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α_1-microglobulin – Therapeutic opportunities in kidney and erythrocyte pathology

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Therapeutic opportunities in kidney and erythrocyte pathology

AMANDA KRISTIANSSON
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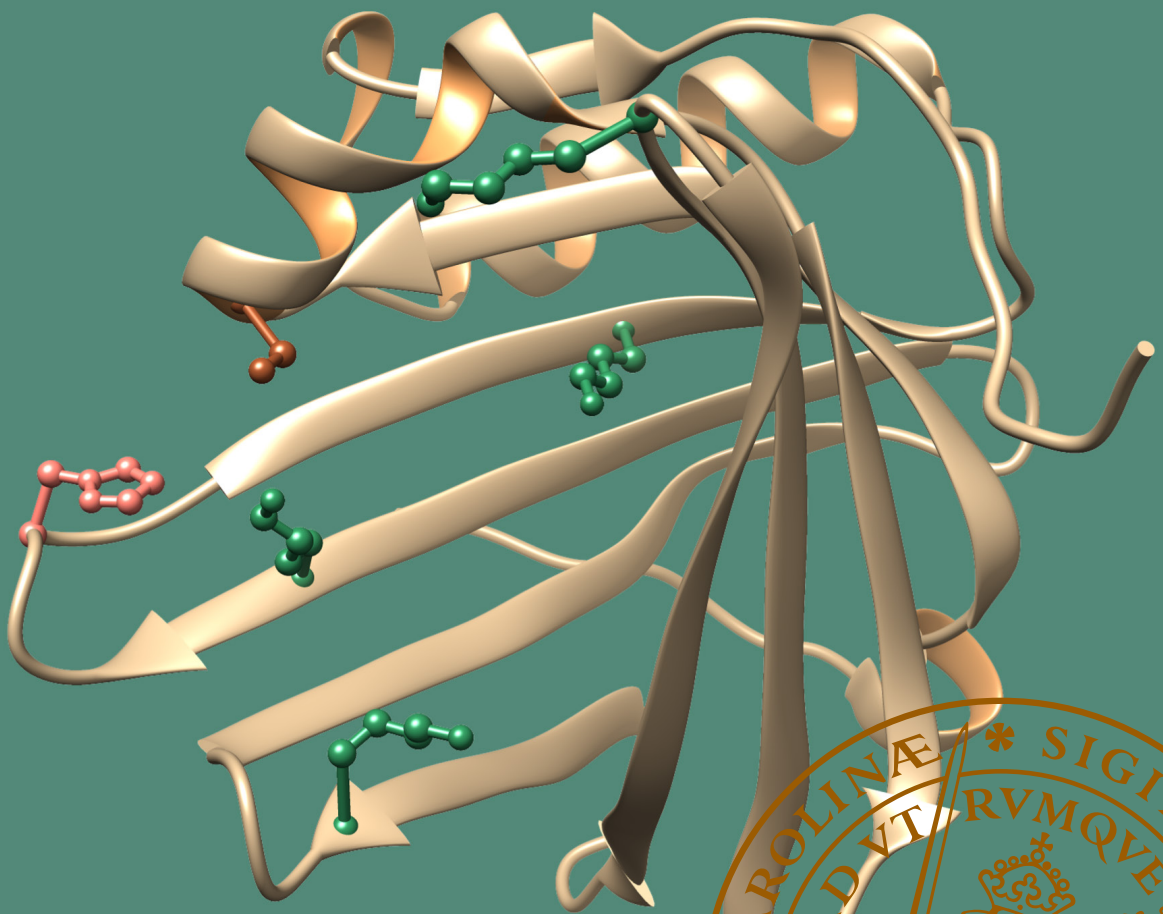

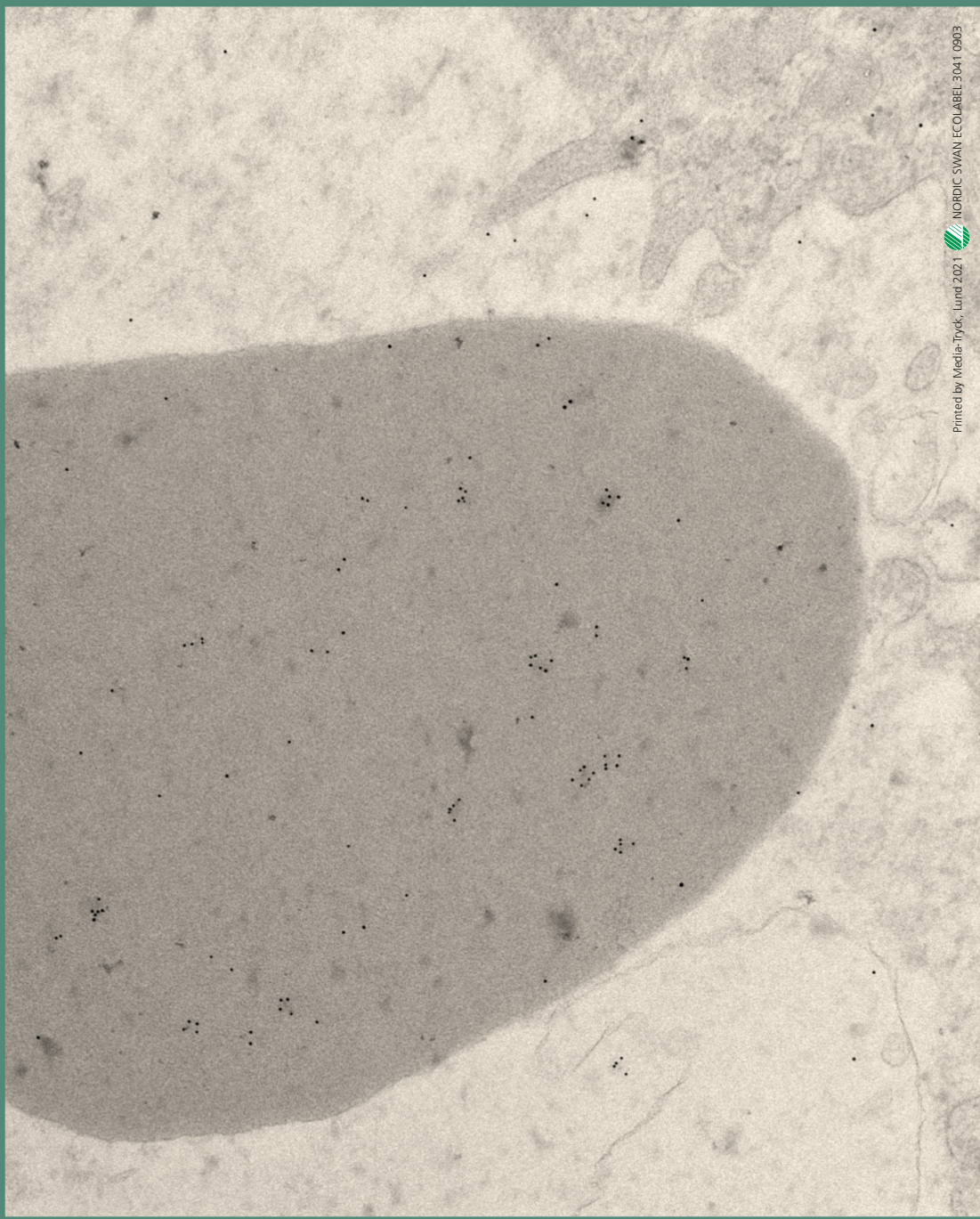

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Amanda Kristiansson

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract

Oxidative stress is defined as an imbalance between oxidants and antioxidants. The reasons behind the condition are diverse, and can result in, or contribute to, the severity of many diseases. The body has different protective mechanisms to reduce the oxidative burden, among them a protein called α_1-microglobulin (A1M). A1M possesses a unique set of molecular properties enabling it to act as a radical scavenger, reductase and heme-binder. Most tissues express *AMBP*, the gene encoding A1M, but the liver is the main site of synthesis. A1M circulates in the blood and is catabolized in the kidneys. Therefore, two interesting sites of action for A1M are the blood and the kidneys.

The aim of this thesis was to investigate new therapeutic opportunities for A1M, with focus on renal and erythrocyte pathology. Paper I studied in vitro heme-induced stress in human proximal tubule epithelial cells. A1M prevented cell death, stress response and mitochondrial dysfunction. In paper II, a ^{177}Lu-DOTATATE cancer treatment mouse model showed that the radiation induced DNA breaks as well as functional and histological damage to the kidneys. The A1M-treated mice, however, showed significantly milder damage suggesting that A1M can be used as a radioprotector. Paper III investigated a ^{177}Lu-PSMA-617 prostate cancer treatment mouse model. Only small alterations in kidney function were detected, nonetheless, they were ameliorated in A1M-injected mice. Moreover, it was concluded that A1M does not interfere with treatment.

Paper IV presents the establishment and characterization of an A1M-knockout mouse. The lack of A1M generated misfolded bikunin, which is co-expressed with A1M in the intact gene. The A1M-knockout mice also showed increased ER-stress and weight-gain. Paper V shows that the A1M-knockout mice had fewer red blood cells (RBCs) which also varied more in size, resembling a macrocytic anemia phenotype. Paper V also concludes that A1M has antihemolytic properties, and can protect adult, fetal and murine RBCs from various stressors. Paper VI discusses the therapeutic potential of the erythrostabilizing and antihemolytic effects of A1M in different hemolytic and erythropoietic conditions.

In conclusion, the results in these studies suggest that the protective effects of A1M towards kidneys and red blood cells may be used in cancer radiotherapy, and in hemolytic and erythropoietic medical conditions.

Key words: α_1-microglobulin; oxidative stress; kidneys; red blood cells; radiation; heme; hemolysis

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Cover photo: A1M structure by Amanda Kristiansson

Back-cover photo: A1M in an erythrocyte in the placenta by Maria Baumgarten and Amanda Kristiansson

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To my family and friends

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Abstract

Oxidative stress is defined as an imbalance between oxidants and antioxidants. The reasons behind the condition are diverse, and can result in, or contribute to, the severity of many diseases. The body has different protective mechanisms to reduce the oxidative burden, among them a protein called α_1-microglobulin (A1M). A1M possesses a unique set of molecular properties enabling it to act as a radical scavenger, reductase and heme-binder. Most tissues express *AMBP*, the gene encoding A1M, but the liver is the main site of synthesis. A1M circulates in the blood and is catabolized in the kidneys. Therefore, two interesting sites of action for A1M are the blood and the kidneys.

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In conclusion, the results in these studies suggest that the protective effects of A1M towards kidneys and red blood cells may be used in cancer radiotherapy, and in hemolytic and erythropoietic medical conditions.

Populärvetenskaplig sammanfattning

Introduktion

Oxidativ stress uppstår när det är obalans i kroppen mellan våra skyddsmekanismer, d.v.s. antioxidanter, och de skadliga fria radikalerna. De fria radikalerna kan bildas i naturliga processer i kroppen t. ex. i cellernas kraftverk mitokondrierna eller i kroppens försvar mot mikrober. Fria radikaler kan dock också bildas genom externa stimuli, t. ex. cigarrettrökning, UV-bestrålning eller radioaktiv strålning som ges i terapeutiskt syfte. Skadorna som uppstår i kroppen vid oxidativ stress kan leda till många olika sjukdomar, som cancer, njurskador och inflammation, men även till åldrande. Kroppen har genom evolutionen utvecklat en mängd skyddsmekanismer för att försvara vävnader och celler mot de fria radikalerna. Kroppen tillverkar t.ex. många antioxidanter själv men vi får också i oss sådana via kosten, såsom Vitamin C och E. En kroppsegen antioxidant är α_1-mikroglobulin, som vanligtvis förkortas A1M. A1M förekommer i fiskar, fåglar och däggdjur, inklusive människan. Det är ett protein som tillverkas i levern och bryts ner i njurarna. Däremellan cirkulerar det i kroppen, både i blodplasman och i andra vävnader. A1M binder fria radikaler, men även skadliga rester från röda blodceller, heme-grupper, och reparerar oxidativa skador som uppstått från fria radikaler.

Avhandlingens syfte

Eftersom A1M kan tillverkas på konstgjord väg möjliggör det att ge A1M till patienter som drabbats av sjukdomar där kroppen själv inte kan ta hand om den oxidativa stressen som uppstår. Syftet med avhandlingen är att undersöka förutsättningarna för att använda A1M som läkemedel för sjukdomar som drabbar njurarna och de röda blodcellerna.

Resultat och diskussion

Artikel I

Eftersom A1M bryts ner i njurarna är förekomsten av A1M hög där. Därför är det intressant att undersöka om A1M:s skyddsmekanismer kan tillämpas i njursjukdomar. Ett första steg är att undersöka detta i njurceller från människor. Njurcellerna utsattes för oxidativ stress i form av heme och sedan undersöktes de uppkomna skadorna genom att mäta celldöd, genuttryck och mitokondriefunktion. Tillsättning av A1M reducerade celldöd och cellerna uppvisade lägre genetisk stressrespons samt bevarad mitokondriefunktion.

Artikel II och III

Radioaktiva ligander kan injiceras i patienter med olika former av cancer, t. ex. neuroendokrina tumörer och prostatacancer. De radioaktiva liganderna letar upp cancercellerna och binder till dem, varefter de tas upp i cellerna så att strålningen kan döda cancercellerna. Problemet med den här typen av behandling är att liganderna utsöndras genom njurarna och kan då, som biverkning, ge njurskador. Därför ges lägre doser, vilket ger sämre behandling och patienter med underliggande njursjukdomar, som tex diabetes, kan behöva uteslutas från behandlingen. Ett alternativ för att kunna ge högre doser är att simultant ge något som skyddar njurarna, som A1M.

Artikel II

I artikel II användes möss där radioaktiva ligander som normalt används för att behandla neuroendokrina tumörer i människor injicerades. I en del av djuren injicerades även A1M, och kontrolldjur fick endast saltlösning. Djuren följdes sedan upp till sex månader och analyserades med avseende på överlevnad, DNA-skador, stressmarkörer och njurskador (både funktionella och histologiska). Skadorna var avsevärt färre i djuren som fått A1M och dessa djur hade också bättre överlevnad.

Artikel III

Precis som i artikel II användes här möss för att studera skador av radioaktiva peptider, men här användes ligander som kliniskt används för behandling av prostatacancer. Strålningsdoserna var här lägre och gav färre och mildare njurskador, vilket endast kunde konstateras med den relativt nya metoden MAG3 som utvärderades i den här studien. De få skador som uppkom var dock färre i mössen som fått A1M.

Resultaten i både artikel II och III, stödda av resultaten i artikel I, tyder på att A1M har en njurskyddande funktion vid olika former av oxidativ stress. Detta innebär att

A1M potentiellt skulle kunna ges till patienter som får behandling med radioaktiva ligander för att minska deras biverkningar alternativt möjliggöra administration av högre doser för bättre behandling.

Artikel IV

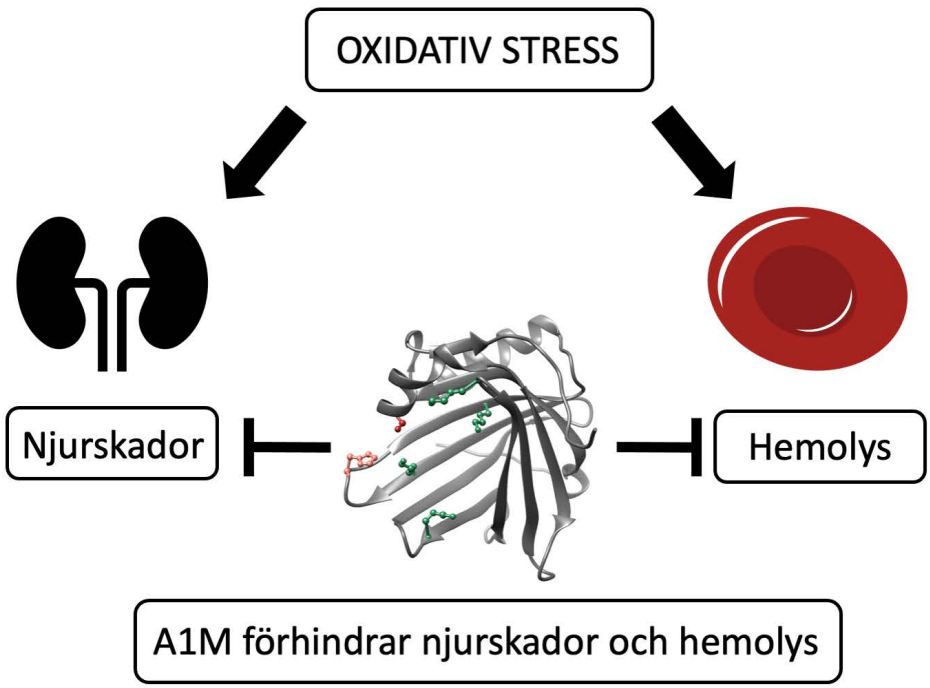
Ett sätt att studera ett proteins roll i kroppen är att klippa bort genen som kodar för det. I studie IV gjordes detta för genen som kodar för A1M i möss, en så kallad knockoutmus. Intressant nog kodar den här genen även för ett annat protein, bikunin, men den delen behölls för att kunna avgöra vilka förändringar som beror på avsaknaden av A1M och inte för bikunin. I mössen som saknade A1M uttrycktes andra antioxidanter i högre grad, antagligen som en kompensationsmekanism. Bikuninnivåerna i plasma sjönk, trots att uttrycket var oförändrat eller högre, vilket talar för att A1M behövs för att bikunin ska kunna utsöndras i sin rätta form. Mössen gick också upp i vikt och var signifikant tyngre än sina motsvarande burkompisar.

Artikel V och VI

Mössen från artikel IV hade också en förändrad blodbild, med färre och större röda blodceller. Detta kan tolkas som att A1M på något sätt reglerar kroppens omsättning av röda blodceller. Därför undersöktes i artikel V om A1M skyddar cellerna från nedbrytning. Röda blodceller från vuxna och nyfödda människor, samt från möss, utsattes för oxidativ stress, både fria radikaler och heme, samt osmotisk stress. A1M förhindrade celldöd vid alla undersökta tidpunkter och mikroskopering visade även att A1M togs in i de röda blodcellerna. Tillsammans med det faktum att A1M-knockoutmössen hade en förändrad blodbild med tendenser till anemi, tyder detta på att A1M skyddar röda blodceller. I artikel VI utvecklades och diskuterades potentialen i detta och A1M föreslogs kunna användas för att behandla sjukdomar där hemolys, destruktion av röda blodceller, förekommer: anemi, blödningar i hjärnans hålrum hos nyfödda, blodtransfusioner, preeklampsi och ateroskleros.

