateral movement of RBCs and thus an increased PBR. Each measurement consists of at least 3000 vascular segments. In our study, 3 consecutive measurements

were performed by an experienced researcher and averaged to generate a mean PBR value per patient to improve data reliability and quality. Unpublished data collected in our ICU population showed that averaging three consecutive measurements improved reproducibility; intraclass correlation coefficient changed from poor to excellent.

Laboratory measurements

Creatinine, C-reactive protein, total hemoglobin, lactate, bilirubin, thrombocytes and leukocytes were obtained from routine clinical chemistry laboratory tests. At the same time as the glycocalyx assessment, blood from an arterial catheter was drawn into tubes containing either 3.2% citrate or EDTA (BD, Franklin Lakes, USA) and immediately centrifuged two times for 12 minutes at 2000g at room temperature.. Plasma samples were stored at -80°C until measurement of established markers of the microcirculation, reflecting increased vascular permeability (Ang-1, Ang-2 and HBP; R&D Systems, Minneapolis, USA) or glycocalyx shedding (syndecan-1; Abcam, Cambridge, UK) by commercial ELISA according to the manufacturer's instructions.

Statistical analyses

For analysis of risk factors predicting mortality, patients were divided in two groups according to hospital mortality. Continuous variables are presented as median with interquartile range [25th, 75th] or mean $\pm$ standard deviation and tested by the Mann–Whitney U test. Proportional data was analyzed using the Chi-squared test. A receiver operating characteristic (ROC) curve was generated to evaluate the discriminative ability of PBR for predicting hospital mortality. To that end the area under the curve (AUC), including a 95% confidence interval (CI), was used. Significance of the AUC was tested against an AUC of 0.5. Correlations between variables were calculated using the Pearson correlation coefficient if these variables were normally distributed, or the Spearman rank correlation coefficient for other variables. Data analysis was performed using SPSS version 24.0 (SPSS, Inc., Chicago, USA). P-values < 0.05 were regarded as statistically significant.

Results

Study population

Baseline characteristics are shown in Table 1. Within our study population, 45% was male and the overall mean age was 67 [61, 76] years, the mean APACHE IV score 99 [60, 136] and the mortality rate 43%. Only mean APACHE IV scores were significantly different between survivors and non-survivors (67 [56, 103] vs. 140 [94, 145], respectively) ($p=0.006$).

Table 1 Baseline characteristics for the entire study population as well as for survivors and non-survivors separately.

	Sepsis patients (n=21)	Survivors (n=12)	Non-survivors (n=9)	P-value
Age (years)	67 [61, 76]	69 [59, 75]	66 [61, 77]	0.92
Gender (male/female)	(9/12)	(6/6)	(3/6)	0.45
Septic shock, n (%)	14 (66.7)	6 (50)	8 (88.9)	0.06
Source of infection, n (%)				0.07
- abdominal	9 (40.9)	7 (53.8)	2 (22.2)	
- respiratory	6 (28.6)	1 (8.3)	5 (55.6)	
- urinary	2 (9.1)	2 (15.4)	0 (0)	
- other	4 (19.1)	2 (15.4)	2 (22.1)	
APACHE IV score	99 [60, 136]	67 [56, 103]	140 [94, 145]	0.006*
SOFA score	8 [7, 11]	8 [7, 10]	10 [9, 14]	0.06
Mechanical ventilation, n (%)	17 (81)	8 (66.7)	9 (100)	0.054
Lactate (mmol/L)[#]	3.4 [1.9, 4.7]	3.1 [1.5, 4.3]	4.0 [2.3, 5.9]	0.27
Heart rate (bpm)	108 [82, 115]	100 [78, 110]	114 [93, 128]	0.07
MAP (mmHg)	66 [62, 76]	71 [61, 85]	66 [62, 68]	0.19-
Creatinine (μmol/L)	105 [74, 224]	120 [72, 195]	101 [74, 235]	0.97
Total hemoglobin (mmol/L)	7.4 [5.7, 8.5]	7.7 [7.0, 8.8]	5.4 [4.9, 8.0]	0.07
SaO₂ (%)[#]	94.7 ± 1.8	94.4 ± 1.4	95 ± 2.1	0.44
Bilirubin (μmol/L)	17.5 [40.1]	20.4 [30.1]	13 [98.3]	0.90
C-reactive protein (mg/L)	233.2 ± 136.4	260.6 ± 158.3	196.8 ± 97.2	0.30
Thrombocytes (x10⁹/L)	196 ± 125.5	209.3 ± 114.4	178.4 ± 144.1	0.59
Leukocytes (x10⁹/L)	13.1 ± 9.4	13.9 ± 9.3	12 ± 10.1	0.67
Norepinephrine, n (%)	17 (81)	8 (66.7)	9 (100)	0.054
Time between PBR measurement and ICU admission (h)	10.9 [5.9, 14.4]	12.3 [7.2, 17.2]	9.3 [2.9, 11.7]	0.11

Continuous data are expressed as median with interquartile range [25th, 75th] or mean ± standard deviation. APACHE: Acute Physiology and Chronic Health Evaluation IV score; SOFA: Sequential Organ Failure Assessment score; ICU: Intensive Care Unit; MAP: Mean Arterial Pressure. Bpm: beats per minute; SaO₂: saturation O₂; PBR, perfused boundary region. [#]Data of these variables were not available for all 21 septic patients: lactate (n=18), SaO₂ (n=19), and bilirubin (n=18). *p < 0.05 non-survivors vs. survivors, Chi-squared test for proportional data and Mann-Whitney U test for other data.

PBR and mortality

The PBR was significantly lower in septic patients who ultimately survived to hospital discharge compared to non-survivors $1.76 [1.59, 1.97] \mu\text{m}$, vs $1.97 [1.85, 2.19] \mu\text{m}$; $p=0.03$) (Figure 1). Admission PBR can be considered a predictor of hospital mortality based on the ROC curve with an AUC of 0.778 (95%CI 0.578-0.977; $p=0.03$) (Figure 2A). Other variables showed an AUC of 0.847 (95%CI 0.657-1.000; $p=0.008$) for APACHE IV score and an AUC of 0.663 (95%CI 0.405-0.920; $p=0.25$) for lactate.

Figure 1. Perfused boundary region (PBR) measurements at admission in sepsis survivors ($n=12$) and non-survivors ($n=9$). Bars indicate median with interquartile range. * $p < 0.05$.

Figure 2. Receiver operating characteristic (ROC) curves of PBR and syndecan-1 for predicting hospital mortality in sepsis. (A) admission PBR (AUC=0.778; 95%CI 0.582-0.977; $p=0.033$) and (B) admission syndecan-1 (AUC=0.781; 95%CI 0.610-1.000; $p=0.035$). Reference lines have an AUC of 0.5.

PBR and circulating microcirculatory markers

We analyzed circulating markers in plasma drawn at the moment of PBR measurements in 17 patients (11 survivors and 6 non-survivors). Syndecan-1 levels were increased in the group of non-survivors as compared to survivors of sepsis ($p=0.04$). The ROC curve of syndecan-1 gives an AUC of 0.781 (95%CI 0.610-1.000; $p=0.04$) (Figure 2B). Ang-1, Ang-2 and HBP did not show statistically significant differences between survivors and non-survivors (Table 2).

Table 2 Biochemical data for sepsis survivors and non-survivors

	Sepsis patients (n=17)	Survivors (n=11)	Non-survivors (n=6)	<i>P-value</i>
Syndecan-1 (ng/mL)	181.4 [56.6, 313.2]	68.8 [36.7, 234.8]	302.7 [160.3, 5332.3]	0.04*
Ang-1 (ng/mL)	1.3 [0.4, 3.5]	1.8 [0.4, 3.9]	1.2 [0.001, 2.8]	0.48
Ang-2 (ng/mL)	17.2 [10.3, 22.7]	13.8 [9.2, 38.8]	17.8 [14.1, 21.4]	0.32
Ang-2/Ang-1	11.0 [5.4, 65.3]	9.3 [2.5, 23.2]	14.2 [7.6, 16717.1]	0.42
HBP (ng/mL)	30.3 [10.0, 88.6]	48.7 [24.8, 91.7]	13.9 [6.8, 214.6]	0.19

Data are expressed as median with interquartile range [25th, 75th]. Ang: angiotensin; HBP: heparin-binding protein. * $p < 0.05$ non-survivors vs. survivors, Mann-Whitney U test.

When studying the correlation between these plasma markers and the sublingual microcirculation as measured by non-invasive SDF imaging, admission PBR showed a positive correlation with Ang-2 levels ($n=17$, $\rho=0.532$, 95%CI [0.069, 0.806], $p=0.03$) (Figure 3) but no statistically significant correlation with syndecan-1 (Figure 4) nor any of the other microcirculatory markers tested (Ang-1, Ang-2/Ang-1, and HBP, all $n=17$, Table 3) or the clinical parameters APACHE IV ($n=21$, $r = 0.101$, 95%CI [-0.345, 0.510], $p=0.66$), lactate ($n=18$, $r=-0.053$, 95%CI [-0.424, 0.507], $p=0.83$) and SOFA score ($n=21$, -0.040, 95%CI [-0.463, 0.398], $p=0.86$).

Figure 3. Correlation between admission PBR and angiotensin-2 (Ang-2) in sepsis (n=17). PBR showed a positive correlation with Ang-2 levels ($\rho=0.532$, 95%CI [0.069, 0.806], $p=0.03$). ρ , Spearman's correlation coefficient.

Figure 4. Perfused boundary region (PBR) measurements at admission versus syndecan-1 (n=17). There was no correlation between PBR and syndecan-1.

Table 3 Correlations between admission PBR and circulating markers

	Correlation (rho)	P-value
Syndecan-1 (ng/mL)	0.087 [-0.411, 0.545]	0.74
Ang-1 (ng/mL)	-0.268 [-0.646, 0.244]	0.30
Ang-2 (ng/mL)	0.532 [0.069, 0.806]	0.03*
Ang-2/Ang-1	0.387 [-0.115, 0.731]	0.13
HBP (ng/mL)	0.181 [-0.328, 0.608]	0.49

Data are indicated as Spearman's rho [lower limit, upper limit 95%CI] for variables that are not normally distributed. All variables are n=17. Ang: angiotensin; HBP: heparin-binding protein. *p < 0.05 indicates a statistical significant correlation with admission PBR.

Discussion

This is the first study that shows that PBR values during the first 24 hours of ICU admission are associated with outcome in critically ill septic patients. Previous studies showed glycocalyx shedding to be associated with edema, microthrombi, inflammation, leukocyte adhesion and low flow [34]. It is plausible that this negatively affects outcome. Whether the relationship is truly causal, however, remains a point of investigation.

In our cohort the PBR measured within the first 24 hours was predictive for outcome. This finding and the cut-off point found should be confirmed in a larger validation cohort. Such a study should investigate the influence of factors such as age and chronic disease on the relationship between PBR and mortality.