Konklusion

I den här avhandlingen presenteras resultat som stödjer att A1M förhindrar njurskador samt hemolys, vilka är vanligt förekommande i många sjukdomar. Resultaten kommer förhoppningsvis ligga till grund för framtida forskning för att utöka användningen av A1M som terapi i kliniken.



Oxidativ stress kan uppstå på många olika sätt. I vissa fall kan det leda till njurskador respektive hemolys och bidra till utvecklingen eller förvärrandet av sjukdom. I den här avhandlingen presenteras resultat som visar att A1M kan motverka dessa skador.

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To start with I would like to thank my supervisors, **Bo**, **Jill** and **Magnus**.

Bo, both as main and co-supervisor, you have always taken the time to help me with everything, most often with your optimistic view. You have been a source of inspiration with your great scientific knowledge that you have shared with me over the years in countless scientific discussions and planning of experiments. Your guidance has made this possible.

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A thank you to all my friends who have been supportive during these years in a multitude of ways even though a bunch of you still don't know what I've been doing. A special thanks to **Victoria** for signing up for courses with me and discussing the ups and downs during the PhD. I would also like to thank **Amanda** and **Cecilia** for, in addition to being great friends, proofreading my popular scientific summary.

A big thank you to my parents, **Eva** and **Per**, for all your support, encouragement and help during both these and all the previous years. Thanks to my sister and brothers, **Helena**, **Markus** and **Andreas**. You have also, in each your own way, given me support during these years.

Albert, thank you for being supportive and patient throughout these years. You are probably the person who knows most about A1M without ever having worked with it due to being forced (?) to listen to all my presentations and read all my articles. I love you and I'm so lucky to have you.

Abbreviations

^{177}Lu	Lutetium-177
^{225}Ac	Actinium-225
5q-MDS	5q-minus Myelodysplastic syndrome
^{90}Y	Yttrium-90
ADT	Androgen deprivation therapy
A1M	α_1-microglobulin
A1M-KO	A1M-knock out
A1M-wt	Wildtype A1M
ABTS	2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)
AKI	Acute kidney injury
AMBP	α_1-microglobulin-bikunin precursor gene
ARE	Antioxidant response element
ATP	Adenosine triphosphate
BFU-E	Burst forming unit-erythroid
BUN	Blood urea nitrogen
CAT	Catalase
CFU-E	Colony- forming unit-erythroid
CHOP	C/EBP homology protein
CKD	Chronic kidney injury
DBA	Diamond-Blackfan anemia
DBS	Double-strand breaks
ECM	Extracellular matrix
EPO	Erythropoietin
ER	Endoplasmic reticulum

ESRD	End-stage renal disease
ETC	Electron Transport Chain
GFR	Glomerular filtration rate
GPX1	Glutathione peroxidase 1
GSH	Glutathione
Hb	Hemoglobin
HbF	Fetal hemoglobin
HO-1	Heme oxygenase-1
HO-2	Heme oxygenase-2
Hp	Haptoglobin
Hpx	Hemopexin
Hsp70	Heat shock protein 70
IPTG	Isopropyl thiogalactoside
IVH	Intraventricular hemorrhage
kDa	Kilodalton
KEAP1	Kelch-like ECH-associated protein 1
KIM-1	Kidney injury molecule-1
Maf	Musculoaponeurotic fibrosarcoma
mCRPC	Metastatic castration-resistant prostate cancer
MCV	Mean cell volume
metHb	Methemoglobin
MPO	Myeloperoxidase
NBT	Nitroblue tetrazolium
NETs	Neuroendocrine tumors
NGAL	Neutrophil gelatinase-associated lipocalin
Nrf2	Nuclear factor erythroid 2-related factor 2
PC	Prostate cancer
PE	Preeclampsia
PRRT	Peptide receptor radionuclide therapy

Prx2	Peroxiredoxin 2
PSA	Prostate specific antigen
PSMA	Prostate-specific membrane antigen
rA1M	Recombinant A1M
RLT	Radioligand therapy
RBC	Red blood cell
RDW	Red blood cell distribution width
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
sstr2	Somatostatin receptor 2
STOX-1	Storkhead box 1
t-A1M	Truncated A1M
Trx	Thioredoxin
UPR	Unfolded protein response
XBP-1	X-box binding protein 1

Introduction

Oxidative stress

Free radicals are molecules or atoms which have one or more unpaired electrons, which make them unstable, short-lived and very reactive (Table 1.). The reactivity comes from reactions with other molecules to attain pairing of the electrons, thereby creating a new radical, and so forth, creating a chain reaction. Oxidative stress refers to the imbalance between prooxidants and antioxidants. In the body, when there is a surplus of free oxidants such as reactive nitrogen species (RNS) and reactive oxygen species (ROS), insufficient defense mechanisms can result in cellular and tissue damage, including proteins, lipids, membranes and DNA. Oxidative stress can have diverse origins and result in a multitude of different diseases or, the other way around, oxidative stress can develop as a consequence of a disease. Therefore, oxidative stress is a potential therapeutic target, both to prevent disease progression or to reduce damage from other treatments, e.g., radiation, resulting in oxidative stress.

Table 1 Overview of free radicals, ROS and RNS [1].

Free radicals	**Chemical formula**
Hydroxyl	$OH^{\bullet}$
Superoxide	$O_2^{\bullet -}$
Peroxyl	$ROO^{\bullet}$
Nitric oxide	$NO^{\bullet}$
Non-radical ROS/RNS	**Chemical formula**
Hydrogen peroxide	H_2O_2
Singlet oxygen	$^1O_2$
Hypochlorous acid	HOCl
Peroxynitrite	$ONOO^-$

Endogenous ROS

The cell organelles mitochondria, peroxisomes and endoplasmic reticulum (ER) are sources of endogenous ROS. In addition, ROS-generating enzymes are activated during the immune response and inflammation e.g., myeloperoxidase (MPO). Another source of oxidative stress is heme, which is elaborated on in the red blood cell section below.

Mitochondria

Mitochondria are the power plants which supply the cell with energy in the form of ATP, and the primary source of endogenous ROS derives from the mitochondrial electron transport chain (ETC). Redox reactions establish an electrochemical proton gradient which generates energy. In the ETC ROS is produced in form of $O_2^{\bullet -}$ in complex I (NADH dehydrogenase) and III (ubiquinone cytochrome c reductase). Mitochondrial super-oxide dismutase converts $O_2^{\bullet -}$ into H_2O_2, which can form highly reactive $OH^{\bullet}$ through the Fenton (through reaction with Fe^{2+}) or Haber–Weiss (through reaction with $O_2^{\bullet -}$) reactions. However, H_2O_2 can also be detoxified by catalase (CAT) and glutathione peroxidase (GPx) [2].

Peroxisome

The peroxisome is found in the cytoplasm of most cells and is involved in catabolism. Peroxisomes have been suggested to have an important role in both production and scavenging of ROS. Peroxisomes have high oxygen consumption and in their respiratory pathway the energy is released as heat (unlike the mitochondria where it results in ATP). ß-oxidation of fatty acids occurs not only in the mitochondria but also in the peroxisomes, contributing to production of H_2O_2 [3]. In addition, peroxisomes produce $O_2^{\bullet -}$, $OH^{\bullet}$, and $NO^{\bullet}$, but also contain antioxidants e.g., CAT to retain balance [4].

Endoplasmic reticulum

The main function of the ER is to fold the newly translated proteins, and prepare them for further trafficking. During formation of disulfide bonds between cysteine residues on proteins, H_2O_2 is formed as byproduct. When disulfide bonds are mispaired in the ER lumen, the antioxidant glutathione can be used for reduction. During ER stress, an increase in mispaired disulfide bonds and breakage can increase ROS. Moreover, parts of the unfolded protein response (UPR), which is signaling pathways in response to accumulating misfolded proteins, can increase oxidative stress. ER stress has also been linked to situations with metabolic overload, disturbed mitochondrial function and enhanced ROS production [5].

Myeloperoxidase

MPO is a heme-containing peroxidase enzyme, most abundantly expressed in neutrophils. MPO catalyzes the formation of bactericidal HOCl from H_2O_2 and Cl^-, but also other oxidants e.g., hypothiocyanous acid (HOSCN) [6]. Together with NADPH oxidase, that produces $O_2^{\bullet -}$ and H_2O_2, ROS are formed that can be used for microbial killing in the phagosome [7]. However, when MPO is not contained and released extracellularly, the generation of e.g., HOCl can damage nearby tissues and cells. Consequently, MPO has been indicated in several diseases e.g., atherosclerosis, cardiovascular and neurodegenerative diseases [8].

Exogenous ROS

Exogenous sources of ROS include pollution, cigarette smoke, UV-light, drugs, heavy metals, pesticides and alcohol, which in the body, are metabolized and results in free radicals [2].

Ionizing radiation is another source of exogenous ROS. Cellular exposure to ionizing radiation can oxidize molecules directly or via interaction with water through radiolysis. The high cellular and bodily content of water makes radiolysis critical to the damage that occurs. During radiolysis, ROS are generated that can target lipids, proteins and DNA [9]. The lethal effects of radiolysis can be used to kill cancer cells in radiotherapy but can also damage nearby off-target cells. The ROS created from radiation can harm cells in close proximity to the radiated cells, bystander cells, and can trigger signalling pathways leading to e.g. inflammation [10].

Damage

Even though excessive ROS are damaging, ROS at normal physiological levels have a role in regulating signalling transduction and gene expression [11]. However, in this thesis the focus is on the damaging effects.

Lipids

Excessive ROS can damage most biological molecules, including lipids, proteins and DNA, by oxidation. Lipid peroxidation, of for example cell membranes, is the result of a reaction between a free radical and a fatty acid, creating a fatty acid radical, an unstable molecule which subsequently reacts with oxygen forming a peroxyl-fatty acid radical. This in turn, can react with another free fatty acid and so on, creating lipid peroxide end products such as malondialdehyde and 4-hydroxynonenal [2,12], which both can be used as markers of oxidative stress [13,14]. When this happens, the membranes lose their fluidity, and the cells can undergo apoptosis. Moreover, the end products can be mutagenic and carcinogenic since they can continue to react with e.g., DNA [15].

Proteins

Proteins are frequently targeted by free radicals due to their high abundance. Damage occurs both at sidechains and backbone, and results in altered hydrophilicity, fragmentation, aggregation, protein unfolding and distorted conformation, disturbed interactions with other target molecules/proteins and shortened lifespan [16]. Furthermore, this can lead to accumulation of modified and improperly degraded proteins in the cell, which is associated with disease and aging [17,18]. If this occurs in the presence of oxygen, peroxyl radicals and peroxides are produced, which in turn can oxidize other proteins and targets, thereby propagating the reaction [16]. A stable, commonly used, biomarker of these oxidative protein lesions are the presence of carbonyl groups [19].

DNA

Oxidative damage to DNA contributes to both ageing and disease, e.g., cancer. ROS can cause several different types of damage to the DNA, including DNA base modification, DNA breaks (both single- and double-stranded), loss of purines, DNA-protein cross-linkage, and alterations in the repair system. $OH^{\bullet}$ is most likely to cause DNA damage, with ionizing radiation as a potential source, which can result in different adducts to the DNA molecule [20]. One of these lesions, an oxidation product called 8-hydroxydeoxyguanosine (8-OHdG), is a marker of such damage [21]. However, the cell possesses a well-established defense system with DNA damage repair pathways including base excision repair, mismatch repair and nucleotide excision repair. Part of the damage caused by ionizing radiation can result in double-strand breaks (DSB), which can be repaired by non-homologous end joining or homologous recombination [22]. At these DSB, phosphorylation of histone H2AX occurs, which enables the use of formation of γ-H2AX as a method to monitor DNA damage [23].

Antioxidant defense

To protect the cells and tissues against oxidative stress the body has developed an arsenal of antioxidants the uphold the balance between oxidants and antioxidants (Figure 1). Focus of this thesis is the antioxidant α_1-microglobulin (A1M), which is described in detail in the last section.

Endogenous

Superoxide dismutase (SOD) produces H_2O_2 and oxygen from $O_2^{\bullet -}$. The activity requires metal cofactors, which are normally bound by SOD. These define the different types and where they are present: zinc and copper, cytoplasm and extracellular (SOD1 respectively SOD3), and manganese, mitochondrial (SOD2) [24]. CAT is responsible for further processing of H_2O_2 into water and molecular oxygen, consequently limiting free radical-induced damage [25]. GPx is another

antioxidant responsible for reduction of H_2O_2. GPx utilizes glutathione (GSH), a thiol tripeptide highly abundant in the body, to accomplish this, which is oxidized to glutathione disulfide (GSSG). GSSG is reduced by glutathione reductase back to GSH [26]. The ratio between GSH and GSSG can be measured as a marker of oxidative stress [27].

Peroxiredoxins are a group of enzymes with conserved cysteine residues used to reduce peroxides e.g., H_2O_2, lipid peroxides and peroxynitrite. In humans there are six different peroxides (Prx1-6). Prx2 is highly abundant in RBCs where it has been suggested to be a marker of oxidative stress [28]. After the cysteine residues have been oxidized, they can be reduced by thioredoxin. Thioredoxins (Trx) are involved in thiol-disulfide exchange reactions, which involve donating electrons to the substrate protein, and in a multistep process reducing the substrate protein and ending up with the formation of a disulfide bond in its oxidized form. Trx is then reduced by Trx reductase, by using the electron-donator NADPH [29].

The antioxidant arsenal also includes non-protein low molecular weight scavengers, for example, uric acid. Uric acid can scavenge singlet oxygen, peroxyl and hydroxyl radicals and, in addition, reduce lipid peroxidation of RBC membranes [30].

Exogenous

Our diet is the main source of exogenous antioxidants such as vitamin C, vitamin E, carotenoids and phenolic compounds. Vitamin C, also known as ascorbic acid, is a ubiquitously present water-soluble antioxidant. It scavenges hydroxyl, alkoxyl and superoxide radical anion, and in addition RNS, throughout the body. Vitamin E is fat-soluble and mostly protects cell membranes from lipid peroxidation by forming a derivative with low reactivity. Beta-carotene and lycopene are examples of carotenoids, and beta-carotene is important in protecting skin and eyes from UV-light damage by quenching singlet oxygen [30]. Resveratrol is a phenolic antioxidant, present in e.g. grapes, that has been shown to bind ROS and modulate enzyme activity, in addition to decreasing lipid peroxidation [31,32].

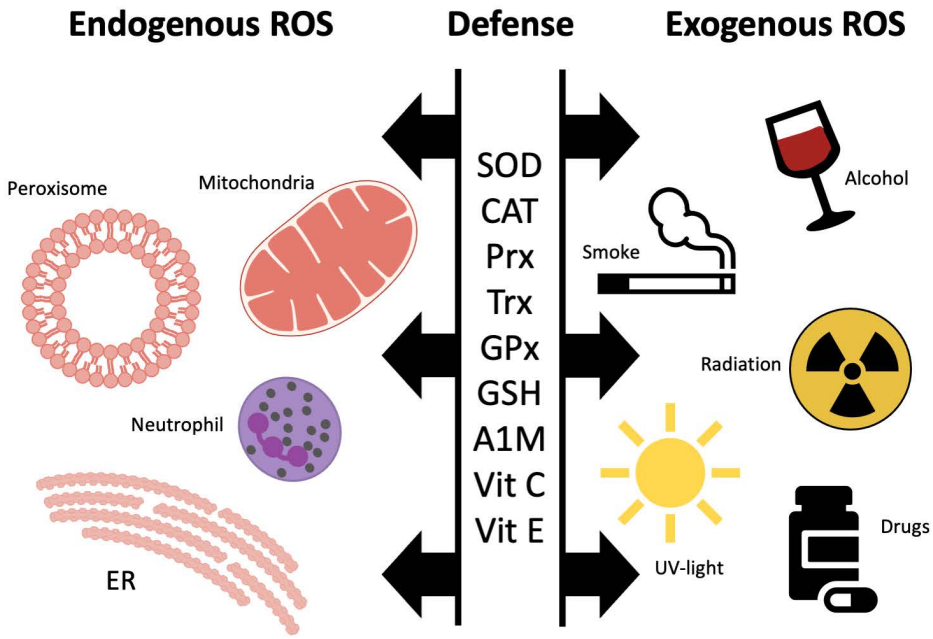

Figure 1. Endogenous production of ROS occurs in e.g. mitochondria, peroxisomes, ER and neutrophils by different cellular processes such as the respiratory pathways of the mitochondria (ATP) and peroxisomes, disulfide formation in ER and the production of bacteriocidal ROS by MPO. Exogenous sources include smoke, alcohol, UV-light, radiation and drugs. The body has an arsenal of antioxidants to help cope with the stress: SOD, CAT, Prx, Trx, GPx, GSH and A1M. Moreover, our diet vitamins, e.g. C and E, also contribute to the antioxidant defense.

Role in disease

Oxidative stress is involved in many diseases and progression of age-related pathologies [33]. Oxidative stress has been highlighted in conditions that become more common as we age, e.g., cardiovascular disease, neurodegenerative disease and cancer (Figure 2). Due to the extensive role of oxidative stress in disease, the role of antioxidants as treatment for such diseases has been suggested. Although few clinical trials have so far been successful, new treatment strategies are currently being investigated [34-36]. One of these is the focus of this thesis, the use of the antioxidation properties of A1M, with focus on erythrocyte and kidney pathology.

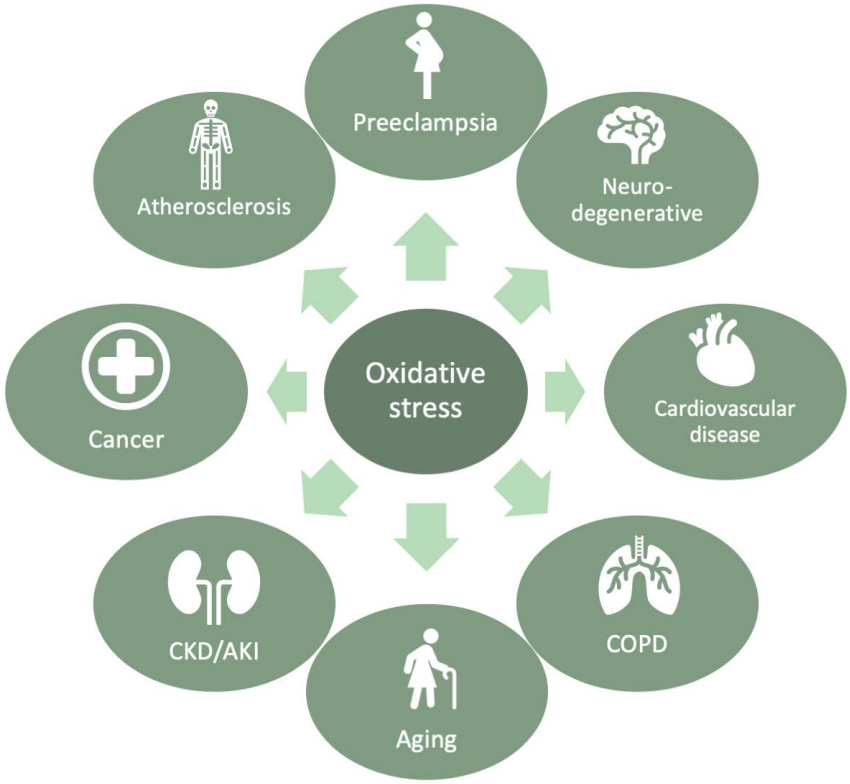

Figure 2. Oxidative stress contributes to the pathophysiology of a wide range of diseases such as preeclamspia, neurodegenerative diseases, cardiovascular disease, chronic obstructive pulmonary disease (COPD), aging, chronic kidney disease (CKD), acute kidney injury (AKI), cancer and atherosclerosis.

Kidneys

The kidney is a complex organ with several different functions that affect the whole body. In the following section a short overview of the kidney physiology and renal diseases are given. Kidney damage resulting from medical therapy from radiation therapy is especially highlighted since it is of central importance to this thesis.

Kidney physiology

The main function of the kidneys is to clear metabolic waste products by filtering the blood. Blood flows to the kidneys through the paired renal arteries and exits through the renal veins, with excretion of waste products occurring with the urine produced. In addition to disposal of metabolic waste products, the kidney regulates electrolyte balance, acid-base balance, fluid volume, blood pressure and secretion of hormones (e.g., erythropoietin, renin and calcitriol).

The kidney is divided into the medulla, the inner part, and the cortex, outer part, with the nephrons spanning both parts (Figure 3). The nephron is the functional unit of the kidneys, with each kidney containing roughly 1 million nephrons (200 000 – 1.8 million), compared to the mouse kidney containing approximately 20 000 [37,38].

The initial filtering occurs through the renal corpuscle, consisting of the glomerulus surrounded by the Bowman's capsule. The high pressure will force the blood to be filtrated through the pores of the endothelium in the capillaries of glomerulus into the extravascular space of Bowman's capsule. Proteins with a molecular weight of up to 15 kDa are freely filtered in the glomeruli; proteins up to 45 kDa, e.g. A1M, are filtered with only minor restriction and proteins between 45 to 60 kDa only restrictively filtered. Plasma proteins above 60 kDa are not filtered through the kidney [39].

From the Bowman's space, the filtrate or primary urine reaches the proximal tubule, loop of Henle and distal tubule where reabsorption of e.g., water, ions, glucose, proteins, peptides and amino acids can take place. In the process, secretion of waste products e.g., urea, metabolites and creatinine also occurs. In the last step the ultrafiltrate leaves the nephron and enters the collecting duct (where some absorption still occurs e.g., water and Na^+) prior to emptying into the ureters. It is from then referred to as urine.

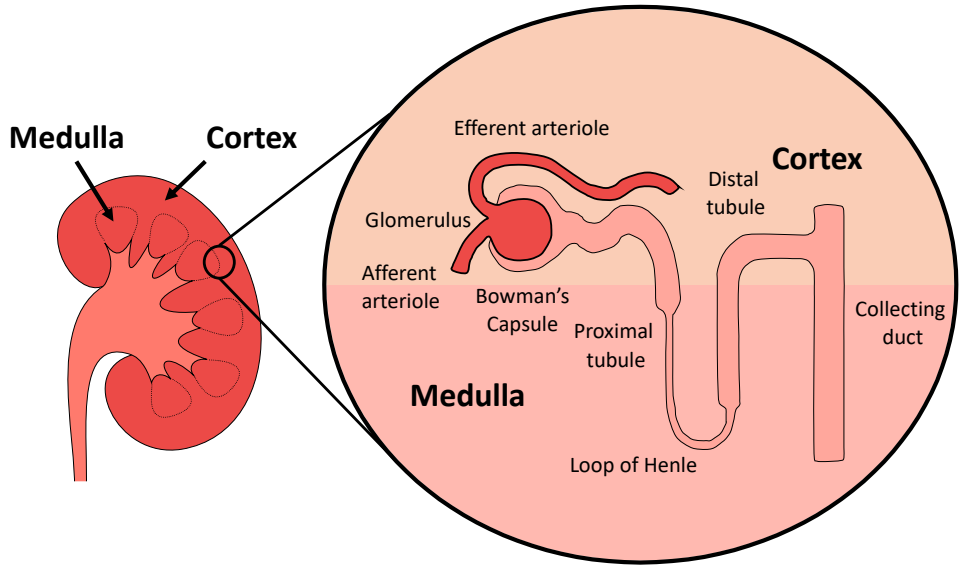

Figure 3. Overview of the renal stucture and the nephron.

Markers of kidney damage

Since A1M is partially filtrated in the glomeruli and partially reabsorbed in the proximal tubuli, the concentrations of A1M in blood and urine can be used as a marker of both glomerular and tubular function, i.e., kidney damage, which is elaborated on below. One of the most commonly used markers for renal injury is albumin. Albumin contributes half of the protein content in human plasma and is mainly a transport protein. With a size of 68 kDa, albumin is not able to pass the glomerular barrier, so its presence in urine indicates that there is a leakage in the filtration [40].

Cystatin C is a small, 13 kDa, protease inhibitor that is filtered, reabsorbed and catabolized by kidney tubules without re-entering the blood stream [41,42]. It is therefore, similar to the commonly used creatinine [43], a marker of glomerular filtration rate (GFR), hence, it estimates the flow rate of filtered fluid through the kidney.

Serum creatinine is a waste product produced from the breakdown of creatine and phosphocreatine, which is filtered freely through the kidneys. Similarly, blood urea nitrogen (BUN), or urea, is a product of protein metabolism, which is cleared almost entirely by the kidneys. Creatinine levels depend on the person's muscle mass and BUN on the diet and hydration level [44], which must be taken into consideration when using its plasma concentration as a marker of GFR. Just as with cystatin C, a reduction in GFR results in increasing plasma levels of these two markers.

NGAL (neutrophil gelatinase-associated lipocalin, also referred to as lipocalin-2) is an iron-transporting protein that is almost entirely reabsorbed by tubules in the normal kidney. NGAL is rapidly and massively expressed in response to acute nephrotoxic and ischemic insults resulting in acute kidney injury. Its presence in urine, therefore, indicates tubular injury [45]. Another marker of tubulointerstitial injury is KIM-1 (kidney injury molecule-1), which is dramatically increased in urine after renal injury in renal proximal tubular epithelial cells [46]. The extracellular part is cleaved and enters tubule lumens which is then excreted in the urine [47]. Although the function of KIM-1 is not fully elucidated, reports suggest that KIM-1 is a phosphatidylserine receptor that transforms epithelial cells to phagocyte-like cells in response to damage or stress [48].

Kidney diseases and conditions

Acute kidney injury and chronic kidney disease

There are many diseases that affect the kidneys, both acquired and congenital. Acute kidney injury (AKI) is defined as a rapid loss of kidney function which can be defined by above-mentioned biomarkers [49]. The triggers and pathophysiology are diverse and can include infections, ischemic injury in connection to major surgery, or exposure to harmful substances such as nephrotoxic drugs and medications [50].

The release of excessive amounts of hemoglobin, myoglobin and heme can result in AKI. Rhabdomyolysis is characterized by myocyte destruction, induced by e.g., trauma, drugs or muscle disease. This is followed by release of cellular content such as myoglobin that contributes to AKI in approximately a third of the patients [51]. Moreover, plasma from patients that develop AKI after cardiopulmonary bypass surgery have higher levels of cell-free hemoglobin and markers of oxidative stress [52]. Therefore, it is believed that generation of ROS by released heme-proteins contributes to the etiology of certain types of AKI.

AKI can, in turn, result in complications including disturbance in fluid balance, uremia, metabolic acidosis, damage to other organs and, in severe cases, death [53]. Moreover, patients with AKI have an increased risk of developing chronic kidney disease (CKD) later in life or aggravating pre-existing CKD, and accelerating the progression to end-stage renal disease (ESRD) [54-56]. CKD is defined by decreased function, often measured by glomerular filtration rate (GFR), or occurrence of kidney damage markers exceeding three months [57]. In addition to previous AKI, diabetes, glomerulonephritis and hypertension are common causes of CKD [58]. For CKD the most common cause of death is cardiovascular disease for example coronary artery disease and congestive heart failure [59].

Diabetes

Diabetes mellitus is a chronic disease characterized by elevated blood sugar levels. It can be divided into type I and II, where the former is characterized by insufficient insulin production and the latter by resistance to insulin. The worldwide incidence is rising rapidly, from 108 millions in 1980 to 422 million in 2014 with cases expected to only keep rising [60]. For both types of diabetes mellitus there are increasing evidence for an association with oxidative stress [61,62].

The microvascular complications of diabetes include diabetic nephropathy, a progressive renal damage, characterized by persistent albuminuria and declining GFR [63]. Insults, both metabolic, (hyperglycemia and dyslipidemia) and hemodynamic (systemic and glomerular hypertension) are the main contributors of progression of the pathology in diabetic nephropathy [64]. There are several sources of ROS in the diabetic kidney, both enzymatic and nonenzymatic, including autooxidation of glucose, Fenton reactions, uncoupling of nitric oxide synthase and advanced glycation. Moreover, mitochondrial production of ROS in response to continuous hyperglycemia may be an early contributor to the pathogenic progression [65]. The excess ROS also contribute to the progression to ESRD by indirectly causing increased expression of extracellular matrix (ECM) genes which contributes to fibrosis [64]. Diabetes is the leading cause of ESRD [66]. Although there is a consensus regarding the role of oxidative stress in diabetes, so far no clinical trials with antioxidant therapy have been successful [67].

Preeclampsia

Pathological hallmarks of Preeclampsia (PE) are albuminuria and high blood pressure. PE affects around 4-5% of pregnancies, and is the most common medical pregnancy complication. Interestingly, PE is a human disease which does not occur in other animals [68].

PE can be divided into two different phases where the first phase is characterized by defective placentation, with decreased utero-placental blood flow as a result. This increases the oxidative stress and aggravates the vascular inflammation, dysfunction and insufficient blood flow in the placenta and maternal organs [69-72]. The second stage is characterized by the clinical manifestation and occurs after 20 weeks of gestation as a response towards placenta-derived factors released through the disrupted placental barrier into the maternal circulation [73]. Other than treating the symptoms, e.g., the hypertension, the only cure is delivery, indicating that it is a placenta-driven disease [74].

Much of the oxidative stress is believed to stem from increased production and accumulation of fetal hemoglobin (HbF) in the placenta, that, later induces damage in the placental barrier and leakage of HbF into the maternal circulation [70,75,76]. As described below, the Hb and heme toxicity can result in ROS, which in PE contributes to vasoconstriction, renal damage, endothelial damage and hemolysis

[77,78]. An aggravated and acute form of PE, HELLP (**H**emolysis, **E**levated **L**iver Enzymes and **L**ow **P**latelets), is especially associated with hemolysis and occurs in approximately 10-20% of severe PE cases [79].

PE affects the kidneys both functionally but also morphologically. Typically, both blood pressure and kidney function normalizes postpartum, however, women with PE have increased risk of developing microalbuminuria and emerging studies also suggests an increased risk of ESRD later in life [80,81]. In addition, women with CKD are ten times more likely to develop PE [82], which highlights the close connection between renal conditions and PE.

Peptide receptor radionuclide/radioligand therapy

Peptide receptor radionuclide/radioligand therapy (PRRT/RLT) is a site-directed therapeutic approach that uses radiolabeled peptides/ligands to accomplish cytotoxic levels of radiation to cancer cells overexpressing specific receptors. The method targets cancer cells with high specificity by binding the ionizing radiation source to the cell (Figure 4). In this thesis PRRT/RLT animal models were used to evaluate kidney damage and the use of A1M as a radioprotector in the treatment of neuroendocrine tumors and metastatic castration-resistant prostate cancer.

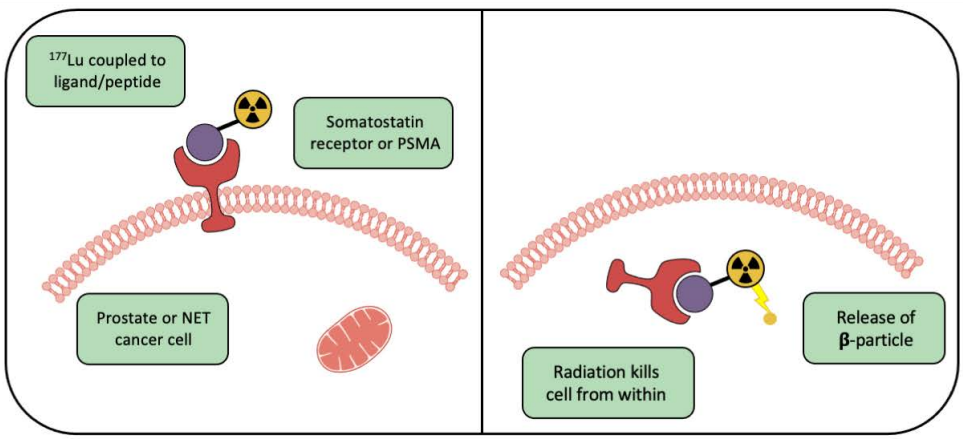

Figure 4. In peptide receptor radionuclide/radioligand therapy, a radiation source (e.g. ^{177}Lu) is coupled to a ligand or peptide that binds to an overexpressed receptor or surface protein on the cancer cell. The complex is internalized and can irradiate the cell from within resulting in death of the cancer cell.

PRRT in neuroendocrine tumors

Neuroendocrine tumors (NETs) are a group of neoplasms derived from the neuroendocrine system accounting for 0.5% of malignancies [83]. The incidence is increasing in both Europe and the USA, from 1.09 per 100 000 people in 1973 to

6.98 in 2012 [84], although numbers may reflect improvements in detection as well. The gastrointestinal tract and lungs are the most common sites for primary tumors [83]. NETs are classified into three categories depending on their mitotic rate and Ki67 proliferative index: NET grade 1, NET grade 2 and neuroendocrine carcinoma [85], where patients with grade 1 and 2 are suitable candidates for PRRT.

Many of these tumors overexpress somatostatin receptors, mainly sstr2 [86]. This is one out of five subtypes in the somatostatin receptor family. In the clinic, inoperable NETs, overexpressing sstr2, can be treated with PRRT using e.g. ^{177}Lu-DOTA^{0},Tyr^{3}]octreotate (lutetium-177 [^{177}Lu]-DOTATATE) and ^{90}Y-[DOTA^{0}, Tyr^{3}]-octreotide (DOTATOC) [87,88], thereby driving the cancer cells into regression and apoptosis. Yttrium-90 (^{90}Y) was suggested to be used in larger tumors due to its higher energy (and therefore longer pathway), but since it also causes greater toxicity to surrounding tissue [89], Lutetium-177 (^{177}Lu) may be preferable.

Acute adverse effects include nausea, vomiting and abdominal pain [90]. Additionally, these peptides are filtered through the glomeruli and are reabsorbed in the tubular cells, due to their small size, which results in significant doses of radiation to the kidneys [91]. Therefore, the kidney is the dose limiting organ. Nephrotoxicity have been reported for patients, particularly when ^{90}Y is the radiation source [92]. Furthermore, known underlying renal conditions such as diabetes and hypertension may limit the activity given to patients or exclude them from treatment altogether. In addition to renal damage, another dose-limiting organ is the bone marrow [92,93].

Co-administering positively charged amino acids (L-arginine and L-lysine) to patients to inhibit kidney retention of the radiopeptides have been shown to reduce absorbed dose in the kidney [94,95]. However, this is associated with acute hyperkalemia and a trigger for hormonal crisis for patients treated with PRRT [96,97].

Radioligand therapy in prostate cancer

Prostate cancer (PC) is the most common cancer diagnosis in males [98]. Although often benign, it is the third-leading cause of cancer death in men [99]. If PC becomes metastatic, androgen-deprivation therapy (ADT) is the preferred therapy with clinical improvements in most patients [100,101]. Nonetheless, ADT is not curative; and after 18−24 months the tumor becomes castration-resistant (CRPC) [102]. Approximately 10-20% of the cases progresses to metastatic castration-resistant prostate cancer (mCRPC) with poor survival rate [103].

Prostate specific membrane antigen (PSMA) is a transmembrane glycoprotein which is overexpressed in prostate-derived tumors compared to the minimal expression seen in other tissues for example, kidney, proximal small intestine and salivary gland [104]. PSMA expression increases with tumor grade, androgen-

independence, metastatic disease, and PC recurrence [105], which makes it both a predictor of disease both also a useful target. PSMA should not be confused with PSA, prostate specific antigen, which is commonly used to determine the extent of PC and to evaluate PC treatment [106].

Small-molecule PSMA inhibitors, e.g., PSMA-617, that can bind to the PSMA on the cancer cells, have been coupled to both the beta-emitter ^{177}Lu and the alpha-emitter Actinium-225 (^{225}Ac) to treat mCRPC. Although ^{225}Ac-PSMA-617 was shown to have reduced blood cell toxicity in one study [107], ^{177}Lu-PSMA-I&T or ^{177}Lu-PSMA-617 are still commonly favored [108,109], due to their short maximal tissue penetration and comparatively long half-life, and have been more extensively studied [110]. A meta-analysis of ten studies, concluded that treatment with ^{177}Lu-PSMA resulted in a PSA decline in almost 70% of patients [111], indicating its clinical usefulness.