Interestingly there was only a limited correlation between glycocalyx thickness as directly visualized using SDF imaging and measured using plasma markers of glycocalyx degradation and microcirculatory dysfunction. Only syndecan-1 was increased in non-survivors as compared to survivors. Our initial hypothesis was that syndecan-1 and PBR would also be correlated, as both are interpreted as indicators of glycocalyx degradation. However, our results did not support this assumption (rho=0.087, p=0.74), though the variability in our study population is rather great. These findings are in line with several other studies in various populations that did not observe a correlation between these parameters in hemodialysis [24,26], CKD [24,25] and sepsis [27]. Discrepancy between PBR and the measured glycocalyx constituent is also observed by Cornelis et al, who measured increased syndecan-1 levels after hemodialysis, whilst PBR did not alter [24]. Moreover, Donati and colleagues also tested other constituents of the glycocalyx and its relation to PBR. Hyaluronan did not show a correlation with PBR and only a minor positive correlation was found for heparan sulfate (HS) [27]. Several explanations can be conceived why glycocalyx thickness measured directly using SDF imaging does not correlate with plasma markers of glycocalyx shedding.

The PBR is defined as the cell-poor layer resulting from the phase separation between the flowing RBC and plasma, including the most luminal part of the glycocalyx that allows cell penetration [33]. HS is a more superficial glycocalyx marker that lines the inside of the glycocalyx, whilst syndecan-1 is the core protein binding HS and is anchored within the cell membrane [35]. It can be hypothesized that the earlier damage will first result in a shedding of HS, and that the more substantial damage which occurs later results in syndecan-1 release. However, Smart and colleagues showed in a cohort of septic patients in the emergency department that hyaluron, which is directly bound to the endothelium in the deepest layer, rose fast whereas syndecan-1 rose slowly [36]. In contrast, Gonzalez Rodriguez et al. found syndecan-1 to be high upon admission at the emergency department in trauma patients [37]. These findings would imply that PBR and syndecan-1 reflect different aspects of glycocalyx damage or that underlying pathologies can affect glycocalyx components differently. Another possible explanation could be a difference in the timing or localization of syndecan-1 release and the measured PBR. Heterogeneity in underlying disease state could have resulted in a variation in the kinetics of syndecan-1 in plasma, with possible differences in release, basal turnover and/or clearance [37–39]. Furthermore, the difference in individual glycocalyx thickness could lead to a greater variation in PBR and/or circulating syndecan-1 levels. Irrespective of the underlying mechanisms, our data indicate that PBR and syndecan-1 could be independent markers of glycocalyx disruption and combining these two markers may enhance discriminative ability. Unfortunately the sample size of this study precluded any such analysis.

PBR did correlate positively with Ang-2 levels. The positive relationship of PBR with Ang-2 supports the idea that glycocalyx degradation is connected to vascular permeability [7], with Ang-2 being able to induce heparanase-mediated glycocalyx degradation [40]. This correlation has also been observed in patients undergoing kidney transplantation [25]. Another permeability marker measured in our study, HBP, was not found to be correlated with PBR. A possible explanation for this difference is that HBP is released by activated neutrophils [41], while Ang-2 originates from Weibel-Palade Bodies in endothelial cells [42]. In our study, we did not specifically look at endothelial activation markers but in a study by Dane et al., PBR and soluble thrombomodulin levels were related to each other [25]. The glycocalyx and endothelium interact with each other in health and disease, and many markers of endothelial activation have been linked with sepsis severity and microcirculatory alterations [35,43,44]. Other groups have shown low levels of Ang-1, and high levels of Ang-2 and HBP to be related with mortality and multiple organ dysfunction in severe infection [45–47] and sepsis [48–50].

A limitation of the present study is that we solely looked at admission levels of PBR and circulatory markers of the microcirculation. It is known that microcirculatory alterations can persist for days, with syndecan-1 remaining elevated up to at least 50 hours in

hemorrhagic shock [29] and 7 days in sepsis [12]. Normalization of these levels are observed in survivors, whilst syndecan-1 remained elevated in non-survivors [12,29]. Syndecan-1 differed between survivors and non-survivors in our sepsis cohort. Other studies also found syndecan-1 levels to be higher in non-survivors of trauma [51] or sepsis [12,13]. Other clinically relevant markers could not distinguish survivors from non-survivors in our cohort of relatively ill septic patients.

The present study was set up in a single center with one expert measuring the glycocalyx thickness in all patients. While this approach limits interhospital and interoperator variability, it may have introduced bias. Additionally, one would ideally correct for possible confounders and covariates, such as APACHE IV and lactate using multivariable logistic regression. The limited sample size, however, did not allow this. It would be interesting to see how confounders and covariates influence the relationship between PBR and mortality. Serial imaging of the microcirculation in critically ill has also indicated that the change in microvessel density and perfusion show good discriminative ability in distinguishing survivors from non-survivors [30,52]. These studies all indicate that temporal improvements in these parameters were typically observed for survivors. Therefore, it would be interesting to investigate the course of serial PBR measurements in sepsis patients and its relation with both circulating markers and clinical outcome.

In conclusion, we show that a thinner glycocalyx layer, as measured by PBR, is associated with increased mortality in ICU patients admitted with sepsis. It remains to be seen whether glycocalyx degradation merely is a marker of an adverse outcome of sepsis or whether it is actually causally related. In the latter case preservation of glycocalyx integrity may become an interesting focus for future therapeutic interventions aimed at reducing multi-organ failure and mortality in sepsis.

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Chapter 6

Design and characterization of novel activated protein C (APC) variants for the proteolysis of cytotoxic extracellular histones

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In preparation

6

Abstract

Extracellular histones can induce various pathological effects *in vivo* including cell death and subsequent organ failure. Neutralization of these proteins is therefore rendered beneficial in various disease states. Activated protein C (APC) exerts such cytoprotective effects by inducing histone proteolysis, which reduces their toxicity. However, as an anticoagulant, APC can increase a patient's bleeding tendency. Molecular engineering of APC provides therefore the potential to optimize treatment of histone-mediated diseases by reducing the anticoagulant effect of APC while enhancing its proteolytic effect towards histones.

We investigated the molecular interactions between APC and histone H3 to design novel APC variants which are intended to have reduced anticoagulant activity but enhanced histone binding and cleaving properties. We then tested the anticoagulant and histone cleaving activity of these variants, comparing them to wildtype APC (WT-APC) and the well-studied 5A-APC with reduced anticoagulant activity.

Through structural bioinformatics experimentation we determined that APC residues Lys37-39, Lys62-63 and Lys74-75, which are involved in APC's anticoagulant effects, are also important for the interaction with histone H3. Reduced anticoagulant 5A-APC (Lys37-39Ala, Arg74-75Ala) showed improved histone binding compared to WT-APC. Other mutations of cationic residues to neutral or anionic residues, rendering 5D-APC (Lys37-39Asp, Lys62-63Asp) and 5D2A-APC (Lys37-39Asp, Lys62-63Asp, Arg74-75Ala), further improved APC-histone H3 interactions. All variants were determined to be less anticoagulant than WT-APC. 5A-APC was found to have the highest histone H3 cleavage rate, followed by both WT- and 5D-APC, and 5D2A-APC.

These novel designed APC variants can be potentially utilized as therapeutics in pathological states associated with elevated extracellular histone levels, whilst not inducing an increased risk for bleeding. Further characterization of these variants should determine their exact properties and efficacy towards extracellular histones.

Introduction

Histones are intranuclear proteins involved in the regulation of gene expression and packaging of DNA into structural units called nucleosomes [1]. Nucleosomes consist of a histone core comprising two copies each of histone H2A, H2B, H3 and H4, wrapped by a DNA helix of 146bp. The linker histone H1 connects these nucleosomes together to form the higher-order structured chromatin, ultimately forming the chromosomes. Once histones are released into the extracellular environment by either activated [2] or dying cells [3,4], they exert a pleiotropy of pathological effects culminating into tissue damage, organ failure and ultimately death [5,6]. The mechanisms by which extracellular histones exert these effects are thought to occur directly through interaction with cellular membranes [7,8] or indirectly through interaction with cell surface receptors (e.g. Toll-like receptors) [9,10], all leading to cell death and additional histone release. Extracellular histones are therefore considered as clinically relevant mediators in disease states such as systemic inflammation [11,12] and sepsis [13,14], in which histone levels correlate with severity and mortality.

Human recombinant activated protein C (APC), a trypsin-like serine protease, is a natural anticoagulant that regulates thrombin formation together with its cofactors protein S and factor V in the so-called protein C anticoagulant pathway [15–17]. Through its three loops in the positively charged exosite on the surface, the 191 loop (alternative chymotrypsin nomenclature: 37 loop) (residues 190-193, alternatively: 37-39), the calcium (Ca^{2+})-binding loop (residues 225-235, (70-80)) and the autolysis loop (residues 302-317, (148-154)), APC is able to bind and cleave the active coagulation factors V (FVa) at Arg506 and VIII (FVIIIa) at Arg336 and Arg562 [17,18]. Residue numbers of APC are mentioned to chymotrypsin numbering.

Besides being an anticoagulant molecule, APC also exerts various cytoprotective effects [19,20]. This includes the stabilization of the endothelial barrier and reduction in inflammation and apoptosis through activation of protease activated receptor-1 (PAR-1) and endothelial protein C receptor (EPCR) [19,21–27]. Another cytoprotective effect of APC is its ability to bind and cleave extracellular histones, thereby reducing their cytotoxic potency [5]. In sepsis, endogenous APC levels were found to inversely correlate with the amount of circulating histones [14]. Recombinant APC was approved by the FDA for human sepsis therapy in 2001 but was withdrawn from the market 10 years later [28] as it failed to show a reduction in mortality and resulted in an increased bleeding risk [29]. It is thought that this APC, by virtue of its residual anticoagulant activities, was not optimized for its cytoprotective effects and was therefore not effective in the large heterogeneous patient group.

Molecular engineered APC variants with reduced anticoagulant activity have since then been developed and successfully tested in pre-clinical disease models. One such APC

variant is the well-studied 5A-APC, which has mutations in three residues of the 37 loop and two residues of the Ca^{2+} -binding loop (Lys37-39Ala and Arg74-75Ala) and was found to possess minimal anticoagulant activity, whilst retaining normal cytoprotective activity [23]. It has been found to be protective in models of sepsis [30], stroke [21,31–33], endotoxemia [34] and ischemia-reperfusion injury [35,36], which can likely be explained by its effects through PAR-1 [30,33].

Preliminary work from our group has indicated that this 5A-APC has enhanced proteolytic activity towards histone H3 as compared to wildtype (WT)-APC (Figure 1A), that this APC-mediated histone cleavage is influenced by Ca^{2+} ions (Figure 1B) and phospholipids (data not shown), and that under these conditions, cleaved histone fragments of lower molecular weight are less cytotoxic to host cells (Figure 1C and D) [37]. Histone H3 cleavage sites were identified to be at the Arg26-Lys27 and at the Arg52-Arg53 peptide bond, with resulting histone H3 fragments of 12.49 kDa (Lys27-Ala135, as indicated in Figure 1E1) and 9.66 kDa (Arg53-Ala135, as indicated in Figure 1E2) in size.

In the current work, we want to extend these findings and design novel modified APC variants which lack anticoagulant activity but are optimized for proteolysis of extracellular histones. These variants could potentially serve as novel therapeutics in histone-mediated diseases without affecting a patient's bleeding risk. To develop these novel APC variants, various computational methods and experimental approaches will be combined and applied. First, we scrutinize the interactions between WT-APC and 5A-APC with histone H3 by applications of protein-protein docking and molecular dynamics (MD) simulations, providing insight into proposed essential interactions between APC and H3. This will then guide protein engineering to design novel APC variants with improved binding and cleaving properties for histone H3. The newly designed APC variants will be produced using recombinant DNA techniques and heterologous protein expression and will be characterized *in vitro* for their anticoagulant and cytoprotective properties.

Material and Methods

Molecular docking and molecular dynamics (MD) simulations of histone H3-APC complexes

The 3D structures of catalytic domain of human APC (PDB code: 1AUT) and human histone H3 (chain A and E of PDB code 1KX5) were obtained from the Protein Data Bank [38]. The possible binding modes between human APC and histone H3 were predicted by application of protein-protein docking method using ZDOCK [39] and the HADDOCK WebServer [40]. All docking solutions were visually inspected and the binding mode that showed interactions in agreement with experimental data (Arg26(P1-subsite)

Figure 1. Histone H3 cleavage by APC and its effect on cytotoxicity towards endothelial cells. (A) + (B). 40 µg/mL histone H3 was incubated for 30 min at 37°C with 50-500 nM WT-APC or 50-500 nM 5A-APC (A) or with 200 nM WT-APC and 0-5 mM CaCl₂ (B). All samples were analyzed with SDS-PAGE followed by Western blot using a rabbit polyclonal histone H3 antibody mapping an epitope at the C-terminus. Western blots are representative images of at least 3 experiments. (C) + (D). 40 µg/mL histone H3 was pre-incubated for 30 min at 37°C with 0-1000 nM WT-APC or 0-500 nM 5A-APC (C) or 0 or 200 nM WT-APC and 0-5 mM CaCl₂ (D). Samples were afterwards incubated for 30 min with EA.hy926 cells, followed by flow cytometric analysis of cell viability with propidium iodide. *p < 0.05 for comparison of WT-APC and 5A-APC in (C) and for comparison to mM CaCl₂ in (D), with n=2-9 per condition. (E) Edman N-terminal sequencing was used to determine the first five N-terminal amino acids of the histone H3 fragments with estimated MW of 12.32 kDa (1) and 9.70 kDa (2). Sequence of bovine Histone H3.1 (UniProt P68432) is shown with the first five N-terminal amino acids of the fragments in bold and underlined. The sequences of both histone H3 fragments are highlighted in grey [37].

and Lys27(P1'-subsite) of histone H3 binding at the binding pocket of APC) was selected. The complexes between WT-PC, the different mutant variants of human APC and histone H3 were subjected to molecular dynamics (MD) simulations and binding free energy (BFE) calculations.

Standard protocols and parameters were applied as described in previous work [41], with the exception that updated Amber14SB force field implemented in AMBER16 program were assigned for APC and histone H3. MD simulations were carried out for 100 ns. Trajectories from 80-100 ns were extracted and utilized for BFE calculations and decomposition (DC) energy analysis using molecular mechanics/generalized Born surface area (MM/GBSA) approach by applying default parameters and generalized Born model 8 for free energy of solvation as previously described [41].

Expression and purification of recombinant human PC

PCR-based site-directed mutagenesis of WT-PC was used to obtain recombinant human 5A-PC, 5D-PC, and 5D2A-PC (see Figure 2C for mutations). The cDNA of these variants was introduced into the eukaryotic expression vector pRC/CMV (Invitrogen, Carlsbad, USA), and then transfected into HEK293 cells (CRL-1573 ATCC) before protein C production and characterization as described before [42,43].

The purity and integrity of hPC preparations were checked using SDS-PAGE. Concentrations of hPC were determined by absorbance measurements at 280 nm using an absorption coefficient of 14.5 (280 nm, 1%, 1cm) and by ELISA. For the hPC ELISA, a polyclonal horse anti-human protein C antibody (1:5000, o/n 4°C, PAHPC-H; HTI, Vermont, USA) was used as a capture antibody and the polyclonal HRP-conjugated sheep anti-human protein C (1:2000, 4 hours at room temperature (RT), ABIN926359; antibodies-online.com, Aachen, Germany) was used as detecting antibody [44].

Activation of PC into APC

For the activation of PC into APC, purified PC fractions were loaded onto a 1 mL HiTrap NHS-Activated HP column (GE Healthcare, Little Chalfont, UK) to which 50 units of carrier-free Protac (Pentapharm, Basel, Switzerland) had prior been coupled. Up to 0.5 mg of PC (WT, 5A, 5D or 5D2A) was loaded onto the column for 1.5 hour at 37°C, was then eluted using 50 mM Tris, 175 mM NaCl buffer and concentrated using 10 kDa Ultra Centrifugal Filter Units (Merck, Darmstadt, Germany) or PD-10 desalting columns (GE Health Care, Little Chalfont, United Kingdom). Full activation was ensured by measuring the amidolytic activity assay of APC using the chromogenic substrate pyroGlu-Pro-Arg-p-nitroanilide (CS-2166; Hyphen Biomed, Neuville-sur-Oise, France) directly and after 1 hour of incubation at 37°C in the presence of additional 0.05 U/mL Protac (final concentration).

The completeness of activation was also checked by SDS-PAGE and subsequent Western blotting (see method Western blotting). APC concentrations were determined using the amidolytic assay or the earlier mentioned ELISA.

Histone H3 cleavage by APC

APC-mediated histone cleavage was studied by incubation of 5 µg/mL (317 nM) purified calf thymus histone H3 (Roche) with 10 nM WT- or variant APC for 30 minutes at 37°C in 25 mM Hepes, 150 mM NaCl, pH 7.4 buffer containing 5 mM CaCl₂. Samples taken at various time points were analyzed using SDS-PAGE followed by anti-histone H3 Western blot analysis (see method Western blotting).

Western blotting

Protein samples were analysed via SDS-PAGE electrophoresis (4-15%) and transferred onto PVDF membranes (Bio-Rad, Hemel Hempstead, UK) via semi-dry blotting. For the detection of APC, membranes were blocked and incubated with a sheep anti-protein C antibody (1:15000, CL20096A; CEDARLANE, Burlington, USA) overnight at 4°C, followed by incubation with a horse radish peroxidase (HRP)-labeled rabbit anti-sheep IgG antibody (1:1000, P0136 DAKO, Glostrup, Denmark) for 1 hour at RT. Commercial APC was used as a positive control (Xigris or drotrecogin alfa; Eli Lilly and Company, Indianapolis, USA).

For the detection of (cleaved) histone H3, membranes were blocked and incubated with a rabbit anti-histone H3 antibody (1:10000, ab183902; Abcam, Cambridge, UK) overnight at 4°C, followed by incubation with a biotinylated donkey anti-rabbit IgG antibody (1:10000, ab97083, Abcam) for 1 hour at RT. Subsequently, the membranes were incubated with StreptABComplex/HRP solution (1:500, Vector Laboratories, Burlingame, USA) for 30 minutes at RT before detection using chemiluminescent ECL substrate (Advansta, San Jose, USA). The cleaving rate constant (k-value) was determined after prior densitometry of the samples taken during a time-course of APC-catalyzed cleavage of histone H3 by ImageJ software (x86, Wayne Rasband), using the one-phase decay function ($Y(t)=Y_0e^{-kt}$) of GraphPad Prism 7 software (GraphPad Software Inc., La Jolla, USA).

Activated Partial Thromboplastin Time (APTT) assay

For this assay, one volume of human pooled plasma from healthy individuals was incubated in the semi-automated coagulation analyzer KC4 (Sigma-Aldrich, St. Louis, USA) for 5 minutes at 37°C with one volume of the APTT reagent (Chromogenix, Milan, Italy) containing purified phospholipids and colloidal silica as a contact activator. The coagulation pathway was then triggered by addition of CaCl₂ (5mM final concentration) in the presence or absence of WT and variants of APC in 50 mM Tris [pH 7.5], 175 mM NaCl buffer with 0.5 mg/mL BSA (0-60 nM final APC concentration), after which the time for

clot formation was recorded. The APC ratio was then calculated as the ratio between the clotting time in the presence of APC and the clotting time in the absence of APC, with the APC ratio of WT-APC serving as a reference value for the anticoagulant properties of the other APC variants.

FVa inactivation by APC

The APC-catalyzed inactivation of FVa was determined through following the loss of FXa-catalyzed prothrombin activation as previously described [45]. First, FV was activated to FVa by addition of a 5 μ L mixture of 175 nM thrombin and 1 mM phospholipid vesicles (10/90 M/M 1,2 dioleoyl-sn-glycero-3-phosphoserine (DOPS)/1,2 dioleoyl-sn-glycero-3-phosphocholine (DOPC)) to 215 μ L of 1/1000 diluted pooled plasma from healthy individuals in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂, 5 mg/mL BSA (buffer I). After 10 minutes incubation at 37°C and full activation of FV to FVa, 10 μ L of buffer I or purified recombinant APC variants (4.6 nM) was added and incubation was continued. 10, 12, 14, 16 and 20 minutes later, 10 μ L of prothrombin (25 μ M) and 10 μ L of FXa (7.5 nM) was added to determine the cofactor activity of FVa. After 1 minute, the reactions were stopped by addition of 10 μ L sample to 990 μ L of 50 mM Tris (pH 7.5 at 37°C), 175 mM NaCl, 0.5mg/mL ovalbumin (Sigma), 20 mM EDTA buffer containing 235 μ M chromogenic thrombin substrate H-D-Phe-Pip-Arg-p-nitroanilide (S-2238; Chromogenix). Thrombin was quantified by using the chromogenic substrate S-2238 [46] and was used to determine the FVa concentration as described earlier [15].

Statistical analysis

Data analysis was performed with GraphPad Prism 7 (GraphPad Software Inc.) using one-way analysis of variance (ANOVA) with Bonferroni post-test for multiple comparisons or independent samples t-tests. Experiments were performed independently at least three times and expressed as the mean $\pm$ standard deviation (SD). Differences were regarded statistically significant at P-values < 0.05.

Results

Design of novel APC variants for cleaving histone H3

The binding mode between human WT-APC and histone H3 was predicted by protein-protein interactions and the selected docking pose was then subjected to MD simulations. The APC-H3 complex derived from the last snapshot of MD simulations (100ns) displayed in Figure 2A as well as DC energy per residue shown in Figure 2B were utilized to scrutinize interactions between WT-APC and histone H3. Results reveal that Arg26 and Lys27 fit well into the substrate binding pocket of APC in agreement with the previous results such

as cleavage site prediction using bioinformatics algorithm [37] and experimental data (Figure 1).

Moreover, inspection of the APC-H3 complex shows that histone H3 also binds to the exosites of APC where Lys37-39 and Lys62-63 are and these residues may have repulsive interactions with Lys residues of histone H3 as demonstrated in Figure 2A. DC analysis confirms that these residues (Lys37-39 and Lys62-63) including Arg74-75 exhibit strong repulsive interactions with histone H3 as indicated by the high positive energy values shown in Figure 2B. Mutations of Lys37-39 and Arg74-75 to Ala (5A-APC) result in improved BFE between APC and histone H3 (-112.78 ± 8.01 kcal/mol) as compared to the WT-APC (-87.19 ± 13.31 kcal/mol), summarized in Figure 2C. Moreover, mutations of Lys37-39 to Ala can significantly reduce repulsive interactions between these residues and histone H3 while mutations of Arg74-75 to Ala in 5A-APC can alter from repulsive to attractive interactions between these residues with histone H3 as shown in Figure 2D. However, residue Lys62-63 in the 5A-APC system still exhibits strong repulsive interactions with histone H3 (Figure 2D). Therefore, to reduce repulsive interactions between APC and histone H3, Lys37-39 and Lys62-63 were in silico mutated to Asp (5D-APC) which might likely result in improved binding affinity with histone H3 which N-terminus contains several positively charged amino acids. Although Arg74-75 are located far from the binding area with histone H3 (Figure 2A), mutations of these residues to Ala such as in the 5A-APC system yield lower binding free energy and can alter from repulsive to attractive interactions between these residues with histone H3.