Among the reported side effects of ^{177}Lu-PSMA-617 RLT are xerostomia (dry mouth), anemia, leukocytopenia, thrombocytopenia and nausea [112]. Surprisingly, considering the renal excretion, so far only low-grade nephrotoxicity has been reported [113,114]. However, there are several risk factors such as pre-existing kidney disease and elevated blood pressure that could increase the risk of developing renal toxicity [113].

In both ^{177}Lu-DOTATATE and ^{177}Lu-PSMA-617 radiotherapy, increased doses correlate with improved therapeutic effect, and therefore, co-administering a radioprotector may allow additional treatment cycles or higher injected activities. A radioprotector could reduce acute and delayed renal toxicity, but also make therapy available for patients with underlying renal dysfunction which today may receive lower activities or be excluded from therapy.

Red blood cells

The red blood cell (RBC), also referred to as erythrocyte, is the most abundant cell in humans [115]. It is responsible for oxygen delivery to cells and tissues throughout the body. In this thesis, the main focus, however, is the toxic side-effects when the RBCs rupture, hemolysis, and the cellular components leak out and instead damage the cells and tissues.

RBC structure and function

Erythropoiesis

In adults the RBCs are produced in the bone marrow, a process referred to as erythropoiesis. During fetal development, the RBCs are first produced in the yolk sac and later in the liver. The RBC lifespan is approximately 115 days in adults, whereas fetal RBCs have a shorter lifespan [116-118] and murine RBCs even shorter (approximately 45 days) [119,120].

Human erythropoiesis is stimulated by erythropoietin (EPO) which is secreted by the kidneys in response to hypoxia and anemia [121]. The process starts with hematopoietic stem cells that differentiate into common myeloid progenitors, which thereafter becomes megakaryocyte-erythroid progenitors (Figure 5). Via burst forming unit-erythroid (BFU-E) and colony forming units-erythroid (CFU-E) the differentiation reaches the pro-erythroblasts. Fast cell divisions of the pro-erythroblasts reduces the cell size drastically and increases Hb content, in addition enucleation occurs, before the reticulocytes can be released from the bone marrow to the blood circulation, where they mature to RBCs [122]. When alterations in the RBC shape, size, surface or deformability are recognized they are broken down in the spleen as the last step of their life cycle [123].

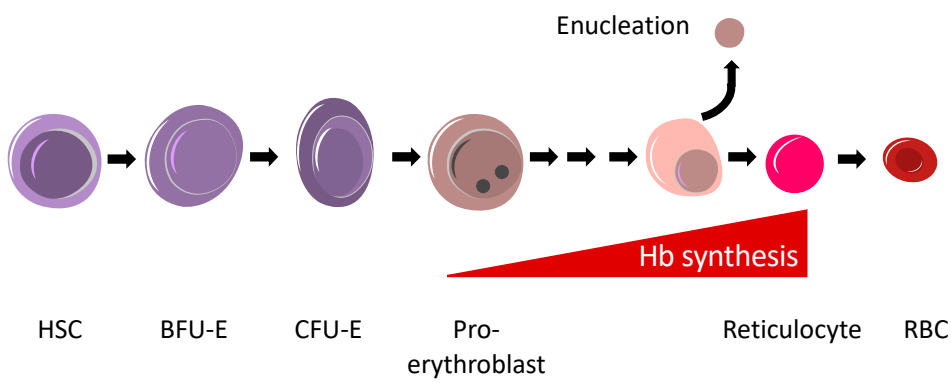

Figure 5. Overview of human erythropoiesis.

Structure

The typical human RBC is a bioconcave disk with a diameter of 7.5-8.7 µm, which is smaller than most other cell types. The small size and the large surface allows it to travel through the capillaries and efficiently exchange gas [124]. The red color is from the heme-bound iron ions in the hemoglobin (Hb), which constitutes a large part of the cell volume. The RBC membrane consists of a phospholipid bilayer with a cytoskeletal network of spectrin molecules underneath. This results in the disc-shape as well as the flexibility of the RBCs [124]. To enable maximum oxygen transportation, RBCs do not contain a nucleus or cell organelles such as mitochondria. Instead, their energy supply comes from anaerobic conversion of glucose generating ATP [125].

Antioxidants

Oxidative stress impairs the oxygen delivery of the RBCs. Therefore, they have an arsenal of antioxidants available, including GSH, ascorbic acid, CAT, SOD, GPx and Prx-2 [126]. External ROS, e.g., from neutrophils and macrophages in the circulation, enter the RBC, and endogenous ROS, constantly generated by auto-oxidation of Hb into methemoglobin (metHb) and superoxide (that dismutates into H_2O_2), are neutralized by these systems. However, the antioxidants are foremost cytosolic and there is a limited capacity the protect the RBC membrane from ROS. The RBC membrane is therefore more vulnerable to oxidative damage, decreasing the formability, and thereby impairing oxygen delivery to cells and tissue, but also to increased hemolysis and, consequently, the extracellular release of Hb and heme.

Hemoglobin, heme and oxidative stress

Structure and function

The RBCs are responsible for oxygen delivery to tissues and cells in the body. To do so, they are packed with Hb, which consists of four globin subunits linked together and each globin chain binding a heme group. In the center of the heme group, an iron atom is coordinated by four nitrogen atoms of a tetrapyrrole ring and a nitrogen of a histidine-imidazole ring (Figure 6). This structure allows heme to reversibly bind oxygen, although carbon monoxide and nitric oxide can also be bound [127].

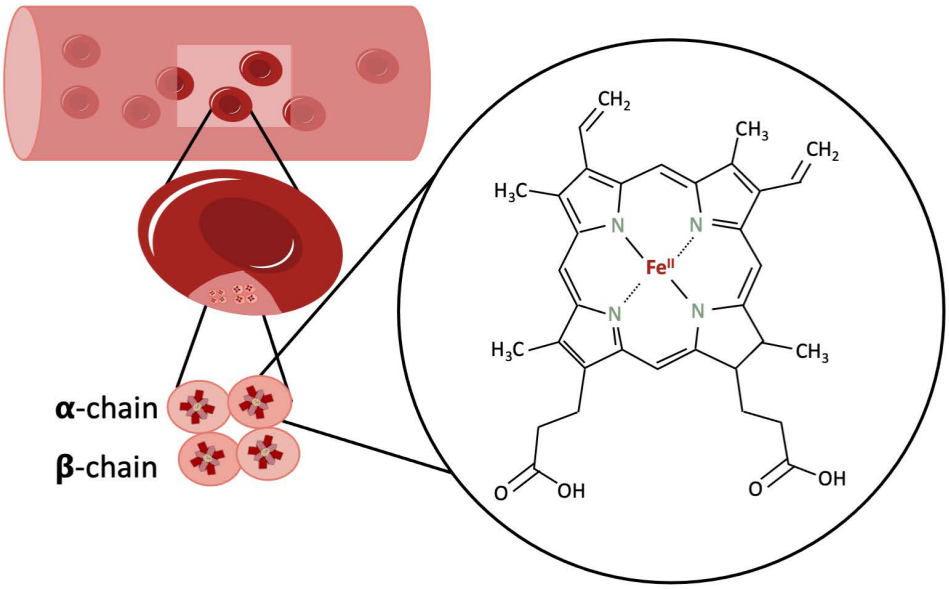

Figure 6. RBCs are packed with Hb, consisting of two α-chains and two ß-chains. Each globin chain binds a heme-group, with an iron atom in the center.

In adults, Hb most commonly consists of two α and two ß-chains ($\alpha_2ß_2$, HbA). During development, the fetal hemoglobin consists of two γ-chains instead of the ß-chains ($\alpha_2\gamma_2$, HbF), which gradually are replaced with ß-chains after birth [128]. HbF has a higher oxygen affinity than HbA to enable oxygen delivery from the mother to the fetus. More than 1800 Hb variants have been described that are associated with hemoglobinopathies and clinical findings, for example in thalassemia and sickle-cell disease [129].

Toxicity/damage

Hb is compartmentalized in the RBCs but can have toxic effect on tissue outside of the RBCs if it leaks out. When intravascular hemolysis occurs, the redox reactivity of the heme iron can result in damage to the surrounding cells and tissues.

In oxyHb, the ferrous iron (Fe^{2+}) can auto-oxidize, resulting in metHb with ferric iron (Fe^{3+}) and the $O_2^{\bullet-}$. The formed $O_2^{\bullet-}$ can then dismutate to H_2O_2, and metHb can act as a peroxidase reacting with H_2O_2, forming ferryl Hb ($HbFe^{4+}$) and ferryl radical Hb ($^{\bullet}HbFe^{4+}$) [130]. In addition to their oxidizing properties, these molecules are proinflammatory and damage the surroundings [131]. Moreover, heme is lipophilic and can intercalate and disrupt cell membranes and the cytoskeleton [132]. Hb can also bind NO, which during increased vascular hemolysis can affect the vasoconstriction and elevate blood pressure [76,133].

Heme and hemoglobin detoxification

To combat heme and Hb toxicity, humans have several detoxification systems (Figure 7). Haptoglobin (Hp) is an acute phase protein mainly synthesized in the liver, which can be increased in response to e.g. inflammatory signaling [134]. Hp binds extracellular Hb in the circulation. Afterwards, clearance of the Hp-Hb complex occurs by binding to the macrophage receptor CD163 [135].

Heme scavenging occurs by hemopexin (Hpx). Hpx is an acute phase glycoprotein which is primarily expressed in the liver [136]. Hpx transports the bound heme to several cell types, including macrophages, expressing the LDL receptor related protein CD91 [137]. The complex is then internalized by receptor mediated endocytosis, whereafter heme can be degraded [136].

Heme oxygenases (HO) participate in heme catabolism [138]. HO-1 expression is upregulated by a range of environmental stress types including free Hb and heme, and HO-1 gene expression can therefore be used as a marker of oxidative stress. HO-2, however, is constitutively expressed [139]. In the first step in heme degradation, HO degrades heme to biliverdin, CO and Fe^{2+} together with microsomal NADPH-cytochrome P450 reductase. Afterwards, biliverdin reductase converts biliverdin to bilirubin [140]. The iron can be stored as Fe^{2+} by ferritin and later be recycled [141].

Albumin has also the ability to bind heme. Since the albumin plasma levels are high, there is a considerate amount that can be bound, making it especially important when Hpx levels are depleted e.g. during increased intravascular hemolysis [142]. Another important heme scavenger is the antioxidant A1M [143]. This is described in detail below.

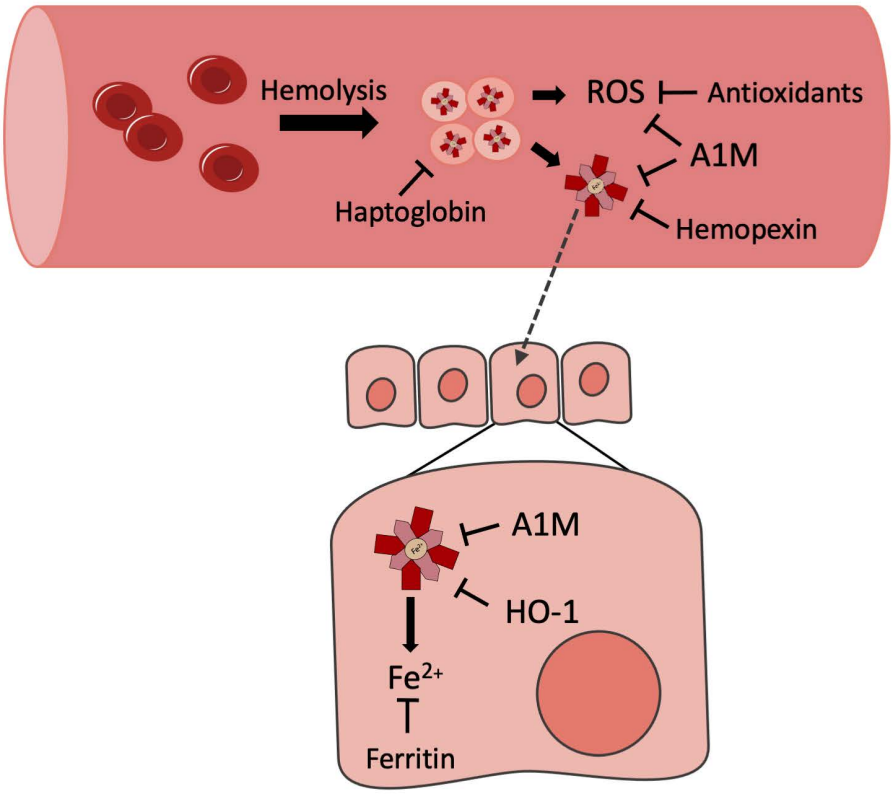

Figure 7. Humans have several protective mechanisms against Hb and heme toxicity. Hp binds Hb, whereas A1M and Hpx bind heme. In addition, A1M binds heme intracellularly and a truncated form, t-A1M, also participates in heme catabolisim. The resulting ROS from hemolysis are detoxified by a number of antioxidants. Heme is catbolized by HO-1 and the Fe^{2+} is recycled by ferritin.

Hemolytic and erythropoietic conditions

Many hemolytic and erythropoietic conditions either stem from RBC dysfunction or inadequate RBC development, and often, hemolysis is a significant part of the pathology. Below a few of these are presented, and are also elaborated on in the last paper of this thesis [144].

Anemia

There are many different forms and origins of anemia, however, they are all defined by a decrease in the total amount of RBCs, often with lowered Hb and subsequently impaired oxygenation of tissues. Anemia affects approximately a third of the world's population [145]. Anemia can be caused by blood loss, decrease in RBC production or increase in hemolysis. Lowered production can be due to impaired

erythropoiesis or nutritional deficiencies. Increased breakdown can be a result of genetic disease, e.g., sickle-cell anemia, or acquired diseases such as malaria.

Macrocytic anemia is defined by larger RBCs, a mean cell volume (MCV) of above 100 fL in adults (normocytic cells 80-100 fL). The underlying causes are diverse and macrocytic anemia can be divided into megaloblastic and non-megaloblastic [146]. Megaloblastic anemia with an increase in red blood cell distribution width (RDW) can be due to folate or B12 nutrient deficiency. Other causes include certain drugs or inborn errors of metabolism. If there is an increase in reticulocytes or reticulocytopenia it is more likely non-megaloblastic. Causes include inherited or acquired errors such as Diamond-Blackfan anemia (DBA), 5q-minus Myelodysplastic syndrome (5q-MDS), Fanconi anemia and hemolytic anemia, but also liver disease and excessive alcohol can be responsible [147].

DBA and 5q-MDS have in common that there is an intracellular accumulation of excessive heme [148]. 5q-MDS is characterized by loss of part of human chromosome 5 in bone marrow myelocyte cells and DBA is a congenital disorder which often presents itself already at infancy. The inactivating mutations occur in ribosomal proteins which results in decreased ribosome synthesis [149-152]. Normally, when there is an intracellular increase of heme, the genes expressing ribosomal proteins are upregulated to equilibrate the globin to heme ratio [153]. It is this unbalance during early erythropoiesis that results in free heme buildup, contributing to the macrocytic anemia and the DBA and 5q-MDS pathology [148,154].

Atherosclerosis

Atherosclerosis is not defined as a hemolytic disease; however, the plaque buildups can have intra-plaque hemorrhages (IPH), which is an important part of the disease progression [155]. IPH have been linked to plaque ruptures, which in turn can lead to myocardial infarction or stroke [156,157]. Atherosclerosis is one of the leading causes of morbidity in the developed world [158], but there is a lack in treatment options beyond statins.

Heme and Hb toxicity are new therapeutic targets that have emerged in atherosclerosis [159]. Supporting this, HO-1 knock-out mice and mice treated with HO-1 inhibitor both have exacerbated plaque formation [160,161], whereas increased expression of HO-1 decreases plaque buildup [162].

Blood transfusions

Blood transfusion is a life-saving treatment, however, in a small percentage of cases (2%) the receiving patient will produce an antibody against a blood group antigen present on the donor's RBC [163]. If the patients are transfused with antigen-positive blood, there is a risk of acute or delayed hemolysis. The pathophysiological pathways triggered by hemolytic transfusion reactions can result in organ damage,

the kidneys are especially at risk [164]. Moreover, in patients receiving chronic transfusions, e.g., patients with sickle-cell disease, antibody evanescence (decrease in antibody titers to below the limits of detection) or previously undetected alloantibodies increases the risk of hemolytic reactions [165].

RBCs destined for transfusion can be stored for up to 42 days in 2–8 °C. However, during storage, physiological changes results in lesions that accumulates over time, due to metabolite depletion/accumulation and oxidative damages [166]. The damaged RBCs stress the recipients clearance capacity and, in addition, contribute to endothelial ROS [167,168].

Intraventricular hemorrhage in premature infants

Intraventricular hemorrhage (IVH) is a major complication in premature infants. Close to half of preterm infants with a birth weight below 1000 g suffer from complications from germinal matrix-IVH and a third of these lesions are severe [169-171], and can be associated with complications later in life such as mental retardation and cerebral palsy [172].

The etiology of IVH is multifactorial and complex. The intrinsic fragility of the germinal matrix vasculature, a richly vascularized collection of neuronal-glial precursor cells in the infant brain, is suggested to be the origin of IVH together with the fluctuation in blood flow [173]. The subsequent rupture of ventricular ependyma progresses into IVH [174]. The leakage of RBCs into the intraventricular space with subsequent hemolysis followed by release of free Hb into the cerebrospinal fluid contributes to the inflammation seen in IVH [175]. Therefore, the hemolytic aspect and Hb and heme toxicity may be an interesting therapeutic target.

A1M

A1M is a small glycoprotein belonging to the lipocalin family. It was discovered in urine almost 50 years ago and researchers have since found several functions in the body of the protein [176]. Due to the reducing potential and many protective properties of A1M it has been suggested that the protein can be used to treat conditions where oxidative stress is a common denominator. This was also the main focus of this thesis – to elucidate new therapeutic opportunities for A1M.

Protein structure

A1M is found in many different species including vertebrates, amphibians, birds and fish [177-179]. Human A1M consists of a 183 amino acid long peptide chain with a molecular weight of 26 kDa, with three oligosaccharides attached [180-182]. A1M belongs to the lipocalin protein family. The lipocalin protein family consist of 40-50 proteins in different vertebrates, invertebrates, plants and bacteria [183]. They have a common protein structure with antiparallel beta sheets formed into a barrel. In many lipocalins, the interior of the barrel functions as a binding site for small hydrophobic compounds [184]. A1M consists of eight beta sheets, folded into the typical β-barrel shape (Figure 8), with one open and one closed end [185].