Thus, these residues (Arg74-75) were in silico mutated to Ala and combined with Lys37-39Asp and Lys62-63Asp (5D2A-APC). These designed novel APC variants (5D-APC and 5D2A-APC) were also subjected to MD simulations and BFE calculations. Results reveal that these novel APC variants gave BFE values lower than the WT-APC and 5A-APC as illustrated in Figure 2C. Furthermore, these mutated residues exhibit attractive interactions with histone H3 (Figure 2D). Thus, these novel APC variants (5D-APC and 5D2A-APC) were proposed for protein purification and consequently investigation of their binding and cleaving properties with histone H3.

Characterization of novel APC variants for histone H3 proteolysis

WT-PC, 5A-PC, 5D-PC and 5D2A-PC were produced in HEK293 cells under serum-free conditions and subsequently activated to APC by using a Protac column. Protac is a fast-acting PC activator isolated from the venom of the copperhead snake *Agkistrodon contortrix*, resulting in the removal of the 158-169 activation peptide of PC [47], which can be immobilized to a chromatography medium. Full activation was then ascertained via an amidolytic assay (> 95%). SDS-PAGE and subsequent Western blot analysis confirmed that > 95% pure APC was produced, as all preparations revealed a similarly sized protein band similar as for the commercially available Xigris APC (Figure 3).

Figure 2. Binding between APC variants and histone H3 and BFE between different APC variants and histone H3. (A) Binding mode of WT-APC (shown in green with white molecular surface) with histone H3 (shown in magenta). The complex is derived from the last snapshot of MD simulation (100 ns). Residues (Lys37-39, Lys62-63, and Arg74-75), which are located at the exosites of APC and are proposed for mutation, are shown as cyan sticks. Catalytic triad residues of APC are displayed as orange sticks. (B) APC residues interacting with human histone H3. Decomposition (DC) energy per residue of APC for interacting with histone H3; positive values indicate repulsive interactions whereas negative values imply attractive interactions. (C) Binding affinities between the different APC variants and human histone H3. Binding free energy (kcal/mol) calculations between different APC variants and human histone H3 are shown (mean ± SD). (D) Comparison of APC mutant residues interactions with human histone H3. Decomposition (DC) energy of WT and mutant residues derived from different APC variants for interacting with histone H3. Residues are indicated using the chymotrypsin nomenclature.

Figure 3. Western blot analysis of produced mutant variants of human APC. Between 75-300 nM APC was loaded on SDS-PAGE gels and analysed with Western blot using an anti-protein C/APC antibody. Commercial APC (Xigris) was compared to the recombinant human WT-, 5A-, 5D- and 5D2A-APC.

Anticoagulant activity of novel APC variants

In order to study the anticoagulant properties of these novel APC variants, the influence of APC on coagulation was tested via two different assays: the APTT assay and the FVa inactivation assay. In the first assay, the effect of APC on clotting time is assessed through initiation of the contact (intrinsic) pathway of coagulation by using colloidal silica as an activation trigger. Figure 4A shows the APC ratios for the various APC variants with increasing concentration.

As expected, at increasing concentrations of WT-APC, the APC ratio increases, which can be explained by a prolongation of the clotting time at higher APC concentration as compared to the clotting time without APC. The ratio was found to increase over 105% for WT-APC when comparing the clotting time for the highest concentration tested (60 nM) with the clotting time for the condition without APC (Figure 4B). The other variants show an increase in the APC ratio between 0 nM and 60 nM APC of 30-53% and are therefore less anticoagulant than the WT-APC. In this assay, the anticoagulant activity did not differ between the engineered APC variants (P-value between 0.07-1) and all APC variants presented partially anticoagulant activities.

In the second assay, the FVa inactivation assay, the anticoagulant effects of the APC variants towards FVa was studied in a relatively pure experimental system through measurement of thrombin mediated substrate cleavage. At the start of the experiment, FV from plasma is activated into FVa by addition of thrombin and CaCl₂. After this activation, the reaction is initially incubated with APC (first 10 minutes in Figure 5) after which prothrombin and FXa are added to form the prothrombinase complex. The effect of APC on the conversion of prothrombin into thrombin is measured as a time course of FVa inactivation (between 10-20 minutes in Figure 5A). FVa is quantitated by its capacity to promote the FXa-catalyzed activation of prothrombin to thrombin and the chromogenic measurement of thrombin via a chromogenic thrombin substrate. The assay thereby shows the inactivation of FVa as a function of time in the presence of APC, as compared to the stability of FVa

formed over time, in the absence of APC. When comparing the level of FVa inactivation between the different variants, the 5A-APC was found to be almost nonanticoagulant as it did hardly affect FVa activity and showed a non-significantly different signal as in the control condition in the absence of APC (Figure 5A). Based on the fitted curves, the initial FVa cleaving rates of all variants were estimated and compared to WT (Figure 5B). The estimated fold change in initial cleaving rate was 229, meaning the FVa cleavage was 229 times slower as compared to WT. The other two variants showed intermediate effects on FVa inactivation, with the 5D2A-APC being slightly less anticoagulant (estimated fold change of 8) than the 5D variant (estimated fold change of 4).

Figure 4. APC ratios of WT-APC and APC variants. APTT analysis was performed to investigate the anticoagulant effects of APC variants. (A) Curves indicate the APC ratios of each APC variant between 0-60 nM APC. The APC ratio represents the ratio between the clotting time in the presence of APC and the clotting time in the absence of APC. (B) Overview of the % ratio increase between 60 nM APC and 0 nM APC for each variant and the comparison between these values of each variant with WT as assessed with the independent sample t-test. Data are indicated in mean ± SD from 3 independent experiments. * $p < 0.05$, *** $p < 0.001$ comparing WT-APC to other variants.

Figure 5. FVa inactivation by APC variants. (A) The fitted curves show the time course of FVa inactivation for the different APC variants, as determined by the FVa assay described in the methods section. (B) *Shows the estimated fold changes between the initial FVa cleaving rates of the APC variants as compared to WT (set to 1), with a higher fold change meaning a lower cleaving rate constant (based on the fitted curves in (A)).

Histone cleavage by novel APC variants

Next, we investigated the histone cleaving potential of the novel APC variants as compared to wild type. For this, 5 µg/mL purified bovine histone H3 was incubated with 10 nM APC for up to 30 minutes. Samples taken at different time points were analyzed for histone cleavage by gel electrophoresis and anti-histone H3 Western blotting (Figure 6). An example of this APC-mediated cleavage of histone H3 is depicted in Figure 6A, where WT-APC is able to fully cleave histone H3 within 30 minutes. Full histone cleavage within this time period was also observed for the 5A and 5D variants, whilst the proteolysis approaches completion for the 5D2A-APC (Figure 6B). The decrease of uncleaved histone H3 over time indicates different cleaving reactions for each individual variant. The calculated cleavage rates or *k*-values were based on the fitted curves presented in Figure 6B and was the highest for the 5A-APC (0.86 ± 0.05), followed by WT-APC (0.13 ± 0.01) and 5D-APC (0.10 ± 0.01), and finally the 5D2A-APC (0.06 ± 0.004), which has the significantly lowest histone H3 cleavage rate (Figure 6C).

Figure 6. Western blot analysis of histone H3 cleavage by variants of APC. (A) 317 nM (5 μ g/mL) Histone H3 was incubated with 10 nM WT-APC at 37°C for up to 30 minutes. Histone H3 cleavage was analyzed using SDS-PAGE followed by Western blotting using an anti-histone H3 antibody. Western blot is representative of histone cleavage of at least 3 experiments. (B) Fitted curves of histone H3 cleavage over time by APC variants from which the cleavage rate constants or k-values are derived and are shown in (C). Data are presented as mean $\pm$ SD. * $p < 0.05$, *** $p < 0.001$ as compared to WT-APC.

Discussion

In this study we aimed to identify key residues involved in the interaction between histone H3 and APC in order to design and produce novel APC variants with improved proteolytic activity. Molecular docking and simulation strategies were applied and these supported the interaction between R26 of histone H3 and APC, proposed as a cleavage site by Xu et al. [5], as this residue was found to fit well into the binding pocket. In addition, the complex between APC and histone H3 revealed that histone H3 also binds to the exosite of APC in which the residues Lys37-39, Lys62-63 and Arg74-75 are located. The minimally anticoagulant 5A-APC has the lysines at residues 37-39 and the arginines at positions 74-75 mutated to alanines, which inhibits the cleavage of FVa [23,24,48] and FVIIIa [49] as well as its binding to thrombin/thrombomodulin (TM) [50]. When comparing the 5A-APC binding to histone H3, the BFE decreases, which indicates improved interactions as compared to WT-APC. This is supported by *in vitro* data that show enhanced histone proteolysis by 5A-APC. Therefore, these mutated residues seem to reduce APC's

anticoagulant actions whilst increasing its cytoprotective activity through stimulation of histone cleavage.

Despite 5A-APC's enhanced interaction with histone H3 as compared to WT-APC, we propose that additional mutations within the APC molecule could further improve its proteolytic effects. For this purpose we mutated residues involved in another potential histone binding, Lys62-63, which is partially involved in APC's anticoagulant properties by stimulating heparin-mediated APC inhibition by PC inhibitor [51,52]. As these lysine residues, and the lysines at residues 37-39, are proposed to have repulsive interactions with the positively charged lysine residues of histone H3, the positive lysines were mutated into negatively charged aspartic acid residues (rendering 5D-APC). In another variant, these 5 lysine mutations were combined with 2 additional mutations in residues 74-75 (also mutated in the 5A-APC) in which the arginines were replaced with aspartic acid (rendering 5D2A-APC). BFE calculations revealed that the interactions with histone H3 were strongest for the 5D2A-APC variant, followed by the less mutated 5D-APC, the earlier described 5A-APC, and finally WT- APC. Therefore, we hypothesized that these variants will show a similar trend in the cleaving rates of histone H3.

The anticoagulant properties of the APC variants were investigated by studying their effect on both clotting time (via APTT) and inactivation of FVa. In the APTT assay, we observed that all variants similarly affected the clotting time but all to a lower extent than WT-APC. However, all variants still retained 30-53% anticoagulant activity. In the FVa inactivation assay, the 5A-APC variant was found to express essentially no proteolytic activity towards FVa. The novel 5D and 5D2A variants both inactivated FVa for about 50% as compared to the anticoagulant WT-APC.

As for the 5A-APC, earlier studies have shown that it exerts <10% anticoagulant activity [23]. In our work, the FVa inactivation assay supports this whilst the APTT experiments indicate a higher anticoagulant activity of 5A-APC. The APTT assay is started by initiation of the contact activation pathway in a plasma-rich environment, whilst the FVa inactivation assay uses highly diluted plasma to study the specific effect of APC on FVa activity. This difference in trigger and reaction environment could result in the differences between both assays. The APTT assay can also be considered a more global clotting test as compared to the specific FVa inactivation assay, with the latter being likely more sensitive in detecting differences between individual APC variants.

In the FVa inactivation assay, only 5A-APC was found to exert almost no anticoagulant activity whilst the other APC variants all remained partially anticoagulant. The cleavage sites of FVa are identified as Arg306, Arg506 and Arg679 [15,16,53]. Earlier studies have shown that the loss of lysines on positions 37-39 inhibits the FVa cleavage at Arg506 considerably, and that the loss of lysines in residues 74-75 also contribute but to a slightly lesser extent [18,48]. This explains the lack of FVa activity from the 5A-APC variant

in our study. The more proteolytically active 5D and 5D2A variants have substituted residues at positions 62-63, which indicates that these mutated residues are still involved in the inactivation of FVa and is likewise reported before [18,48].

Nevertheless, we observed that APC's anticoagulant functions are not needed for histone cleavage and that the reduced anticoagulant mutants could be protective in histone-diseases, like has been studied *in vivo* [34,35]. However, additional assays should be performed to decipher the full range of activities towards the coagulation system. These assays could include the activity towards FVIIIa, another important substrate of APC [17], the influence of protein S, the inhibition of APC by protein C inhibitor (PCI), or the effect towards plasminogen activator inhibitor-1 (PAI-1) in fibrinolysis [54].

Based on the histone proteolysis experiments, 5A-APC was found to be the most cytoprotective as it had the highest cleavage rate towards histone H3. This was followed by both WT- and 5D-APC, and finally 5D2A-APC, which had a significantly lower cleavage rate and did not result in complete histone cleavage after 30 minutes. The other variants were less efficient in cleaving extracellular histone H3 in our setup, which seems counterintuitive based on our MD simulations. We hypothesize that several explanations may add to these observations. One potential explanation is that an *in vitro* biological setting is simply more complex and essentially different than *in silico* calculations, which are mere predictors of interactions between enzyme and substrate based on a specific set of interaction parameters, which are mostly enthalpic by nature. Therefore, proposed interactions might be different in their extent between the enzyme and substrate. Another explanation for the lack of correlation between the strength of H3-APC interaction and the potency of APC-mediated histone H3 cleavage is that the novel APC variants may in fact bind too firmly to their substrate or reaction products. For the 5A-APC, due to the lower binding energy and the enhanced cleaving rate as compared to the other variants, histones are most likely bound and instantly cleaved, thereby facilitating a fast release from the molecule. For the other variants, especially the 5D2A-variant carrying an extra mutation, histones are most likely bound rapidly and strongly by APC and may not release quickly from the binding site after cleavage. The negatively charged aspartic acid groups in these mutants induce better electrostatic interactions with the lysines on histone H3 than the uncharged alanine groups in the 5A-APC. This makes that for the 5D- and 5D2A-variant, histone could present itself as a substrate with high k_{on} but very low k_{off} , thereby effectively preventing the enzyme from catalyzing more proteolytic events. The binding affinities and kinetics of these interactions should be studied in more detail to substantiate this hypothesis and gather information on the exact mechanism of histone binding and proteolysis.

The APC-catalyzed cleavage of histones can be influenced by many factors, as can the functionality of the APC variants. Both can be attributed to different sensitivities

towards (circulating) plasma proteins, which may bind histones and/or APC [19,55]. Likewise, different forms of histones (e.g. free histones or complexed) or specific histone subtypes could also be of influence on the nature of their interactions with proteases, as histones can for example exert subtype specific effects in terms of cytotoxicity (own observations and [5]) and TM activation [56]. Therefore, additional studies should focus on the identification and characterization of these determinants of APC-mediated histone cleavage to further improve the design and efficacy of therapeutics in disease.

Taken together, we have identified key residues involved in the interaction between APC and histone H3, which were also involved in APC's anticoagulant functions. This knowledge was used for the protein engineering of novel APC variants with proposed enhanced histone binding and cleaving abilities. These mutants were rendered less anticoagulant and were able to cleave histone H3 with variable efficacy. Additional studies should investigate this APC-mediated proteolysis in more detail and assess the therapeutic potential of these variants in histone-mediated diseases.

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Chapter 7

General discussion

7

Histone proteins serve vital functions in evolutionary biology. Their involvement in the regulation of gene activity and gene expression renders them crucial proteins in normal development [1]. Along with these physiological functions, histones can exert pathological effects once present extracellularly [2,3].

This thesis describes studies that were performed on the presence of extracellular histones in several conditions associated with tissue damage, to elucidate their role in these processes. Furthermore, studies are described that investigated methods for neutralization of histone-mediated toxicity. Particularly, we aimed (I) to provide an extensive overview on the anticoagulant and nonanticoagulant functions of heparin, (II) to study the release of extracellular histones during kidney preservation and its implication for organ function after transplantation, (III) to investigate histone release in the context of ischemia in which the effects of the histone-binding polysaccharide heparin are assessed, (IV) to determine parameters of microvascular injury in sepsis and study their association with circulating histones as well as clinical outcome, and (V) to assess and optimize histone proteolysis by the serine protease activated protein C (APC). In this chapter, the present findings will be discussed in the view of recent literature whilst translating them into a clinically relevant context.

Functions of heparin beyond anticoagulation

Heparin is a heterogeneous mixture of linear polysaccharides with varying chain length that are composed of different repeating sugar units and degrees of sulfation [4]. The negatively charged sulfate groups are important for heparin's functions [5], which can be anticoagulant and nonanticoagulant in nature. The anticoagulant functions of heparin have been described for many decades (Chapter 2). The nonanticoagulant functions, however, have not been recognized until more recently. Heparin's anti-metastatic, anti-inflammatory and anti-apoptotic properties make it a potential therapeutic in various pathological disease states. Yet, application of heparin may be associated with an increase in anticoagulant effects as well. For this reason, deciphering the exact properties and structural requirements of heparin will aid in the design of optimized heparin formulations for particular applications.

The first alterations of the traditional heparin drug (unfractionated heparin or UFH) were made via chemical or enzymatic fragmentation [6]. This yielded heparin preparations of lower molecular weight (LMWH) with improved bioavailability and a more predictable dosing than UFH [7].

Later it was recognized that not all heparin molecules within UFH preparations possessed anticoagulant properties. Roughly 70% of the heparin molecules in UFH are

not able to bind antithrombin (AT) (termed “inactive heparin”) [8], which is required for its anticoagulant effects towards coagulation factors thrombin and Xa. The remaining 30% or “active” material is thought to be the predominant mediator of anticoagulant effects of heparin. Heparin-associated side effects are not that frequent and only minor in nature, which indicates that the largest part of heparin molecules (the “inactive” heparin) may either be inert or contribute to the overall beneficial effects of UFH.

Our group has employed the possibility of separating the two heparin fractions and specifically isolated the heparin fraction that is devoid of anticoagulant activity [9]. By means of affinity chromatography using AT that is immobilized to a column matrix, UFH can be fractioned into AT binding (anticoagulant) and non-binding (nonanticoagulant) heparin. The nonanticoagulant heparin has been tested in various pre-clinical models of inflammation and tissue injury, in which we have shown that it has pharmacological activity by neutralizing histone-mediated toxicity and diminishing organ damage [9]. Our findings have led to the initiation of a clinical development program to obtain a GMP-grade nonanticoagulant heparin product that may be applied for the treatment of patients suffering from fulminant inflammation such as sepsis.

Extracellular histones in human kidney transplantation

Histones can be released into the vasculature under pathological conditions characterized by immunoactivation and cellular injury, and function as inflammatory, thrombotic and cytotoxic agents [2,3]. Likewise, histones are implicated in conditions associated with ischemia such as stroke [10,11], vascular necrosis [12] and acute organ injury [13,14]. Ischemia is also likely to occur in donor kidneys during the period between collection and transplantation. Hypothermic machine perfusion (HMP) of donor kidneys is believed to limit the deleterious effects of ischemia on stored kidneys.

In chapter 3 we investigated the presence of extracellular histones in the perfusate of human kidneys preserved with HMP before transplantation. Cells that are affected by ischemia are prone to cell death, which can result in the release of intracellular material [15]. As expected, extracellular histones were detected in ischemic human kidneys that were preserved by HMP (Chapter 3, [16]). The extent of histone H3 release after 4 hours of perfusion was found to correlate with kidney dysfunction after transplantation. The primary non-functioning grafts (PNF) and delayed functioning grafts (DGF) had higher perfusate levels of extracellular histone H3 than primary functioning grafts (Chapter 3, [16]). Not only did these levels correlate with the post-transplantation parameters graft function, graft survival and 3-month serum creatinine, they also correlated with the pre-transplantation factors warm ischemia time (WIT) and donor serum creatinine (Chapter

3, [16]). Therefore, initial damage inflicted upon the organ during the time between donor death and organ procurement (e.g. WIT), likely induces a sequence of events that progressively deteriorates kidney function. Based on these results we propose that circulating histones are predictive biomarkers in organ donation.

Previous studies have shown that histones are toxic to endothelial [17], renal [13,18] and glomerular cells [12] *in vitro*. These histone proteins seem to contribute to the decrease in glomerular filtration rate and albuminuria in an *in vivo* model of glomerulonephritis, as anti-histone strategies significantly improved renal function [12]. Injection of anti-histone immunoglobulins immediately after 30 minutes of bilateral renal artery clamping prevented post ischemic tubular damage [13]. Although we did not measure histone levels before or after machine perfusion, the studies indicate that histones can contribute actively to deterioration of kidney function and might also play a role during other stages of the donation process.

The predictive value of extracellular histone H3 levels on PNF and DGF was found to be comparable to that of other reported biomarkers such as neutrophil gelatinase-associated lipocalin (NGAL), liver-type fatty acid-binding protein (L-FABP), kidney injury molecule-1 (KIM-1) and lactate dehydrogenase (LDH) [19–21]. However, only extracellular histones appeared to be independent predictors of delayed graft function when data were adjusted for relevant confounders. Histone presence within the perfusate correlated inversely with one-year graft survival (Chapter 3, [16]). This significant negative association with one-year graft survival seems to be determined by the initial rates of PNF, leading to an immediate drop in graft survival rate in the high histone H3 level group. It further suggests that circulating histones likely compromise graft function after transplantation. The association with serum creatinine 3 months after transplantation suggests histones induce a prolonged reduction in kidney function.

Nevertheless, the predictive value of extracellular histones in our cohort was rather limited and could therefore not justify yet their use as biomarkers to select donor kidneys for transplantation. Additional studies from independent cohorts are required to determine the value of extracellular histones as stratifying biomarkers. Furthermore, future studies should elucidate the nature of the relationship between circulating histones and graft function. Insights into this relationship will help to understand the value of extracellular histones both as biomarker and therapeutic target.

Extracellular histones in an *ex vivo* ischemic kidney perfusion model

Chapter 4 describes the use of an *ex vivo* porcine kidney perfusion model with ischemically damaged kidneys to study the release of extracellular histones in more detail. In this model we observed an exponential release of histone H3 into the perfusate when kidneys were perfused at 28°C (subnormothermic perfusion or SNMP) whilst there was no substantial release when the perfusion was performed at 4°C (HMP). These findings illustrate the ischemia- and time-dependent release of extracellular histones during machine perfusion. This also supports the association between WIT and perfusate histone H3 that we observed in our human study (Chapter 3, [16]).

Remarkably, we measured extracellular histones in cold perfused human kidneys but not in cold perfused porcine kidneys. This discrepancy likely was the result of the fact that the porcine kidneys were extensively flushed before application of HMP. Extensive flushing removes more radically blood leukocytes from the kidney suggesting that leukocytes are the initial source of extracellular histones. This is also suggested by a small animal study using a pig-to-baboon xenotransplantation model [22]. Animals displaying consumptive coagulopathy and infection had the highest histone levels after transplantation, although the groups were small and histone levels were highly variable [22]. In our *ex vivo* porcine perfusion model, we extensively flushed and perfused the retrieved organ with decellularized kidney perfusion solution (KPS-1) to remove most intravascular (immune) cells (Chapter 4). It does not, however, eliminate any residual immune cells that are present within the tissue of the organ.