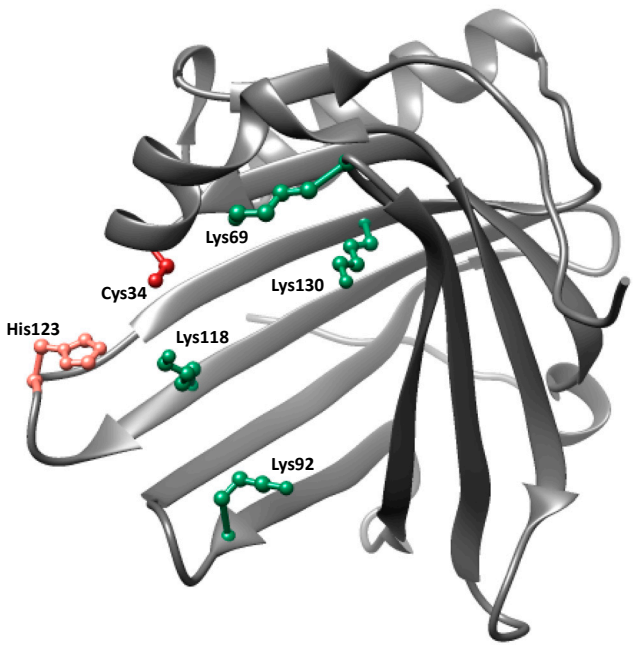

Figure 8. Structure of A1M. Important side-chains Cys34, His123, Lys69, Lys92, Lys118 and Lys130 are highlighted.

Located on the edge of the open end of the lipocalin pocket is the Cys34 sidechain, believed to be vital for many functions of A1M, described in detail below. This has also been supported by experiments with a mutant form where the cysteine was exchanged for a serine, which showed that A1M lost some of its enzymatic functions [186]. In addition to Cys34, A1M purified from urine contains covalent modifications on Lys69, Lys92, Lys118 and Lys130 which are believed to be connected to the radical and reductase abilities of A1M and responsible for the color and charge heterogeneity seen after purification [187,188].

AMBP gene

A1M is expressed by the *AMBP* gene (α_1-microglobulin-bikunin precursor gene) found in a lipocalin gene cluster in the 9q32–33 region in humans and chromosome 4 in mice [189-191]. Interestingly, this gene also encodes the protease inhibitor bikunin [182], with exons 1-6 encoding A1M and 7-10 encoding bikunin [192]. This construct is conserved in all species, indicating its biological importance.

The liver is the main site of synthesis, although A1M is found in most tissues, including kidney, placenta and skin [193,194]. Expression is upregulated in the presence of oxidative stress or heme [195-197]. One route of regulation of A1M expression, although it may not be the only one, is through the nuclear factor erythroid 2–related factor 2 (Nrf2)-pathway. The Nrf2-pathway is a key pathway that has been linked to antioxidant response e.g. upregulation of *HO-1* and *SOD* transcription [198]. Normally, Nrf2 is bound to KEAP1 homodimers in the cytosol that facilitates degradation of Nrf2. When intracellular oxidative stress levels increase, KEAP1 is covalently modified and Nrf2 escapes ubiquitylation [199]. Nrf2 then relocates to the nucleus where it binds small Maf proteins and to ARE (antioxidant response element) gene regulation sites, which then initiates transcription of *AMBP* among other cytoprotective genes [200-202].

After translation to a precursor protein, A1M and bikunin are kept together by a linker tripeptide (in humans VRR and mice ARR) while modifications occur [203]. A1M is glycosylated, as mentioned above, and bikunin is modified by attachments of a chondroitin sulfate chain, which subsequently is linked to protein heavy chains, forming the protein complexes inter-α-inhibitor (composed of heavy chains 1 and 2) and pre-α-inhibitor (composed of heavy chain 3) [204,205]. One function of the conserved co-synthesis is for A1M to act as a chaperone for bikunin and thereby suppress misfolding of bikunin and aid in formation of the bikunin complexes [206]. The linker peptide is cleaved in the Golgi apparatus before A1M and the bikunin complexes are released into the circulation separately [207] (Figure 9).

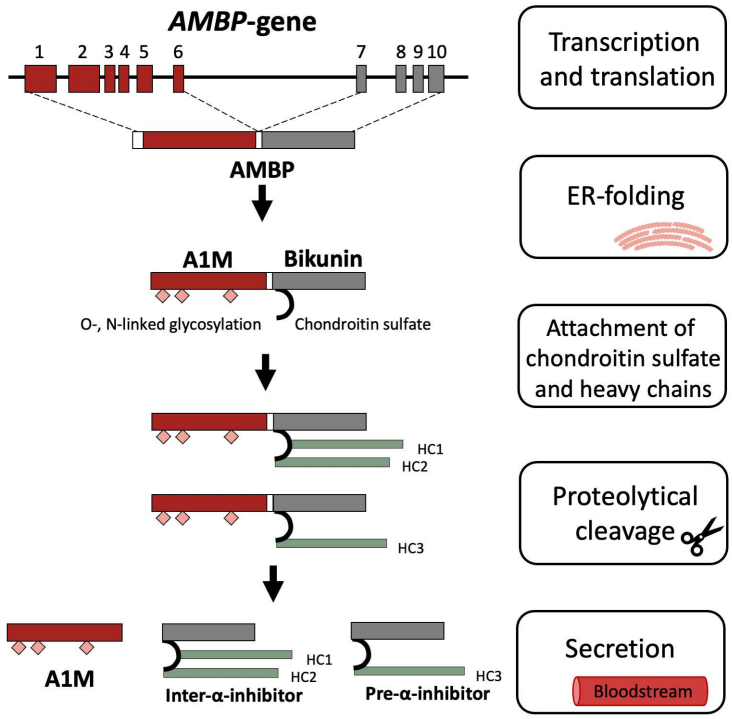

Figure 9. A1M is expressed by the *AMBP* gene that also encodes bikunin. After translation to a precursor protein, consisting of both A1M and bikunin, A1M is glycosylated and bikunin is modified by attachments of a chondroitin sulfate chain, which is subsequently linked to protein heavy chains, and forms different protein complexes. The linker peptide keeping A1M and bikunin together is cleaved in the Golgi apparatus and afterwards A1M and the bikunin complexes are released into the blood circulation.

Biodistribution and lifecycle

After synthesis and release into the blood circulation, A1M exists either in free form (50%) or bound to IgA, with some bound to albumin (7%), or prothrombin (1%) [208]. The plasma concentration of A1M is 20-50 mg/l (1-2 μM) with a relatively short half-life around 2-3 minutes [193,209,210]. A1M is extravasated into the extravascular space and, in recent years, A1M has also been shown to internalize into different cell types, including kidney cells, blood cells, keratinocytes and liver cells [197,211,212]. It has, however, not been established through which receptor(s) this takes place.

Plasma A1M can pass through the glomerular membrane and be taken up by the tubular cells where catabolism occurs [213]. A small amount of A1M is also excreted to the urine and a rise in the urinary levels is used as a sensitive biomarker of kidney damage [181], which is elaborated on below. Urinary A1M has a yellow/brown color when purified, which is explained by the binding of different chromophores to lysyl

sidechains and Cys34 [188,214]. Although not completely understood, this has been suggested to be a consequence of the radical and heme-binding activity of A1M.

Thus, A1M is rapidly equilibrated between blood plasma, extravascular spaces and the interior of cells, freely or through unknown transfer mechanisms, performing its protective roles (see below) mainly in the extravascular space and cell interior (Figure 10). Its half-life in blood is short and the turnover rate is rapid, allowing quick clearance of consumed A1M and rapid supply of fresh A1M.

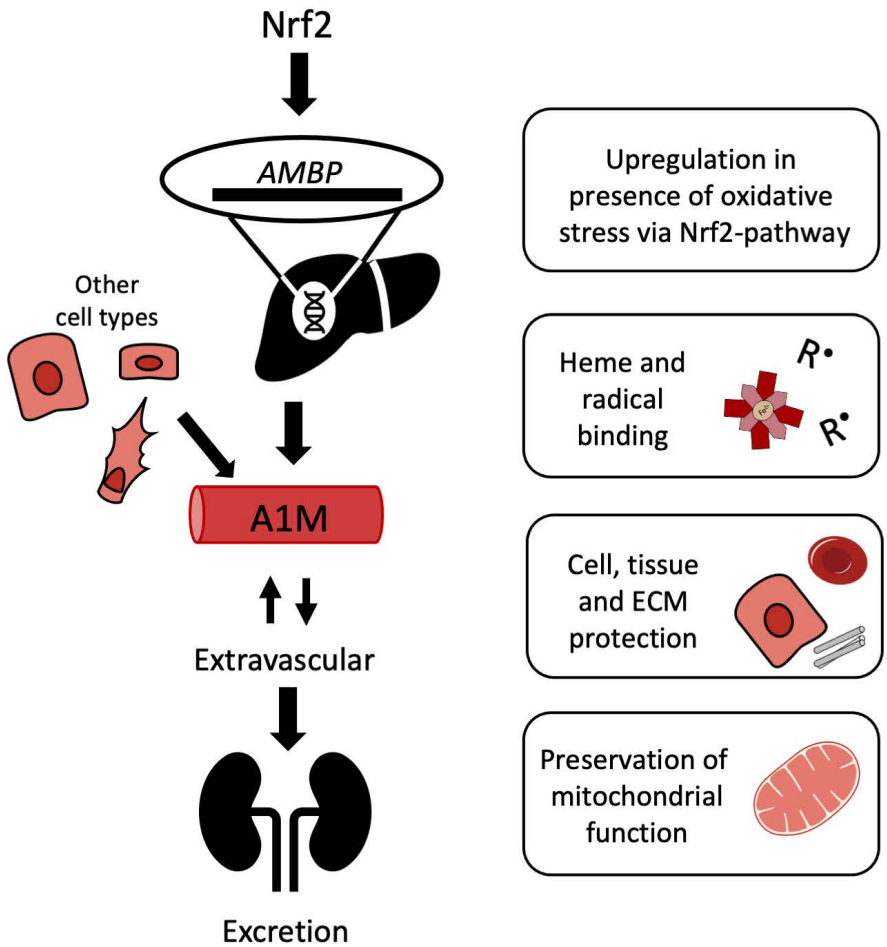

Figure 10. Lifecycle of A1M. Expression of *AMBP* is regulated through the Nrf2-pathway, and can be upregulated in response to heme or oxidative stress. The main site of synthesis is the liver but most cell types express *AMBP*. After translation A1M reaches the bloodstream from where it is extravasated, and can also be internalized into cells. In the different compartments A1M acts as a heme and radical scavenger, protector of cells, tissue and ECM, and preserver of mitochondrial function. A1M passes through the glomerular membrane and is taken up by the tubular cells in the kidneys where catabolism occurs.

Molecular properties and protective functions

A1M has been referred to as a household protein due to its capacity to bind heme-groups and free radicals and clear them from cytosol and the extravascular space (Figure 11). In the following section the protective properties of A1M are described, which is what makes it such an interesting protein.

Radical scavenger

One definition of a radical scavenger is a molecular entity that can bind free radicals. This was shown to be the case for A1M by Åkerström et al [187]. Using the synthetic radical ABTS (2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)) it was demonstrated that 8-9 ABTS radicals could be eliminated by each A1M-molecule, whereof 6 molecules were reduced and, 2-3 ABTS molecules were covalently trapped. Both the reduction and covalent trapping reactions were shown to occur with recombinant A1M (rA1M) as well as urine and plasma A1M. The rA1M-wildtype (rA1M-wt) also possessed radical scavenging activity but when using the mutant form lacking the thiol-group, a significant drop in the reduction rate was seen. The covalently trapped ABTS-molecules were attached to tyrosine sidechains on A1M. Several lysine sidechains, earlier described to be modified in urinary A1M [214], were also identified as potentially involved in the trapping.

Reductase

A1M has reductase and dehydrogenase activity. The reduction has been shown for a variety of substrates including cytochrome c, NBT (nitroblue tetrazolium), ferri-cyanide, ABTS, metHb and oxidized collagen [186,187,215], suggesting that A1M is rather nonspecific in its reduction ability. The reduction rate was slightly higher for metHb than cytochrome c, NBT, and ferricyanide. Addition of the electron donors NADH, NADPH or ascorbate enhanced the reduction capacity indicating that A1M can use them as cofactors. Moreover, GSH also increased the acceleration rate, proposing that A1M may not have a specific cofactor, but, more likely, A1M interacts with a range of different cofactors in vivo [215].

The Cys34 sidechain, containing the free thiol group, is involved in the redox reaction. It is, however, also dependent on the three lysyl residues located on the rim of the lipocalin pocket (Lys92, 118 and 130). It was speculated that the lysyl residues could be involved by creating an electronegative environment or by transferring electrons to the thiol on the Cys34 [215]. Interestingly, rA1M had stronger negative redox potential than urine and plasma A1M, which may reflect the fact that purified A1M contains chromophores that decrease its reductase ability by blocking the involved sidechains.

Heme-binder

The heme group from Hb in RBCs has a potentially damaging effect in the body when not contained inside the RBCs. A1M is part of the defense against heme toxicity by its ability to bind heme with $K_d = 10^{-6}$ M^{-1} [143,216]. This is conserved in a wide range of species, including mouse, rat, guinea pig, cow and fish [216]. Each A1M-molecule can bind two heme groups, and three such A1M-heme molecules form a complex $[(heme)_2(A1M)]_3$ [217]. Using site-directed mutagenesis and molecular simulation it was concluded that the side groups of Cys34, Lys92, Lys118 and Lys130 and His123 were involved in the heme binding and it was suggested that the first site is located in the lipocalin pocket and the other binding site between the loops 1 and 4 [218].

A truncated form of A1M, t-A1M, that lacks four amino acid residues in the C-terminal end (Leu-Ile-Pro-Arg, LIPR), was generated after full length A1M reacted with lysed RBCs or Hb in vitro [143]. In addition to binding heme, t-A1M was also able to catabolize heme. Furthermore, this truncated form has been found in urine, placenta and skin in vivo [76,219,220], but, so far, not in plasma [76,221].

MPO production of HOCl and HOSCN can lead to modifications of LDL, which is believed to contribute to development of endothelial dysfunction and atherosclerosis [222,223]. Interestingly, A1M can react with MPO, resulting in formation of t-A1M, the heme-catabolizing form of A1M [224]. CAT, another heme-containing enzyme, did not induce this cleavage suggesting that this reaction is specific for Hb and MPO [224]. In the same study, A1M inhibited MPO-induced oxidation of LDL and may therefore have a role in reducing damage to bystander tissue after neutrophil oxidative bursts.

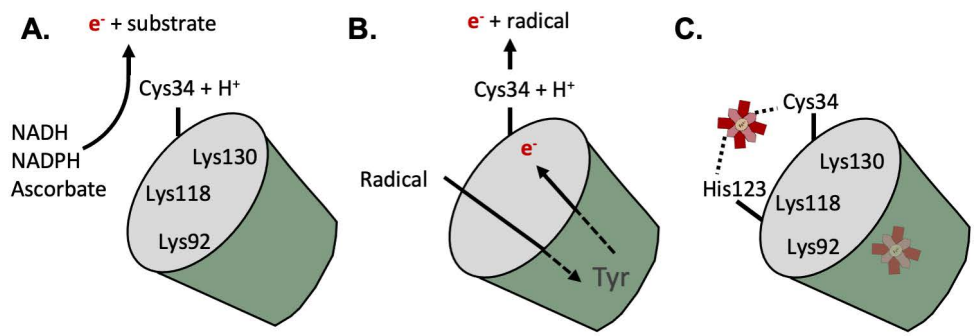

Figure 11. Molecular mechanisms of A1M. (A) Reductase ability. The pKa of the Cys34 is lowered by the proximity of Lys92, Lys118 and Lys130. The Cys34 can reduce substrates by electron transfer. The reduction becomes faster and catalytic in the presence of electron-donating cofactors (NADH, NADPH, and ascorbate) which regenerate the thiolyl radical. (B) Radical scavenging. The Cys34 transfers an electron to a radical. With no electron-donating cofactors present, the thiolyl radical is regenerated by intramolecular reactions with tyrosyl side chains, resulting in tyrosyl radicals. The tyrosyl radicals then forms stable Tyr-radical adducts by reacting with other free radical substrates. (C) Two heme-binding sites have been described for A1M. One site involves His123 and Cys34 and is located superficially. The second site is found within the lipocalin pocket, in close proximity to Lys92, Lys118 and Lys130.

Mitochondrial protection

Studies have shown that A1M localizes to the mitochondria and binds with high affinity to mitochondrial Complex I of the respiratory chain [211]. In keratinocytes, blood and liver cells, A1M was found to internalize during apoptosis and prevent swelling of the mitochondria and reverse severely reduced ATP-production. Preservation of mitochondrial function was later also shown in kidney cells [197]. It may be speculated that A1M have a role in neutralizing mitochondrial ROS, and, in addition, ensure that the mitochondria are able to maintain energy delivery during apoptosis.

Cell, organ and animal models

Over the years, numerous studies have been performed in vitro and in vivo which have unraveled the many mechanisms of A1M-actions and elucidated the potential therapeutic effect. These are presented briefly below.

Cell culture

In response to heme- and oxidative stress-induced damage, many cell lines, including hepatoma (HepG2), erythroid (K562), histiocytoma (U937) and keratinocytes as well as human primary kidney cells, upregulate the expression of *AMBP* [195-197]. The upregulation of *AMBP* is further supported by in vivo data: increased amounts of mRNA are found in the placenta of preeclamptic women, a condition associated with oxidative stress [76].

In addition to protecting erythroid K562 cells from cell death and intracellular oxidation in response to heme, H_2O_2 and hydroxyl radicals (by the Fenton reaction), A1M was also able to clear cells from heme [225]. Silencing of the A1M expression also increased intracellular oxidation. Similarly, addition of heme, Fenton reactants or water to induce oxidative or osmotic stress in RBCs resulted in increased cell death, measured by LDH leakage from cells, which was counteracted with A1M [212]. This effect was seen in RBCs of different origin: human adult and fetal RBCs as well as murine.

Since infused A1M accumulates in the kidneys, due to glomerular filtration and tubular uptake [226], its potential as a kidney protector has been evaluated. A1M added to the culture medium reduced both cell death and upregulation of apoptotic and stress genes in HK-2 and human primary kidney cells in response to addition of heme [197,227]. In the HK-2 cells, A1M also preserved mitochondrial respiratory function [197].

Molecular interactions between A1M and the photo-induced riboflavin radical or the synthetic radical ABTS (see above) resulted in proteolytic cleavage of A1M in the N-terminal part of the protein [228], which is in contrast to the reactions with lysed RBCs, resulting in a C-terminal cleavage [143,187]. One may speculate that

the cleavage site may depend on the molecular mechanism involved: heme-binding or radical binding. Moreover, illuminated riboflavin and FAD (flavin adenine dinucleotide) can bind specifically to A1M, and it was hypothesized that A1M may be involved in protection against riboflavin-induced radicals in light exposed tissue [228].

Oxidative stress in the endoplasmic reticulum (ER) can trigger protein modifications leading to accumulation of misfolded and damaged proteins. This, in turn, can lead to ER-stress and subsequent activation of processes called unfolded protein response (UPR) [229]. Since heme is a known cause of oxidative stress, Gáll et al sought out to test if heme could induce the UPR response in human aortic smooth muscle cells [230]. Pathways of ER-stress, including C/EBP homology protein (CHOP) and X-box binding protein-1 (XBP1) were found to be activated upon heme addition. However, addition of either Hpx, a well-known heme-binder, or A1M dampened the ER-stress. In the A1M-knock out (A1M-KO) mice a similar trend was seen; the liver had upregulated expression of CHOP and XBP1, indicating that A1M is protective against ER-stress in vivo as well [206].

Irradiation does not only kill directly hit cells, but is also known to damage nearby cells, possibly through generation of oxidative stress and cell debris from dead cells [10]. A low dose of alpha-particle irradiation increased oxidation markers, cell death and expression of stress response genes in a hepatoma cell (HepG2) culture in both directly hit cells and bystander cells [231]. When A1M was added cell death (estimated at 95% in the irradiation center and 50% in the periphery) dramatically decreased, with a reduction of 50% for cells in the irradiation center and down to non-irradiated levels in the periphery. A halt in the upregulation of HO-1, SOD, CAT and GPx1 was also seen after addition of A1M, together with reduced lipid peroxidation and carbonyl formation, both markers of oxidative stress. In a follow-up study, the Cys34 side-chain was shown to be an absolute requirement for the irradiation-protection after a site-directed mutation exchanging the cysteine for a serine [186].

Skin, muscle and placenta explants

A1M is associated to cells and matrix components in the epidermal and dermal layers of the skin and has been found in chronic leg ulcers [219,232,233], indicating a protective role in skin. Histochemical analysis of skin explants revealed that A1M is present in both dermis and epidermis, and, furthermore, that the gene expression of *AMBP* was upregulated in cells exposed to heme or hydroxyl radicals (by the Fenton reaction) [196]. A1M was also able to protect the skin explants from ROS and heme-induced damage, which was estimated by cell death and expression of known stress genes. In addition, electron microscopy analysis revealed the binding of A1M to collagen fibrils. Interestingly, A1M was not only able to protect the collagen fibrils from damage but could also restore oxidation-induced damage

suggesting that A1M may have a role in restoring the extracellular matrix (ECM) components.

The effect of heme toxicity on contractile force in skeletal muscle fibers was studied in human tissue samples [234]. Heme increased the protein thiol group oxidation which lowered the contractile force and, as a consequence, increased muscle rigidity. This was not seen in muscle fibers that were treated with A1M. Moreover, the authors suggested that heme may be at fault for skeletal muscle wasting [234], frequently seen in anemic patients [235,236].

Structural deformation of the placenta is believed to be a central part of the disease development in PE. HbF has been shown to increase in the maternal circulation during PE [237] and overexpression of HbF has been suggested to be one of the drivers of the pathophysiology in PE [70]. To show the impact of Hb, human placentas were perfused ex vivo with Hb in the fetal circulation resulting in morphological damage and upregulation of genes involved in inflammation and apoptosis [75]. Simultaneous perfusion of A1M in the maternal circulation reduced the pathological response and, moreover, A1M also induced upregulation of ECM genes, once more highlighting its role in the ECM.

Animal models

Before initiation of the work in this thesis, many of the in vivo models had focused on A1M as a potential treatment in PE (Figure 12). Knowing the harmful potential of free Hb and its potential pathogenic role in the disease, researchers showed that systemic infusion in a rat model resulted in increased glomerular permeability, which was reversed when A1M was co-injected [238]. Moreover, HbF injections in a pregnant rabbit model were used to mimic the proteinuria and structural damages to placenta and kidneys seen in PE patients and, similarly, A1M ameliorated the damages [239].

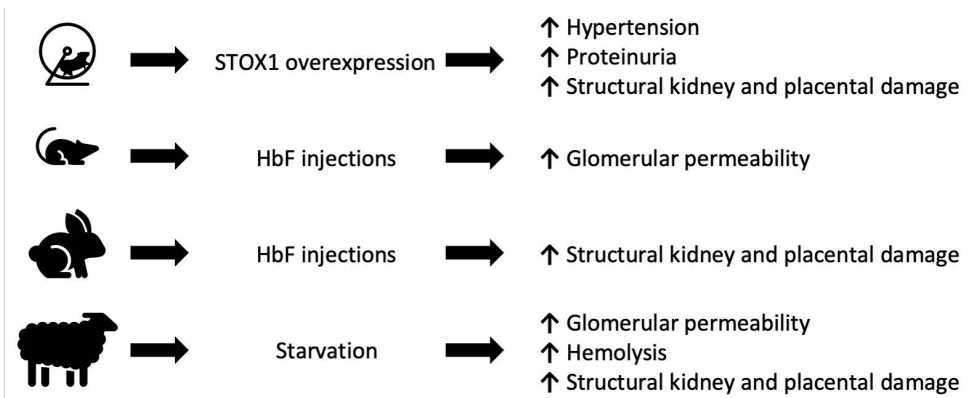

Figure 12. Summary of PE animal models and the damage that was ameliorated with administration of A1M. From the top: mouse model with overexpression of STOX1, rat and rabbit models with HbF injections and starvation of pregnant ewes.

Starvation of pregnant sheep can result in hemolysis-induced PE-like symptoms, which were measured by increased bilirubin levels (a marker of hemolysis), structural damage to placenta and kidneys as well as increased GFR [240]. When treating the sheep with multiple doses (2 x 1.8 mg/kg) of rA1M, several of the symptoms (high blood pressure, compromised placental and renal histology and defect renal filtration barrier) were ameliorated. In addition, the sheep model showed, for the first time, that clinically active doses of rA1M were well-tolerated and non-toxic in living animals.

Female pregnant mice developed PE symptoms, such as hypertension, proteinuria, renal and placental structural impairments, when overexpressing the transcription factor Storkhead box 1 (STOX-1) gene in a transgenic mouse model [241]. Therefore, this animal model is used to study a severe form of early onset PE. In a recent study, increased hypoxia and nitrative stress were also reported and, together with above mentioned symptoms, were significantly reduced when A1M was given as a treatment [242].

Glycerol-induced rhabdomyolysis resulted in a mild acute kidney injury (AKI) in a mouse model, with stress genes HO-1 and Hsp70 increased substantially in renal tissue [227]. As the release of myoglobin, radicals and heme are major contributors of the AKI, A1M injections were given 30 minutes after glycerol to counteract the toxicity and significantly lowered the stress response.

PRRT with ^{177}Lu-DOTATATE is used to treat cancer. However, these radiopeptides are excreted through the kidneys and may therefore cause unwanted side-effects in the kidneys through irradiation when retained. A1M has a similar biodistribution and pharmacokinetics as DOTATATE. This was shown by injecting ^{125}I- and non-labelled rA1M as well as the ^{111}In- and fluorescence-labelled somatostatin analogue and examining the co-localization in vivo histologically and at molecular level. The biodistribution and pharmacokinetic data indicate a high renal uptake for both A1M and octreotide, with the maximum uptake between 10 to 20 minutes after injections. SPECT and autoradiography imaging further supported these findings, and showed that both molecules have a preferential localization to the kidney cortex, and immunohistochemistry and fluorescence microscopy further revealed a distinct co-existence in proximal tubules [226]. Therefore, A1M has been suggested to be a suitable radioprotector in PRRT [243]. In a ^{177}Lu-DOTATATE mouse model A1M reduced DNA-damage, increased overall survival and reduced renal damage, both histologically and as measured by urinary albumin [244]. In a later study, it was concluded that A1M does not interfere with the tumor treatment itself [245].

Another condition where the therapeutic effect of A1M has been studied is intraventricular hemorrhage (IVH) in preterm rabbit pups [246]. Intracerebroventricular administration of A1M was given 24 h after induction of IVH and the protein was found to be highly co-localized with Hb in periventricular white matter

regions. Moreover, animals receiving A1M had lower levels of structural damage and decreased upregulation of proinflammatory response genes in the brain tissue.

To better understand the biological role of A1M in vivo, A1M-deficient mice were studied for up to 12 months [206]. In addition to the increased liver ER-stress mentioned above, the mice had upregulated expression of other antioxidant genes in the liver. Surprisingly, the mice also gained more weight and were significantly heavier suggesting a role in appetite regulation or fat metabolism for A1M. In addition, the mice had a tendency towards a macrocytic anemia phenotype [212], supporting a role for A1M in erythroprotection (reviewed in [144]). Moreover, blood pressure and heart rate was significantly elevated, and pregnant A1M-deficient mice displayed signs of compromised kidney and placenta structure, as well as reduced fetal birth weight [247].

Clinical potential

Biomarker

Urinary A1M is a sensitive biomarker of tubular damage in many renal pathological conditions in both children and adults [213,248-250]. It can also be used to differentiate between subgroups of CKD and as a predictor of development of AKI in hemorrhagic fever [251,252]. Another disease where A1M has gained attention as a clinical marker is in PE, where women with PE have higher levels in plasma and urine [76]. A1M, together with HbF, can therefore be a first or early second trimester, predictive biomarker of PE [237,253]. Moreover, in the late second trimester, severe PE is associated with higher A1M levels than non-severe cases [254]. In contrast, lower levels of A1M were found in cord blood from growth restricted fetuses, with or without mothers with PE, compared to normal growth fetuses [255].

In patients with rhegmatogenous retinal detachment, vitreous samples showed an increase in A1M levels [256] and in patients with acute inflammatory arthritis, the knee synovial fluid and serum levels contained high A1M concentrations [257], indicating that A1M can be a useful biomarker in a wide range of diseases linked to oxidative stress.

Rationale behind therapeutic use

Oxidative stress is part of many diseases and conditions where it contributes to the pathological development and progress. Therefore, it has been suggested that the addition of different antioxidants could be beneficial. However, so far, the number of antioxidants used in the clinic are very limited [258].

The protective abilities of A1M, and its molecular mechanisms: reduction of oxidized molecules, heme- and radical-scavenging, cellular uptake, binding to

mitochondria and protection of mitochondrial function, makes it a promising therapeutic candidate. Being ubiquitous and rather unspecific in regard to substrate, its potential target applications can be rather diverse. However, since the natural route in the body localizes A1M to the kidneys, most animal studies, as described above, therefore have focused on protection of the kidneys. In animal models of PE, PRRT and rhabdomyolysis, intravenous administration of A1M has reduced kidney damage significantly. In addition, in a rabbit model of IVH, infusion of A1M decreased brain damage induced by heme and heme-metabolites, suggesting that administrating A1M is beneficial also in non-kidney related conditions.

Compared to antioxidants such as vitamin C and E, A1M has several advantages. To start with A1M is naturally synthesized in the body and therefore has routes of distribution and clearance, which also applies to the recombinant versions of the protein [226]. Secondly, A1M has a higher capacity of binding/neutralizing radicals than vitamin C and E, and can, in addition, act as a heme-binder, thereby eliminating another important generator of ROS and oxidative stress. Lastly, after binding radicals, A1M is electroneutral and as such does not expose the surrounding tissue to oxidation. In contrast, most low molecular weight antioxidants, cf. vitamin C and E, become oxidants themselves after reacting with radicals, ROS and other oxidants.

Recombinant A1M

For most of the work in this thesis A1M was expressed recombinantly in *E. coli.* as first described by Kwasek et al. [259]. In short, the *AMBP* gene was ligated into a vector, with an amino acid elongation in the N-terminal containing eight histidines (His-tag). Competent *E. coli* bacteria were then transformed with the plasmid and the expression was induced by IPTG (Isopropyl thiogalactoside). The rapid expression resulted in accumulation of cytosolic aggregates, inclusion bodies, which were harvested from the bacteria, washed and dissolved in Guanidine-HCl buffer. The sample was applied on a Ni-NTA column whereafter the bound, unfolded A1M-molecules were eluted with imidazole. Refolding was then achieved by adding reduced GSH to the eluate and dripping the solution into a folding buffer containing oxidized GSH. The sample was dialyzed and separated by ion-exchange chromatography [227,259].

As mentioned above, human plasma A1M is glycosylated. Glycosylation increases the stability and solubility of the protein. However, expressing recombinant wildtype A1M (rA1M-wt) in *E. coli* results in a protein lacking glycosylation. To improve solubility and stability properties of A1M and hence its clinical usefulness, a new variant was designed with amino acid substitutions referred to as rA1M-035 (Figure 13) [260]. The variant has the same N-terminal His-tag extension followed by the human A1M sequence with three amino acid substitutions (N17D, R66H, and N96D). These substitutions increase stability and solubility of rA1M-035 without affecting the protective properties of the rA1M-wt [212,260].

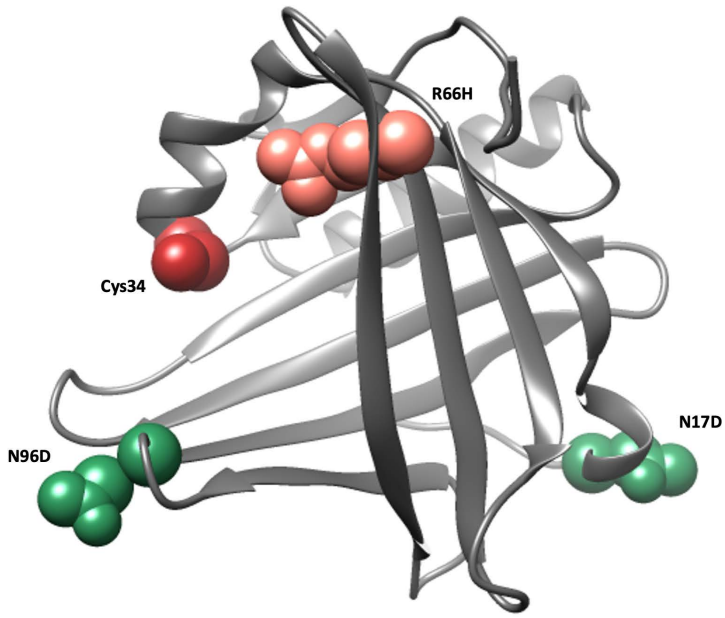

Figure 13. Structure of A1M highlighting the substitutions in rA1M-035 (N17D, R66H and N96D) and rA1M produced in *Nicotiana benthamiana* (N17D and N96D).

A recent study described a new way of expressing rA1M in the leaves of *Nicotiana benthamiana*, which resulted in functional rA1M [261]. The rA1M-variant contained two of the substitutions (N17D and N96D) associated with the increased solubility and stability of the rA1M-035 variant (Figure 13). Interestingly, the plants also showed a potential to express glycosylated forms of rA1M [262]. Expressing A1M in plants is a scalable option and holds great potential in the future and may be an alternative option for large-scale production.

During spring 2019 the first clinical trials with rA1M-035 in humans were initiated by Guard Therapeutics International as a phase I study with healthy adults. The phase I results concluded that rA1M-035 have a favorable safety profile [263]. Furthermore, a phase II study is planned with patients undergoing cardiac surgery, where reperfusion injuries to the kidneys is a major concern. As suggested in this thesis, there are other opportunities for A1M as a therapeutic option if the clinical studies go as planned.

Aim

The overall aim of this thesis was to explore the possibilities of therapeutic use of A1M in different medical conditions and diseases. The specific aims of the papers were as follows:

I. Study the protective effect of A1M in kidney cell lines exposed to heme-induced oxidative stress.

II. Investigate the protective effects of A1M in early and late stages after ^{177}Lu-DOTATATE injections in a mouse model.

III. Examine the protective effects of A1M after ^{177}Lu-PSMA-617 injections in mice and tumor bearing mice.

IV. Characterize A1M deficient mice (A1M-KO) and thereby increase the understanding of the biological role of A1M in vivo as well as finding new pharmaceutical targets.

V. Investigate erythroprotective effects of A1M in vivo and in vitro.

VI. Review new therapeutic opportunities for A1M in erythropoietic and hemolytic conditions.

Results and discussion

Cell protection

Previously, it has been shown that A1M can prevent several different cell lines from death and stress response induced by various insults. In paper I and V the protective effects of A1M were studied in three different additional cell types: the cell line HK-2, human primary RPTE cells and RBCs.

Human kidney cortex proximal tubule epithelial cells

Oxidative stress is a contributing factor in many kidney diseases. In paper I, human kidney cortex proximal tubule epithelial (HK-2) cells were exposed to heme-induced stress in different concentrations and time points with or without rA1M. There was a dose-dependent increase in cellular death, as measured by LDH leakage and the cell proliferation agent, WST-1, that was counteracted with rA1M (Figure 14A-B). As a control, the Hb-binding protein haptoglobin did not confer any protection. These results indicate that the heme-induced cell death reduction is specific for A1M (Figure 14C).

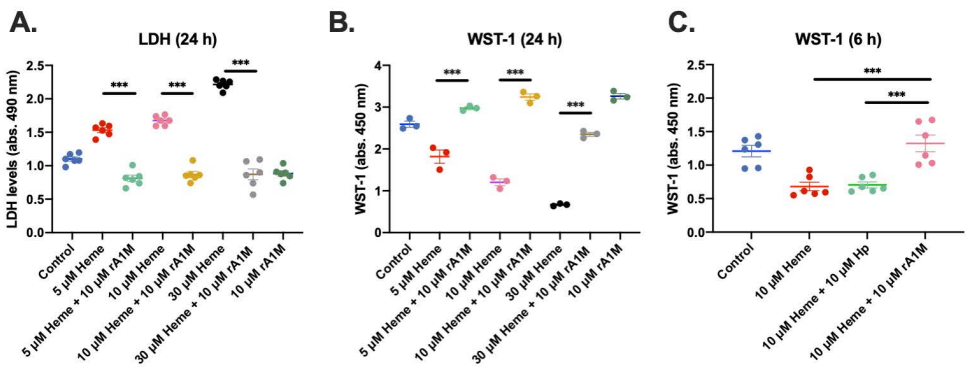

Figure 14 Cell viability after incubation with heme, with or without rA1M. (A) LDH levels after 24 h. (B) WST-1 levels after 24 h. (C) Comparision of WST-1 levels after incubation with rA1M or haptoglobin. Figure adapted from [197].