Electron microscopic tissue analysis of these ischemic kidneys indicate that host cells (e.g. renal tubular cells and endothelial cells) are another likely source of extracellular histone release during machine perfusion (Chapter 4). We observed that histones were localized both in the cytosol and near or at the cell surface of these cells, implying that cellular injury leads to the release of histones into the perfusate (Chapter 4). Indeed, cell death is more profound in kidneys with more extensive ischemic damage and apoptosis is thought to be evident after reperfusion (Chapter 4 and [23]). The time-dependent, exponential release of histones in machine perfused kidneys seems to suggest that progressive ischemic damage contributes to this release. Cytotoxicity of extracellular histones, likely results in a self-sustaining cascade of (active) cell death and additional histone release [24]. In our *in vitro* experiments, this histone cytotoxicity appears to be decreased at 4°C, suggesting that lowering of the machine perfusion temperature is beneficial for stored kidneys.

Heparin has shown a clear benefit *in vitro* and *in vivo* through binding of extracellular histones (Chapter 2 and [25]). We hypothesized that heparin would reduce the amount of circulating histones in our machine perfused kidneys and potentially improve kidney

viability. Administration of heparin at the start of machine perfusion did however not reduce the total amount of extracellular histones released into the perfusate (Chapter 4). Remarkably, we demonstrated that the appearance of extracellular histone H3 was altered in kidneys perfused with heparin. Low molecular weight histone H3 (LMW-H3) was detected earlier and more pronounced in the heparin group.

The appearance of LMW-H3 in the circulating perfusate could be the result of either histone proteolysis and/or histone modification. Protease factor VII-activating protease (FSAP) was identified as a nucleosome releasing factor and can be activated or inhibited by heparin depending on the presence of serine protease inhibitors [26]. Interestingly, histones can induce FSAP activation which then stimulates their own degradation by the protease [27,28]. Histones bound to immobilized heparin possibly still possess the ability to bind plasma proteins, which could render them as bridging or crosslinking molecules and enhance their own proteolysis by simultaneously binding multiple molecules [32]. Other enzymes such as APC (Chapters 4, 6 and [17]), elastase (Chapter 4 and [29]), cathepsin L [30] and granzyme A [31] are able to cleave histones as well.

In our perfusion model, the generation of LMW-H3 could be initiated as such. Unfortunately, we were not able to deduce whether a protease is indeed involved in this process. What we did find is that the LMW-H3 seems to be truncated somewhere within the N-terminus, although we could not specify its exact sequence (Chapter 4). The N-terminal part of histones is prone to proteolysis [33,34] and modifications [1]. Both acetylated histone H3 and acetylated high mobility group box-1 (HMGB1), another intranuclear protein with pathological effects [35], were found to be upregulated in ischemically damaged livers [36]. Moreover, the acetylated form of HMGB1 was found to be released from *ex vivo* and *in vitro* ischemic hepatocytes. Therefore, it is possible that the LMW-H3 appearing in the perfusate is a modified form of histone H3 with different gel-electrophoretic properties. We did not detect phosphorylation or citrullination of histone H3 in our samples (unpublished data). However, there are many other histone modifications that could be tested and that could explain the observed change in apparent molecular weight. More sophisticated analysis can hopefully determine the exact size and origin of LMW-H3 and demonstrate whether heparin plays an active role in its appearance within the perfusate.

In some preliminary experiments, we were not able to show a reduction in apoptotic cell death within biopsies of heparin treated kidneys (unpublished data). These findings are somewhat contradictory to the benefit of heparin in reducing toxicity *in vitro* and *in vivo* [25,37]. Supposedly, heparin can bind to the cell surface within hypothermically perfused porcine kidneys and protect renal cells after normothermic oxygenated reperfusion [38]. The high perfusion temperature used in our setup might inflict irreversible kidney damage or mask less pronounced effects of heparin treatment during machine perfusion.

More sensitive analysis of cellular function could give a more definite answer regarding these issues.

All in all, extracellular histones appear to be involved in organ perfusion and their release is related to ischemic damage. Heparin administration did not appear to reduce release of histones and presence of circulating histone levels or show a clear benefit on graft viability. Heparin administration did, however, induce the release of a lower molecular weight histone H3 variant, the implications of which should be investigated further. Our work further suggests that kidneys are best preserved cold in order to reduce ischemia and histone release. Whether extracellular histones are active contributors towards organ damage in these settings remains to be investigated.

Extracellular histones, microcirculatory dysfunction and mortality in sepsis

Global disease burden and death by sepsis remain high due to inadequate diagnosis, monitoring and treatment [39]. An important mediator of disease severity and organ failure in sepsis is the endothelial glycocalyx of the microcirculation [40]. The glycocalyx is damaged in sepsis, leading to the shedding of its constituents into plasma [41,42]. There they are able to bind [43] and neutralize circulating histones [44,45]. In our study we examined the association between circulating markers of microvascular dysfunction and parameters of non-invasive microcirculatory imaging at an early stage in sepsis (Chapter 5). A second aim was to investigate the relation between extracellular histones and glycocalyx integrity.

We found that the perfused boundary region (PBR), as an inverse parameter of endothelial glycocalyx thickness, was significantly higher in non-survivors as compared to survivors of sepsis within 24 hours of intensive care unit admission (Chapter 5). Also increased shedding of syndecan-1 was observed in non-survivors. Surprisingly, none of the other circulating markers (angiopoietin (Ang)-1, Ang-2 and heparin binding protein (HBP)) were significantly associated with mortality. This can most likely be attributed to the relatively small sample size of 21 patients. PBR and syndecan-1 did not correlate with one another in our study, which is in agreement with earlier work [46–49]. An explanation for this lack of correlation could be that syndecan-1 and the PBR reflect different aspects of the glycocalyx. Syndecan-1 is a core protein imbedded in the impermeable layer of the glycocalyx and reflects more profound shedding of the glycocalyx [4]. PBR on the other hand, might reflect the superficial layer of the glycocalyx still permeable for circulating red blood cells [50]. Heparan sulfate (HS) is a more superficial glycocalyx constituent through its linkage with core proteins like syndecan-1 [4]. In one study, HS was found to have a

weak association with PBR [49]. Interestingly, not the absolute HS levels but changes in HS levels over the course of red blood cell transfusion correlated with changes in PBR. Serial imaging of the microcirculation in critically ill has indicated that the change in microvessel density and perfusion show good discriminative ability in distinguishing survivors from non-survivors [51,52]. Therefore, we propose that changes within microcirculatory parameters may be more indicative of ongoing glycocalyx impairment than single point measurements.

Both increased PBR and syndecan-1 were associated with elevated Ang-2 levels. Other markers of vascular permeability (Ang-1 and HBP), however, did not correlate with PBR or syndecan-1 (Chapter 5). This is in concordance with some [53], but not all studies [47,48] and underlines the complexity of the relationship between these markers. In addition, we were not able to correlate extracellular histone levels to microcirculatory parameters as we only detected plasma histone H3 in 2 out of 21 sepsis patients (data not shown). This was an unexpected result, given that extracellular histones are regularly correlated with disease severity and clinical outcome in sepsis [54–57]. We can only speculate why the number of patients positive for extracellular histones was low.

First of all, sepsis is known to be a very heterogeneous disease state. This is apparent from the reported extracellular histone levels, which vary between the ng/mL and µg/mL range [54,57–59]. Although our study included predominantly septic shock patients (68%), plasma levels of histones do not always correlate with clinical injury scores such as SOFA or APACHE II [55]. In a previous study from our group, 10 out of 43 patients were negative for plasma histone H3 [57], and also in another study not all septic patients had detectable extracellular histones [59]. It could be that the majority of patients had plasma histone H3 below our detection limit of 50 ng/mL.

Another explanation could potentially be in the methods of pre-analytical sample processing. Although we processed our blood samples within 1 hour and minimized the time between plasma thawing and analysis, histones are found to be instable in spiked plasma [54]. This instability could be attributed to the binding of histones to other proteins [32] or (plastic) surfaces (own observations). Therefore, subtle differences between protocols and materials used might influence the detection of extracellular histones in stored plasma samples. The small cohort size unfortunately precludes any solid conclusions on the contribution of any pre-analytical, clinical or laboratory parameters to the low numbers of detectable plasma histone H3.

In conclusion, glycocalyx integrity is compromised in sepsis patients within 24 hours of admission. PBR measurements and syndecan-1 levels were able to predict sepsis mortality, whilst other invasive markers reflecting glycocalyx shedding or vascular permeability were not. Microcirculatory monitoring by measurement of PBR should be studied in additional cohorts to determine its use in clinical practice. Also, the potential relationship between

glycocalyx integrity and extracellular histone levels should be investigated in patient groups as this currently remains unknown.

Activated protein C variants and their effects on histone proteolysis

APC is a serine protease that exerts both anticoagulant and cytoprotective properties [60]. Its ability to proteolyse extracellular histones reduces the histone-mediated toxicity towards cells (Chapter 6 and [17]) and provides a potential treatment strategy in histone-mediated diseases. At present however, the clinical use of APC is considered unsafe due to the associated risk of bleeding. Modulating APC into a nonanticoagulant molecule could therefore reduce these complications whilst maintaining its cytoprotective effects. One such variant is 5A-APC [61], which has <10% anticoagulant activity [62] and was found to be protective in stroke [63], endotoxemia [64] and ischemia-reperfusion injury [65]. 5A-APC is able to cleave extracellular histones significantly better as compared to wild type (WT)-APC (Chapter 6 and [66]). Mutation of five positively charged residues (Lys37-39 and Arg74-75) into neutral alanines supposedly improves the binding and subsequent proteolytic neutralization of histones.

In our study we aimed to further improve the proteolytic capacity of APC towards histones by specifically mutating key residues involved in the interaction of APC with histone. In this way, novel APC variants with minimal anticoagulant activity and improved histone proteolytic capacity can be created. Molecular simulation studies identified Lys37-39, Lys62-63 and Arg74-75 as key residues of APC interacting with histone H3. These positively charged amino acid residues are thought to have repulsive effects towards the positively charged lysine groups in histone H3. Specific mutations of these residues were introduced by recombinant DNA technologies to improve the electrostatic interactions between APC and histones. 5A-APC (Lys37-39Ala, Arg74-75Ala), 5D-APC (Lys37-39Asp, Lys62-63Asp) and 5D2A-APC (Lys37-39Asp, Lys62-63Asp, Arg74-75Ala) all showed reduced anticoagulant activity as compared to WT-APC (Chapter 6). The 5A-APC variant completely lost its anticoagulant activity in a FVa inactivation assay whilst the other APC variants remained partially anticoagulant. Earlier studies showed that replacement of lysines on positions 37-39 inhibits FVa cleavage at Arg506 considerably, whilst the replacement of lysines in residues 74-75 contributes to a lesser extent [67,68]. Also mutations at positions 62-63 are important in the inactivation of FVa [67,68]. These findings can explain the residual FVa activity in presence of 5D- and 5D2A-APC, and the lack of FVa activity with 5A-APC.