Analysis of gene and protein response indicated an ameliorated response in the presence of rA1M, with lower levels of HO-1 (both protein and gene) and *HSP70* among others (Figure 15). These data suggest that heme-induced cellular stress is normalized or decreased with co-incubation of rA1M.

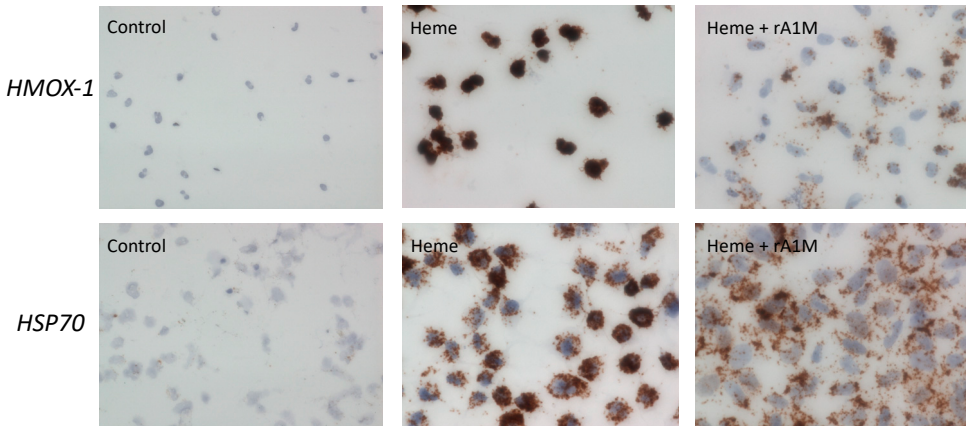

Figure 15. In situ visualization of mRNA levels of *HMOX-1* and *HSP70* after incubation with heme, with or without A1M (representative light microscopy images with 20X magnification). Figure adapted from [197].

Human primary renal proximal tubule epithelial cells

In addition to HK-2 cells, human primary renal proximal tubule epithelial (RPTE) cells were studied in paper I. Similarly, rA1M reduced heme-induced cell death and stress gene upregulation (Figure 16A-C). Protein levels, measured by Western blot, indicated that heme activated the Nrf2-pathway and in response *AMBP* (A1M) and *HMOX-1* expression were increased (Figure 16D). This is in line with previous studies that have concluded that *AMBP* expression, and genes encoding other detoxification enzymes, are regulated through the Nrf2-pathway [200-202], which is activated during increased stress conditions.

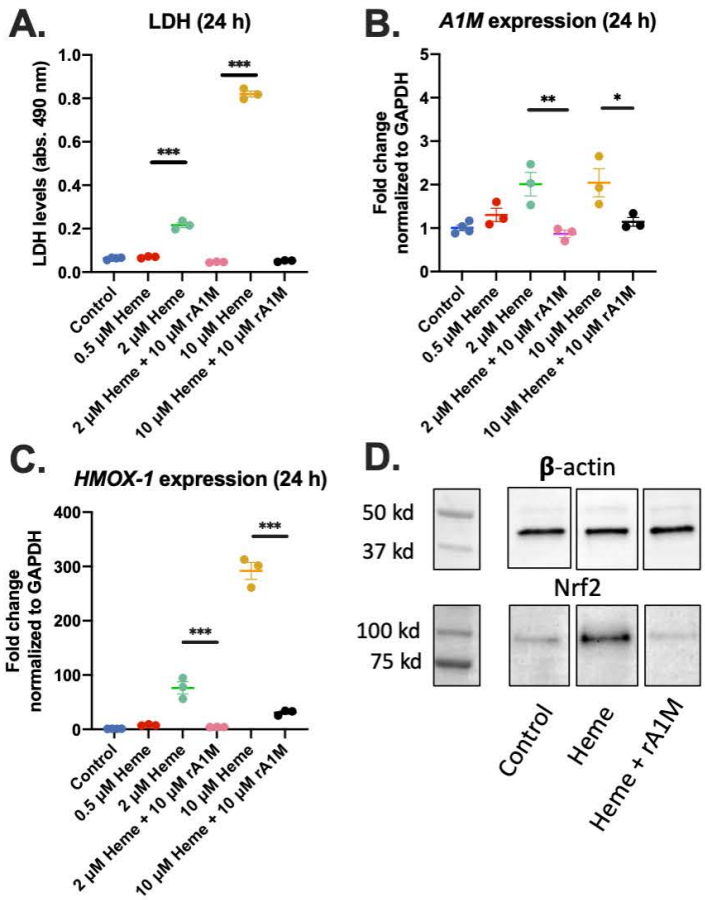

Figure 16. Viability and stress response in RPTE cells. (A) LDH levels after 24 h after incubation with heme with or without rA1M. (B) *A1M* expression and (C) *HMOX-1* expression after 24 h incubation with heme, with or without rA1M. (D) Western blot indicating levels of Nrf2 from cell lysates following exposure to 2 µM heme, with or without co-treatment of 10 µM rA1M. Figure adapted from [197].

Red blood cells

When hemolysis occurs, Hb and heme leak out of the RBCs and damage the surrounding cells and tissues. In paper V we studied the protective effect of rA1M when RBCs were exposed to different types of stress. rA1M was able to reduce cell death initiated by heme, radicals and osmosis, in addition to spontaneous stress (incubation and rotation). The erythroprotective effect was specific to A1M, since neither the heme-binding protein Hpx nor the antioxidants Trolox (Vitamin E) and Ascorbic Acid (Vitamin C) conferred a similar protection. (Figure 17A-D) Moreover, it was not a lipocalin-related function since orosomucoid, another lipocalin, could not reduce cell death in a similar manner (Figure 17E-F).

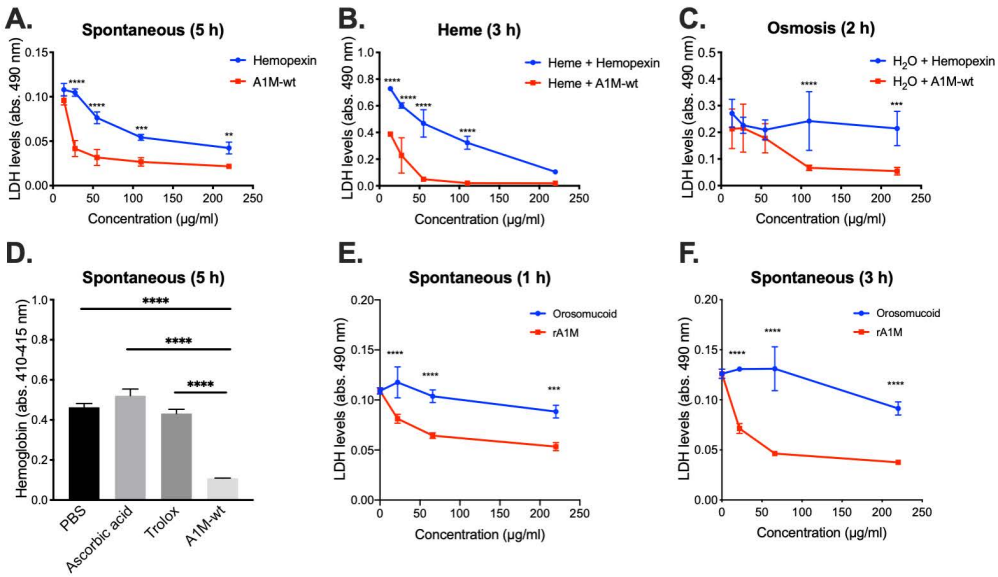

Figure 17. Lysis of RBCs after exposure to different forms of stress. LDH levels in supernatant after exposure to (A) spontaneous stress for 5 h, (B) heme-induced stress for 3 h and (C) osmotic stress for 2 h with either rA1M or Hpx. (D) Hemoglobin levels in supernatants after 5 h of incubation with ascorbic acid, Trolox or rA1M. Spontaneous stress after incubation with either rA1M or orosomucoid after (E) 1 h and (F) 3 h. Figure adapted from [212].

To investigate if A1M also could protect other sources of RBCs, fetal and murine cells were investigated. Fetal RBCs were more sensitive both to spontaneous and heme-induced stress than their maternal counterparts (Figure 18A-B). This could result from immaturity and having a shorter lifespan in addition to containing a lower number of antioxidants [116,264,265]. Murine RBCs also have a shorter lifespan [120]. Nevertheless, both fetal and murine RBCs had lower LDH release when incubated with rA1M (Figure 18A-D), suggesting that the erythroprotective function is universal.

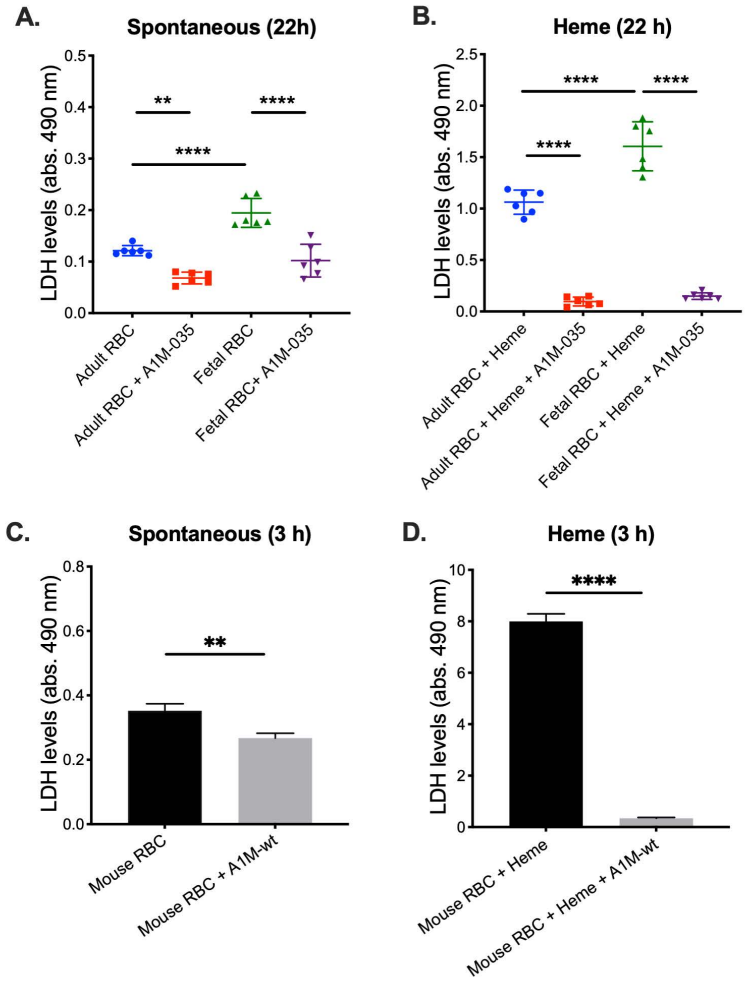

Figure 18. LDH levels in the supernatant after human fetal and adult RBCs were incubated with or without rA1M for 22 h of (A) spontaneous or (B) heme-induced stress. LDH levels in the supernatant after murine RBCs were incubated with or without rA1M for 3 h of (C) spontaneous or (D) heme-induced stress. Figure adapted from [212].

Hemolysis can be caused by numerous stressors, and is part of many diseases, but here rA1M decreased lysis irrespective of insult. The many molecular properties of A1M may come into play during protection: heme-scavenging, binding and reduction of radicals. Furthermore, after lysis the released components, such as heme, can accelerate the destruction of other RBCs hence creating a feed-forward loop. Therefore, there may be therapeutic opportunities for A1M in several diseases which have a pathology driven by hemolysis and heme toxicity.

Cellular uptake

Previously, various cell types, e.g., keratinocytes, liver and blood cells, have been shown to internalize A1M from the surrounding medium [196,211,225,231]. In paper I RPTE cells were shown to internalize A1M, which was measured by radio-immunoassay. In the same study, mitochondrial function was preserved when HK-2 cells exposed to heme were co-incubated with rA1M (Figure 19A-C). A possible explanation is that, in addition to binding extracellular and intracellular heme, rA1M could protect mitochondrial integrity intracellularly. This agrees with previous studies where A1M has been shown to bind to a Complex I subunit in the respiratory chain [211]. Furthermore, Nrf2 is important in protecting mitochondrial integrity and function during stress [266], and, as mentioned, expression of A1M is regulated through the Nrf2-pathway suggesting that increased oxidative stress might mediate an upregulation of A1M to preserve mitochondrial function.

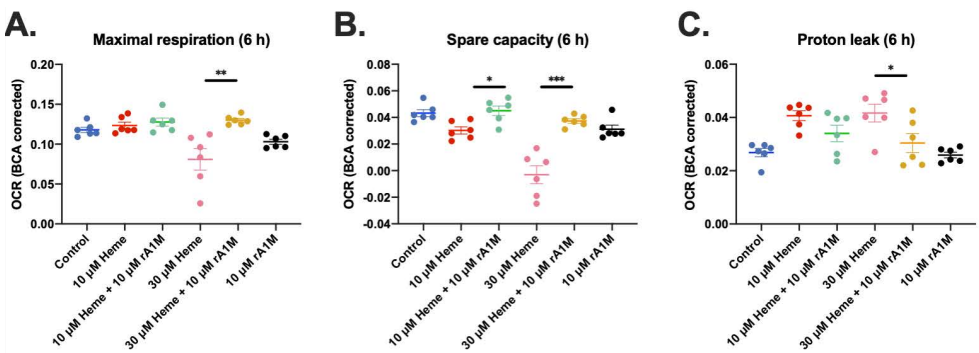

Figure 19. Mitochondrial function of HK-2 cells. Mitochondrial parameters after incubation for 6 h with heme, with or without rA1M. (A) Maximal respiration, (B) spare capacity and (C) proton leak. Values are corrected for differences in protein content. Figure adapted from [197].

RBCs were also shown to internalize A1M in paper V. In vivo uptake by the RBCs was shown in the placenta, visualized with electron microscopy (Figure 20A). In vitro, rA1M was taken up and could be found both intracellularly but also in the plasma membrane of the RBCs (Figure 20B). Although the uptake mechanism is still unknown, these results indicate that A1M has an intracellular role in protecting cells and may also protect plasma membranes from oxidative stress.

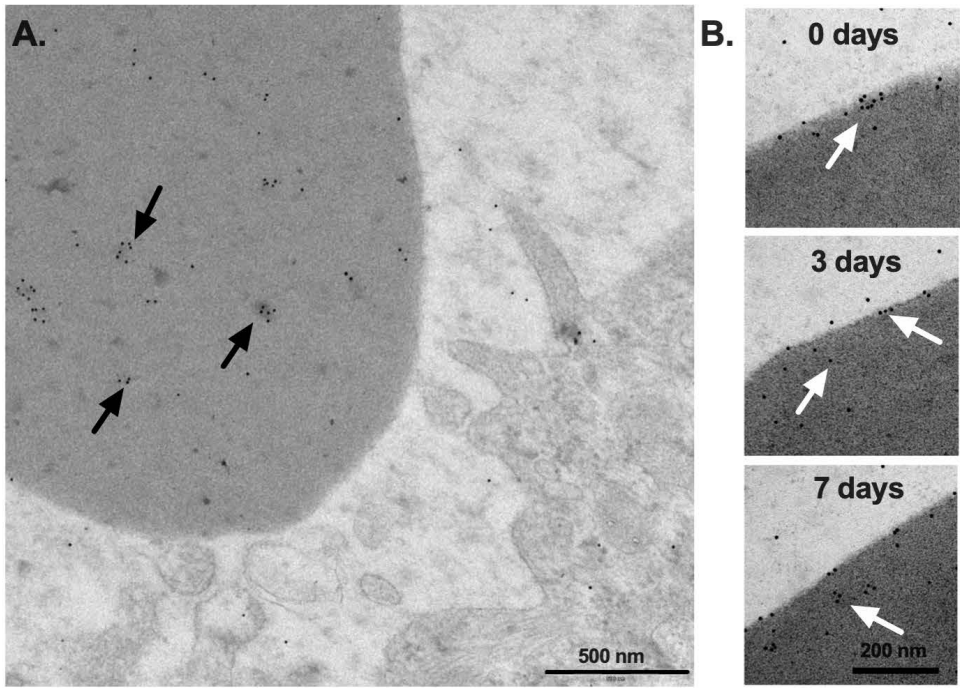

Figure 20. In vivo and in vitro uptake of A1M, indicated by arrows. Sections and RBCs were stained with anti-A1M and gold-labelled secondary antibodies. (A) Visualization of A1M occurence in RBCs of the placenta in vivo. Scale bar indicates 500 nm. (B) Washed RBCs were incubated with rA1M-035 for 0 (1 h), 3 or 7 days. A1M is localized to both the plasma membrane and intracellularly. Scale bar represents 200 nm. Figure adapted from [212].

Renal protection in mouse models

A1M has previously had success in many animal models, mainly reducing placental and renal damage in different disease models. In paper II and III we focused on a new area, radiation, and investigated A1M as a radioprotector of the kidneys.

^{177}Lu-DOTATATE

In paper II, the protective effect, focused mainly on the kidneys, of A1M was examined in a ^{177}Lu-DOTATATE radiation mouse model. The kidneys are especially exposed to the radiation from ^{177}Lu, since the peptides are retained in the kidneys.

The mice that received co-injections of A1M had a tendency towards prolonged survival compared to animals receiving only ^{177}Lu-DOTATATE, which more often had swollen/pathological internal organs and their deaths, therefore, were classified as radiation-induced.

DNA-damage often occurs relatively early after radiation. In our model, the maximum DNA-damage in the cortex occurred after 4 days, as measured with the marker γ-H2AX in kidney sections, but was almost completely reduced to control levels in rA1M-injected animals (Figure 21). The expression of genes related to apoptosis was also significantly reduced in the rA1M-injected animals, suggesting that rA1M protects the kidneys against early radiation damage, most likely by reducing the ROS generated by ^{177}Lu.