5A-APC, however, did show >50% anticoagulant activity with the APTT assay. This discrepancy with the FVa inactivation assay is likely based on methodological differences.

The APTT is performed in plasma with a strong contact activation pathway trigger. The FVa inactivation assay is performed in highly diluted plasma, which is essentially an assay composed of purified assay components. This assay provides a small amount of FV, which is then activated into FVa, that is subsequently inactivated by the addition of APC [69].

Next, all created APC variants were studied for their proteolytic activity towards extracellular histone H3. For this, 10 nM APC was incubated with 317 nM histone H3 for 30 minutes at 37°C and subsequent analysis of proteolysis by SDS-PAGE and Western blotting (Chapter 6). 5A-APC showed the highest histone cleavage rate as compared to the other variants. 5D-APC and WT-APC had comparable cleaving efficiencies, whilst the 5D2A-APC variant was not able to fully cleave the substrate within 30 minutes. We expected 5D2A-APC to have the fastest proteolysis rate, followed by 5D-APC, 5A-APC and WT-APC, respectively. Interestingly, the predicted binding affinities of APC with histone H3 did not correspond to the cleavage assay results. The most logical explanation would be that increased binding affinities result in a reduced dissociation of either the substrate or product after binding the 5D- and 5D2A-APC. This would impede the overall catalytic efficiency of these APC-variants. The correctness of this hypothesis could be tested in more sophisticated binding assays. Using proteolytic inactive equivalents of each APC variant, the mode and strength of binding can be assessed for each variant and these experiments are currently being prepared.

The newly designed and produced APC variants show reduced anticoagulant activities compared to WT-APC and are able to induce histone H3 proteolysis. Future work should focus on the further *in vitro* characterization of these variants in order to assess their potential use as biologicals to specifically proteolyse extracellular histones.

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Appendices

A

Summary

Summary

The present thesis aimed at studying the role of extracellular histones in processes of tissue injury, with a particular focus on organ preservation and inflammation. A second aim was to investigate the neutralization of extracellular histones in the aforementioned conditions. We specifically looked at activated protein C (APC) and heparin, which were engineered such as to reduce their anticoagulant activities whilst retaining or improving their affinity towards extracellular histones.

Chapter 1 introduces the main topics of this thesis. It gives a general overview on the functions of histone proteins inside and outside the cell. The different modes of extracellular histone release as well as pathological consequences of this release are discussed. APC and heparin, two biomolecules with anticoagulant and cell protective properties, are likewise described. Both are also able to inactivate cytotoxic histones. Reducing the anticoagulant properties of these biomolecules, whilst retaining their cell protective characteristics, could result in safer treatment strategies in several disease states. Finally, the outline and aims of this thesis are given.

Chapter 2 summarizes the diverse properties of the polysaccharide heparin. This compound has been employed as an anticoagulant since its initial discovery. Over the last decades also many nonanticoagulant functions of heparin have been described and these are summarized here. The anti-metastatic, anti-apoptotic and anti-inflammatory functions of heparin provide a variety of applications in, for example, cancer and inflammatory conditions. Elimination of the anticoagulant properties of heparin could circumvent a potential bleeding risk that is associated with the use of conventional heparins in various diseases and result in a safer heparin formulation.

Chapter 3 studies the presence of extracellular histones in donation after circulatory death (DCD) kidneys that were subsequently transplanted. These kidneys are preserved till the time of transplantation using machine perfusion, during which circulating markers can be measured in the machine perfusate. We measured the levels of histone H3 in this perfusate after a 4 hour perfusion period to investigate its potential as a pre-transplant viability marker. We found that higher histone H3 levels were associated with increased rates of non-functioning or delayed functioning kidneys after transplantation. Elevated perfusate histone H3 was also found to be an independent risk factor for one-year graft survival. In addition, these histone levels also correlated with warm ischaemia time, donor creatinine and 3 month serum creatinine. These findings suggest that extracellular histones could potentially be used to assess post-transplantation outcomes.

Chapter 4 investigates the presence of extracellular histones in ischemically damaged kidneys in more detail. For this we first validated an *ex vivo* porcine kidney perfusion model. In this model, different extents of ischemic damage were inflicted upon two

kidneys from the same animal by perfusion at 4 degrees (hypothermic machine perfusion or HMP) or 28 degrees (subnormothermic machine perfusion or SNMP). Using this model, we observed that the appearance of extracellular histones in the perfusate is both time- and ischemia-dependent, with higher levels in the SNMP kidneys than in the HMP kidneys. Overall morphology and brush border integrity in nephrons, as analyzed by microscopy, after 4 hours did not differ between both groups. Immunohistochemical analysis suggested that the extent of cell death was higher in SNMP kidneys as compared to HMP kidneys. The application of heparin during SNMP did not result in a change of total extracellular histone H3 levels in the perfusate. It did, however, result in earlier and increased perfusate levels of a lower molecular weight form of histone H3 as compared to untreated kidneys. The exact origin and implications of this histone product remain to be determined.

Chapter 5 describes glycocalyx degradation in sepsis within 24 hours of intensive care admission. We studied the glycocalyx using non-invasive imaging and analysis of circulating plasma markers. An increase in glycocalyx degradation was observed in non-survivors of sepsis as indicated by both an increased perfused boundary region (PBR) and plasma syndecan-1. The PBR correlated with the permeability marker angiopoietin-2 (Ang-2), but not with syndecan-1 or other markers. PBR and syndecan-1 are therefore independent predictors of sepsis mortality in this cohort. Additional studies should investigate the potential value of the PBR in clinical patient monitoring.

Chapter 6 explores the use of novel APC variants for the proteolytic inactivation of cytotoxic histones *in vitro*. *In silico* docking and molecular dynamics simulations revealed the key residues of APC that are involved in its interaction with histone H3. The interaction between wildtype-APC and histone H3 was then optimized to produce new APC variants that were predicted to have enhanced proteolytic properties towards histones. In these variants, simultaneous mutations were introduced that were predicted to reduce APC's anticoagulant activities. Indeed, the produced APC variants all had reduced anticoagulant activity as compared to wildtype-APC. However, the new APC variants were no better at cleaving histones as compared to both wildtype-APC and the better known 5A-APC variant. This can possibly be explained by the fact that the new APC variants bind too strongly to their substrate or reaction products, resulting in an overall reduced proteolytic activity. Further characterization of these novel variants should assess their potential as therapeutics in histone-mediated diseases.

Finally, the results are critically discussed in Chapter 7. In this chapter, the clinical relevance of the main findings of this thesis are debated in the context of existing literature and current practice.

Samenvatting

Samenvatting

Het onderzoek beschreven in dit proefschrift was gericht op het bestuderen van de rol van extracellulaire histonen in condities geassocieerd met weefselschade, met een specifieke focus op orgaanpreservatie en ontsteking. Een tweede doel was om de neutralisatie van extracellulaire histonen in de bovengenoemde situaties te onderzoeken. We hebben specifiek gekeken naar geactiveerd proteïne C (APC) en heparine, die moleculair veranderd waren om hun antistollende activiteiten te verminderen, terwijl de affiniteit voor extracellulaire histonen behouden of verbeterd werd.

Hoofdstuk 1 introduceert de belangrijkste onderwerpen van dit proefschrift. Het geeft een algemeen overzicht van de functies van histoneiwitten binnen en buiten de cel. De verschillende manieren van extracellulaire histon afgifte evenals de pathologische gevolgen hiervan worden besproken. APC en heparine, twee biomoleculen met antistollende en celbeschermende eigenschappen, worden ook beschreven. Beiden zijn ook in staat cytotoxische histonen te inactiveren. Het verminderen van de antistollende eigenschappen van deze moleculen, terwijl ze hun cel beschermende eigenschappen behouden, zou kunnen resulteren in veiligere behandelingen voor verscheidene aandoeningen. Tenslotte worden de hoofdlijnen en doelstellingen van dit proefschrift besproken.

Hoofdstuk 2 beschrijft de diverse functies van de polysaccharide heparine. Deze stof wordt al sinds z'n ontdekking gebruikt als antistollingsmiddel. De afgelopen decennia worden echter ook verschillende niet-antistollende functies aan heparine toegeschreven, welke in dit hoofdstuk zijn samengevat. De anti-metastatische, anti-apoptotische en ontstekingsremmende functies van heparine zorgt voor een diversiteit aan toepassingen in bijvoorbeeld kanker en ontstekingsziekten. Het wegnemen van de antistollende eigenschappen van heparine dat gepaard gaat met het gebruik van conventionele heparines zou daarom een eventueel bloedingsrisico in deze ziektebeelden kunnen omzeilen.

Hoofdstuk 3 bestudeert de aanwezigheid van extracellulaire histonen in nieren van donoren die zijn overleden na een circulatiestop en welke vervolgens zijn getransplanteerd. Deze nieren worden tot transplantatie bewaard met behulp van machine perfusie, waarbij circulerende markers gemeten kunnen worden in het machine perfusaat. Wij hebben histon H3 levels in dit perfusaat gemeten na 4 uur machine perfusie en onderzochten de bruikbaarheid ervan als marker van orgaankwaliteit vóór transplantatie. Wij vonden dat hogere histon H3 levels geassocieerd waren met het niet of vertraagd functioneren van een nier na transplantatie. Een verhoogd perfusaat histon H3 level vormde ook een onafhankelijke risicofactor voor het 1 jaar overleven van het getransplanteerde orgaan. Daarnaast correleerden deze histon levels ook met warme ischemie tijd, donor creatinine,

en serum creatinine levels na 3 maanden. Deze bevindingen suggereren dat extracellulaire histonen mogelijk gebruikt kunnen worden om zowel functie als overleving van het orgaan na transplantatie te voorspellen.

Hoofdstuk 4 onderzoekt de aanwezigheid van extracellulaire histonen in ischemisch beschadigde nieren in meer detail. Hiervoor hebben we eerst een *ex vivo* varkensnier perfusiemodel gevalideerd. In dit model werden verschillende niveaus van ischemische schade toegebracht aan twee nieren van hetzelfde dier, door de perfusie uit te voeren bij 4 graden (hypotherme machine perfusie of HMP) of bij 28 graden (subnormotherme machine perfusie of SNMP). Met behulp van dit model hebben we waargenomen dat het verschijnen van extracellulaire histonen in het perfusaat zowel tijds- als ischemieafhankelijk is, met hogere levels in SNMP nieren dan in HMP nieren. De algemene morfologie en brush border integriteit in nefronen na 4 uur perfusie, zoals geanalyseerd met behulp van microscopie, verschilden niet tussen beide groepen. Immunohistochemische analyse suggereerde dat de mate van celdood wel hoger was in SNMP nieren in vergelijking met HMP nieren. De toepassing van heparine tijdens SNMP veranderde niet de totale hoeveelheid van extracellulaire histonen in het perfusaat. Het resulteerde echter wel in eerdere en verhoogde perfusaat levels van een histon H3 vorm met een lager moleculair gewicht. De exacte oorsprong en implicaties van dit product moeten nog worden onderzocht.

Hoofdstuk 5 beschrijft de afbraak van de glycocalyx in sepsis binnen 24 uur na opname op de intensive care. We hebben de glycocalyx bestuurd met behulp van niet-invasieve beeldvorming en analyse van circulerende plasma markers. Een toename van de glycocalyx degradatie werd waargenomen bij niet-overlevenden van sepsis zoals blijkt uit een verhoogde perfused boundary region (PBR) en verhoogde syndecan-1 in plasma. De PBR correleerde met de permeabiliteitsmarker angiopoëtin-2 (Ang-2), maar niet met syndecan-1 of andere markers. PBR en syndecan-1 zijn dus onafhankelijke voorspellers van sepsis mortaliteit in dit cohort. Aanvullende studies zullen de potentiële waarde van de PBR in het klinisch monitoren van patiënten verder moeten onderzoeken.

Hoofdstuk 6 verkent het gebruik van unieke APC-varianten voor de proteolytische inactivatie van cytotoxische histonen *in vitro*. *In silico* moleculaire simulatie studies onthulden de belangrijkste residuen in APC die betrokken zijn bij de interactie met histon H3. De interactie tussen wildtype-APC en histon H3 werd vervolgens geoptimaliseerd om nieuwe APC-varianten te produceren waarvan werd voorspeld dat ze verbeterde proteolytische eigenschappen hadden met betrekking tot histonen. In deze varianten werden gelijktijdige mutaties geïntroduceerd waarvan werd voorspeld dat ze APC's antistollende activiteiten zouden verminderen. De geproduceerde APC-varianten hadden allen inderdaad een verminderde antistollende activiteit in vergelijking met wildtype-APC. De nieuwe APC varianten waren echter niet beter in het knippen van histonen in vergelijking met zowel wildtype-APC als de bekende 5A-APC variant. Dit kan mogelijk

worden verklaard doordat de nieuwe APC-varianten grofweg te sterk binden aan hun substraat of reactieproducten, wat resulteert in een algeheel verminderde proteolytische activiteit. Verdere karakterisering van deze nieuwe varianten zou daarom hun potentieel als therapeutica in histon-gemedieerde ziekten moeten beoordelen.

Tot slot worden de resultaten kritisch bediscussieerd in Hoofdstuk 7. In dit hoofdstuk wordt ook de klinische relevantie van de belangrijkste bevindingen van dit proefschrift besproken in de context van bestaande literatuur en de huidige praktijk.

Valorization

Valorization

The social and economic relevance of academic research is becoming increasingly important for its impact on current societies. Researchers are continuously requested to critically evaluate the validity and value of their work, keeping in mind the potential translation of scientific knowledge into novel products, processes and services. Although sometimes the direct utilization of research may not be that clear, unraveling fundamental principles of physiological and pathological processes is essential for the development of future clinical applications.

The work in this thesis describes the potential use of extracellular histones as both biomarkers and therapeutic targets in several conditions associated with tissue damage. The rationale was to not only verify the presence of histones in these conditions, but also test approaches aimed to reduce their potential pathological effects.

The initial discovery that nuclear histones can be released into the extracellular space and there serve different pathological functions, has since revealed a vast array of pathological conditions associated with these extracellular proteins [1]. One strategy to neutralize the toxicity of extracellular histones is the use of heparin. Heparin exerts both anticoagulant and nonanticoagulant effects, which are summarized in **Chapter 2**. The diversity of heparin's functions has led to its use in a wide range of clinical applications. The differential functions of heparin can be separated by sophisticated production methods to render heparin formulations that are specifically tailored for certain applications. Providing a detailed and up-to-date overview of heparin's functions may offer peers and those who are new to the study of heparin new insights for the use and optimization of this versatile molecule.

The number of people on the waiting list for kidney transplantation in the Netherlands at the end of 2017 was 673, with a median waiting time of 2.3 years since start of dialysis (annual report 2017 Nederlandse Transplantatie Stichting). To reduce these numbers, there is a need to make better use of the sparse donation pool and increase the number of transplantations with (suboptimal) donation after circulatory death (DCD) kidneys. As of January 2017, all kidneys from DCD donors in the Netherlands are preserved using hypothermic machine perfusion due to increased graft and patient survival rates as compared to cold storage in the absence of perfusion [2]. However, still 33% of all registered DCD kidneys get rejected for transplantation as compared to only 10% for donation after brain death (DBD) kidneys or kidneys from living donors (numbers Nederlandse Transplantatie Stichting). This difference can likely be explained by the fact

that DCD kidneys suffer from increased and inevitable periods of ischemia compared to the other kidney groups. Therefore, understanding and optimization of machine perfusion might increase both the use and transplant success of DCD kidneys.

A major advantage of machine perfusion is that it allows the direct measurement of circulating markers and assessment of organ quality from the perfusion liquid. Many different markers have been detected in machine perfusates, but none of the individual biomolecules was shown to accurately predict short-term graft function [4]. Given that ischemia is associated with processes of cell death, we investigated the presence of extracellular histones in machine perfused DCD kidneys (**Chapter 3**). Our initial goal was to determine its use as a pre-transplantation viability marker that could potentially aid in the decision to transplant DCD kidneys. We found that histone H3 levels circulating within the machine perfusate correlated negatively with graft function after transplantation. Also, survival rates of the transplanted grafts were higher for DCD kidneys with lower circulating histone levels during preservation. The predictive value of extracellular histone H3, however, was limited and it did not perform better than other perfusate markers. As we are the first to describe the presence of extracellular histones in ischemically damaged machine perfused kidneys, additional independent studies should investigate the (additive) value of measuring histones during machine preservation. In order to become a better biomarker candidate for both monitoring and diagnostic purposes, more rapid and accurate quantification of extracellular histones is desirable. Preferably, this would entail a real-time and easily performed measurement that would help guide current clinical practice. This test would not only be convenient in the context of organ donation, but any clinical condition associated with extracellular histones.

The presence of potentially cytotoxic histones in human machine perfused kidneys prompted us to investigate this in more detail (**Chapter 4**). For this, we first set up an *ex vivo* porcine kidney perfusion model in which the extent of ischemic damage was varied between both kidneys from the same animal. The use of porcine kidneys serves a great alternative to human kidneys, do to their comparable size and function and ease of obtaining such kidneys from slaughterhouses [5]. We observed that the release of extracellular histones into the perfusate over time was both time and ischemia dependent. This confirms the results of our human study and stresses the importance of minimizing the length of ischemia in terms of histone release.

Machine perfusion also provides a platform for testing both diagnostics and therapeutics. Our research group has experience with a nonanticoagulant heparin fraction, the use of which is accompanied by a reduced risk of bleeding but a similar cytoprotective effect as unfractionated heparin [6]. This heparin formulation has already been successful in reducing histone-mediated organ damage in pre-clinical inflammation models [6], and is

now in clinical development. Therefore, we aimed to use this nonanticoagulant heparin to alter extracellular histone kinetics by reducing their levels in machine perfused kidneys.

It is important to note that in our study we perfused both the heparin treated and untreated kidney at 28°C. Our goal was therefore to ascertain tissue damage and thus, presence of perfusate histones. This gave us the opportunity to study the potential effects of the administration of heparin on histone perfusate levels. Overall levels of perfusate histone H3 were found to be similar in both groups. Interestingly, the addition of heparin at the start of perfusion did result in the gradual appearance of an alternative histone product. This product originated earlier in time for the heparin-treated kidneys as compared to the untreated counter kidneys. The exact implications of these findings remain to be investigated, as this is not the current practice in organ preservation. Perfusion at higher temperatures are often performed using autologous blood or under oxygenic conditions [7]. The consideration could therefore be made to test whether histones are also present in these different conditional setups and might be a target for neutralization by heparin.

As for now, the results from both studies seem to indicate that perfusion at low temperatures is beneficial in terms of ischemia and histone release. Both these parameters are linked to adverse outcomes in terms of short-term graft function after transplantation. Hence, careful consideration for both the choice of organ preservation method and transplantable ischemic kidneys is paramount.

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection [8]. Sepsis is estimated to globally affect over 31 million people every year, leading to over 5 million deaths worldwide [9]. Both extracellular histones and microcirculatory dysfunction have been linked to disease severity and mortality in patients with sepsis [10,11]. Our initial aim in **Chapter 5** was therefore to study the potential relationship between extracellular histones and microcirculatory markers in sepsis. Due to yet undefined reasons, we did not detect plasma histones in the majority of our sepsis patients. This has been observed in other cohorts as well, which revealed up to 23% of the patients having no plasma histone H3 [10]. This observation displays the challenges in determining extracellular histones as well as the complexity and diversity of disease in sepsis. On the other hand, we did find that microcirculatory dysfunction was increased in patients who died of sepsis, as reflected by an increased degradation of the glycocalyx. Both the perfused boundary region (PBR), as an inverse indicator of glycocalyx thickness, and the plasma glycocalyx constituent syndecan-1 could discriminate between survivors and non-survivors. The assessment of PBR occurs through direct non-invasive imaging of the sublingual microcirculation. This could serve as an alternative for the use of plasma markers, which requires a longer, more invasive analysis method. The additional value of PBR for clinical monitoring the microcirculation should be investigated in larger

populations of sepsis patients or patients with any other pathological state associated with alterations in the microcirculation. Future studies should also focus on assessing the potential relationship between both histones and glycocalyx, and its role in pathological conditions.

APC is an important protein involved in the protein C anticoagulant pathway. It also possesses cytoprotective effects that include the proteolytic cleavage of extracellular histones [12], such as present in sepsis [10]. For sepsis, mortality rates remain as high as 30% since there is no adequate treatment strategy besides providing supportive therapy [9]. This number underlines that improvement of treatment of sepsis could have a great impact on society. The use of APC in sepsis, however, is complicated by its associated bleeding risk and the resulting discontinuation of the marketed Xigris-APC (an Eli-Lilly product) in 2011 [13].

For this reason, we designed novel APC variants with limited anticoagulant properties but retained or increased histone cleaving properties (**Chapter 6**). These APC variants (termed 5D-APC and 5D2A-APC) were indeed less anticoagulant than wildtype APC, but did not show an improved proteolysis of extracellular histones *in vitro*. As APC and extracellular histones can be associated with many other molecules [14], we believe that additional studies should unravel important determinants of histone cleavage by APC. A more complex *in vivo* environment could provide such factors that are important for APC's proteolytic functions, with mutations within APC possibly affecting the rate of substrate proteolysis. Our current study should thus be considered as a series of pilot experiments, providing the basis for further characterization of novel APC variant(s). Our APC variants are therefore candidate molecules for the development of an effective and safe treatment strategy in sepsis and other histone-mediated diseases.

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Curriculum Vitae

Curriculum Vitae

Daniëlle Beurskens was born on the 1st of March in 1991 in Venlo, the Netherlands. She graduated from secondary school in 2009 at Bisschoppelijk College Broekhin in Roermond. Afterwards she started her university studies at the Radboud University in Nijmegen. She obtained her bachelor degree in Biomedical Sciences in 2012, and continued at the same university with the Toxicology and Human Pathobiology track of the corresponding master program. During this master she performed internships at the Institute for Cell and Molecular Biosciences in Newcastle upon Tyne (United Kingdom) and the Tumor Immunology department of the Radboud Institute for Molecular Life Sciences in Nijmegen. In November 2014 she started her PhD research at the department of Biochemistry at Maastricht University under the supervision of Prof. Chris Reutelingsperger and dr. Gerry Nicolaes. Her project focused on the extracellular histone H3 as both marker and therapeutic target in conditions of tissue injury.

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- van Smaalen TC, **Beurskens DMH**, Polonia R, Reutelingsperger CPM, van Heurn LW, Nicolaes GAF. Extracellular histones: a new biomarker in kidney transplantation. 15th Congress of the European Society for Organ Transplantation (ESOT), Brussels, Belgium, September 2015.
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Dankwoord

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