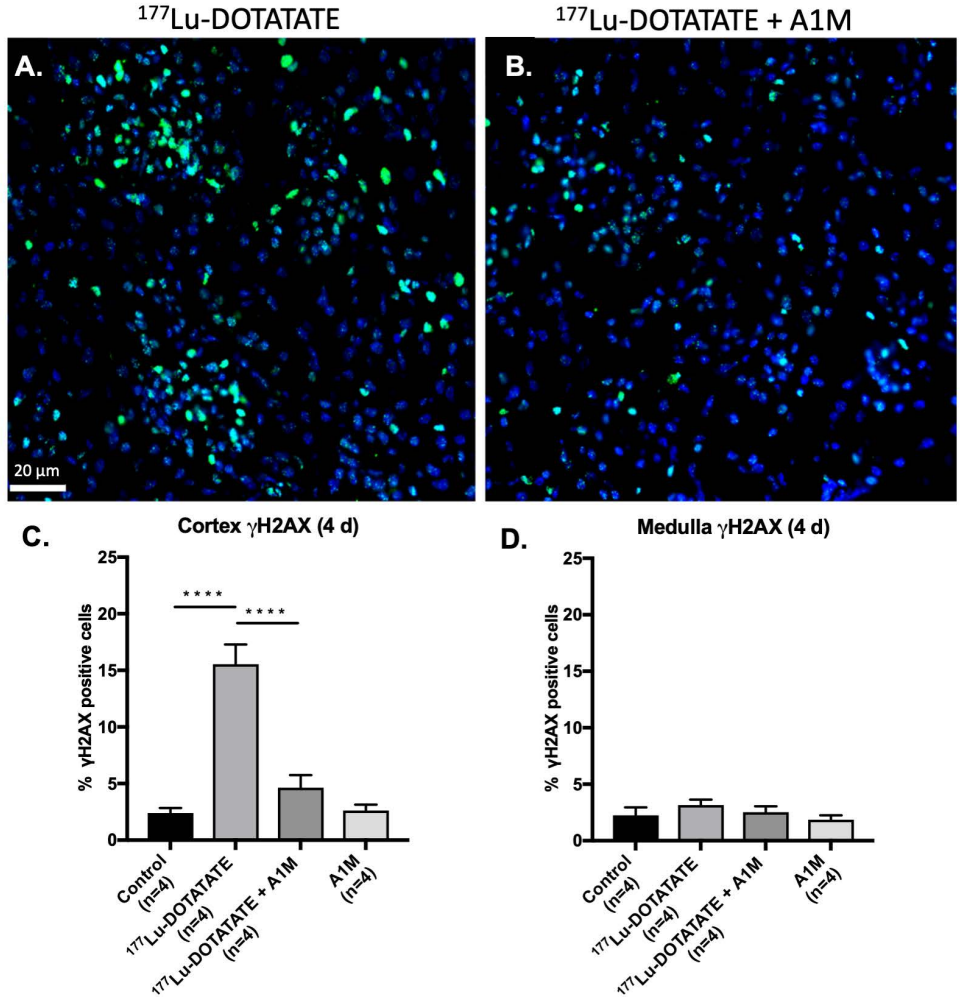

Figure 21. Kidney damage assessment 4 days after injections of ^{177}Lu-DOTATATE with or without co-injection of rA1M. DNA-damage in kidney tissue highlighted with immunofluorescence-labeled γ-H2AX (green) and cell nuclei (blue) in (A) ^{177}Lu-DOTATATE and (B) ^{177}Lu-DOTATATE + rA1M animals. Scale bar indicates 20 µm. Percent γ-H2AX positive cells in (C) cortex and (D) medulla. Figure adapted from [244].

Histological examination suggested a time-dependent development of kidney damage, with the highest number of lesions after 24 weeks. At this timepoint there had in addition been a significant decrease in glomeruli in the ^{177}Lu-DOTATATE group. Functional analysis indicated an earlier decrease in kidney function in the ^{177}Lu-DOTATATE group already at 4 weeks, as measured by an increase in urinary albumin (Figure 22).

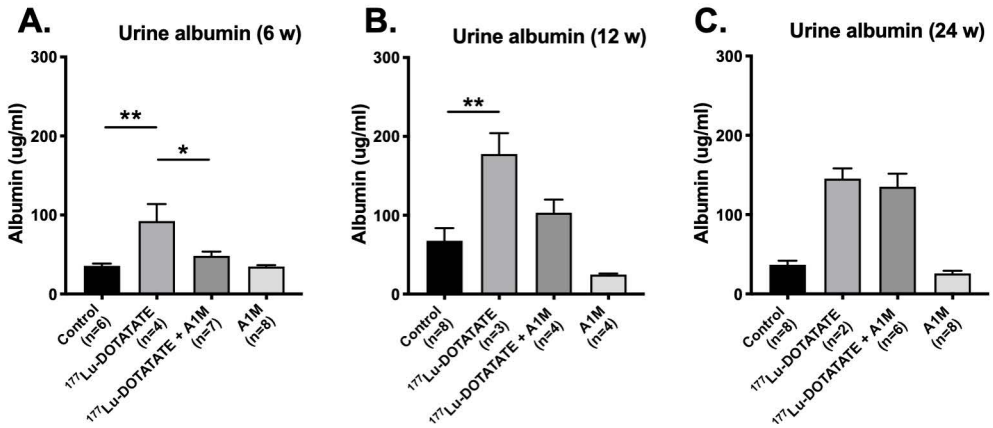

Figure 22. Albumin levels measured in urine in animals injected with ^{177}Lu-DOTATATE with or without co-injections of rA1M. Measured in mice (A) 6 weeks, (B) 12 weeks and (C) 24 weeks after injections. Figure adapted from [244].

Results suggest that co-administering rA1M to patients receiving PRRT may be beneficial and reduce both initial damage and the renal loss of function that develops over time. It could also allow inclusion of patients with underlying renal conditions and/or increase dosage for improved therapeutic outcome.

^{177}Lu-PSMA-617

In paper III, similarly to paper II, rA1M was used as a kidney radioprotector, but here in a ^{177}Lu-PSMA-617 radiation mouse model. Surprisingly, the standard urine and plasma damage markers, e.g., albumin and BUN, and histological examination showed no increased kidney damage.

However, alterations could be detected with ^{99m}Tc-MAG3 imaging. ^{99m}Tc-MAG3 imaging uses a radiolabeled pharmaceutical agent injected into the blood to monitor the flow through the kidney with a gamma camera and can thereby be used to evaluate kidney function. From the renograms the slope of initial uptake (SIU) can be determined, which is a central measurement as it reflects three key factors: perfusion, uptake and retention. The SIU is defined as the inclination during the first 5-35 seconds. The SIU indicate that rA1M ameliorates the kidney damage after exposure to ^{177}Lu- PSMA-617 after 3 months (Figure 23A). The excretion from the kidney deteriorates over time in all groups, which could reflect renal or urethral blockage (Figure 23B). It is difficult to say if this reflects radiation damage or aging since it is a similar trend in all groups, with the exception of ^{177}Lu-PSMA-617 that received the highest dose (100 MBq) and in which, unexpectedly, the excretion increases between 3 and 6 months.

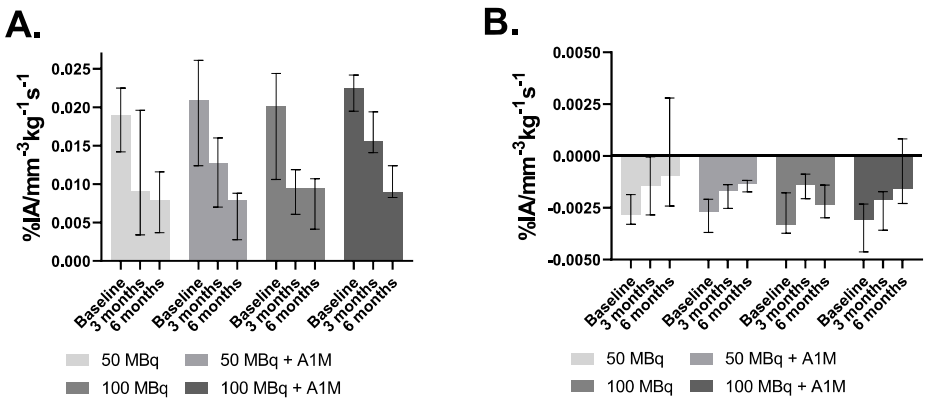

Figure 23. ^{99m}Tc-MAG3 renograms after injections with ^{177}Lu-PSMA-617 with or without rA1M co-injections. Graphs indicate (**A**) the SIU and (**B**) the excretion in all groups at baseline, 3 months and 6 months.

In the same study, it was examined if rA1M would affect tumor treatment. In a tumor mice model, where the mice received ^{177}Lu-PSMA-617 with or without co-injections of rA1M, tumor regression and growth were monitored (Figure 24A). Tumor uptake of ^{177}Lu-PSMA-617 after 24 h were also measured (Figure 24B). Data showed that rA1M did not interfere with tumor treatment or uptake; mice had a similar tumor regression and growth. This is in line with previous ^{177}Lu-DOTATATE data that shows that, rA1M-injections do not protect the tumor [245].

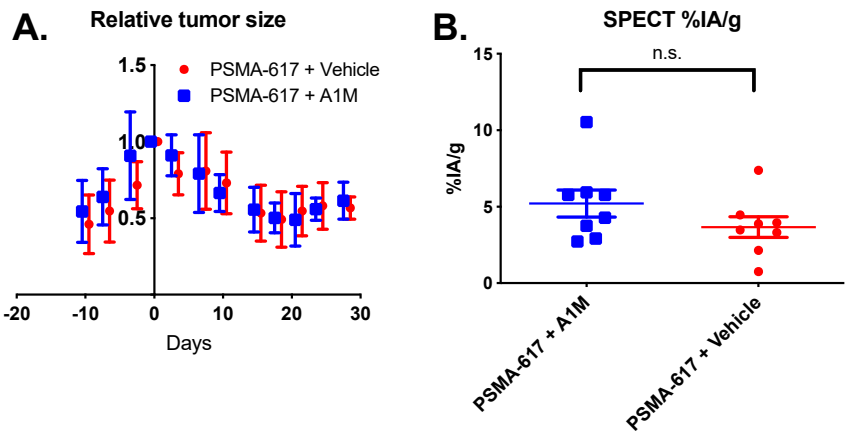

Figure 24. Tumor treatment efficiency and uptake. (A) Relative tumor size comparing ^{177}Lu-PSMA-617 injected animals with or without rA1M co-injections. (B) ^{177}Lu-PSMA-617 uptake in tumor.

From the dosimetry we concluded that animals received in the range of one fraction given to patients in the clinic, thus not close to the clinical limit. This could explain why no damage could be detected with traditional plasma and urine markers. However, the small damage detected with ^{99m}Tc-MAG3 imaging could be expected to accumulate with each fraction. Therefore, it could be of interest to do a follow-up study with multiple fractions to test the efficiency of rA1M in a clinically relevant setting.

Knock-out of A1M

Creating a knock-out mouse can give new insights to the physiological role of a protein. In the case of A1M, the reason behind the evolutionally conserved co-expression with bikunin has not been fully understood. Therefore, creating an A1M-KO mouse with the codons encoding bikunin intact, was the aim of paper IV. To do so the Cre-loxP system was used to remove exons 2-6 coding for A1M in the *AMBP* gene (Figure 25). This resulted in mice completely lacking A1M, both at the gene expression and protein levels. Interestingly, bikunin complexes decreased in plasma although bikunin expression was upregulated in the A1M-KO mice, suggesting that the presence of A1M is needed for correct modifications of bikunin. Without A1M, misfolded bikunin accumulated in the liver and activated the UPR pathways and resulted in increased ER-stress.

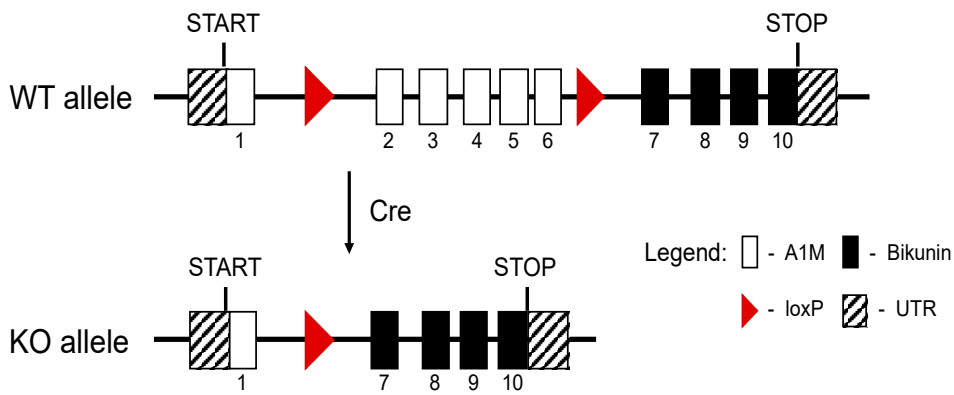

Figure 25. Overview of the *AMBP* gene with exons 1-6 encoding A1M and 7-10 bikunin. The Cre-loxP system enabled deletion of exons 2-6, thereby eliminating A1M expression but keeping bikunin expression. Figure adapted from [206].

The loss of *A1M* expression led to an upregulation of other antioxidants, among others, thioredoxin, HO-1, CAT and SOD. This is not surprising since when one antioxidant is removed from the system, other antioxidants may compensate its function to maintain the redox balance [267].

A completely new biological role for A1M may be in fat or hunger regulation since A1M-KO gained more weight than their cage mates and also a tendency towards increased fat accumulation in the liver (Figure 26A-C). Although weight is commonly affected in KO-mice it is more common for a weight decrease to occur [268]. The pathways that regulate weight and metabolism are complex, and exactly how absence of A1M resulted in weight gain was not determined, although the ER-stress could be a contributing factor [269,270].

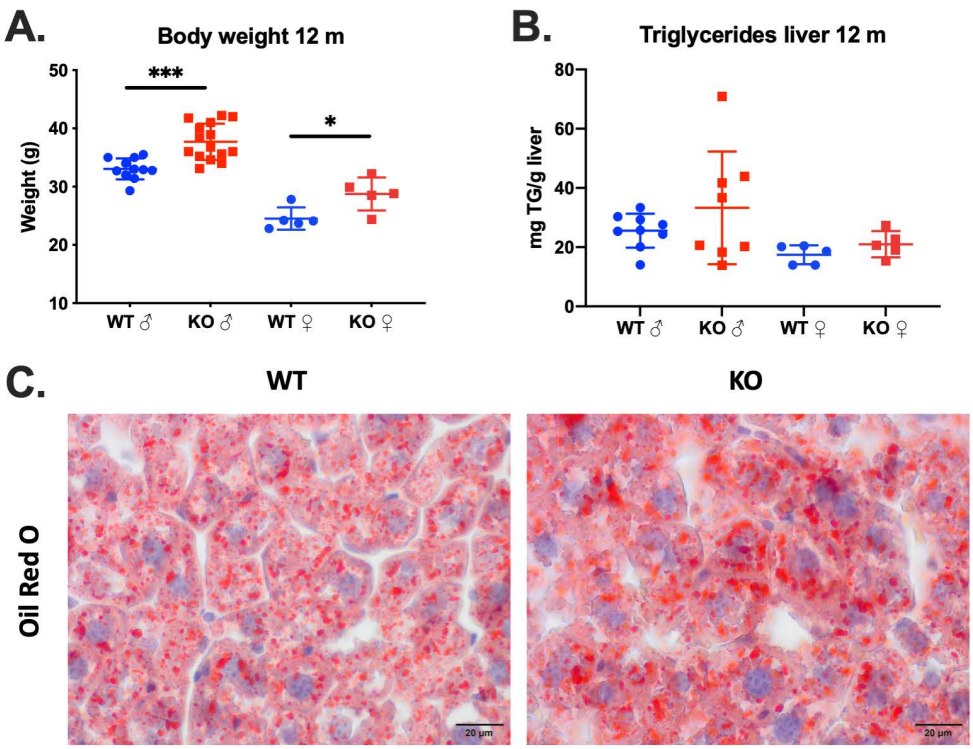

Figure 26. (A) Body weight of 12-months old WT and KO mice (male and female). (B) Triglycerides in liver after 12 months in male respectively female mice. (C) Liver sections from 12-month-old males stained for lipids and triglycerides with Oil Red O (Scale bars indicating 20 µm). Figure adapted from [206].

Erythrocyte stability

In paper V the blood from the A1M-KO mice (paper IV) was examined. The mice had a tendency towards a lower number of RBCs and the RBCs varied more in size, as suggested by a significant increase in both MCV and RDW. These changes in the RBCs indicate a macrocytic anemia phenotype due to the lack of A1M. Several bikunin-KO models have been described of which neither have reported and changes in blood composition [271-273]. The changes in composition indicate that A1M could have a role in erythropoiesis, RBC turnover or stabilization.

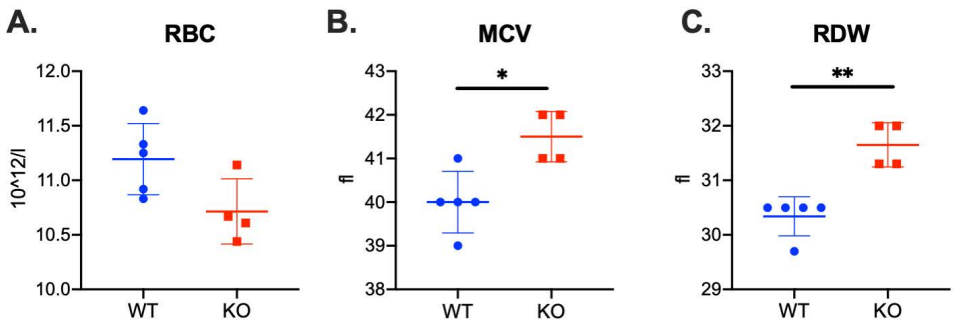

Figure 27. RBC morphology in A1M-KO and A1M-WT mice. (A) RBC count, (B) MCV and (C) RDW. Figure adapted from [212].

The findings in paper V motivated paper VI, which describes the background of A1M, including previous studies, and continues to discuss new possible therapeutic targets involving RBCs. The use in the following diseases and conditions were discussed: Diamond-Blackfan anemia (DBA), 5q-minus myelodysplastic syndrome (5q-MDS), blood transfusions (including storage), intraventricular hemorrhage (IVH), preeclampsia (PE) and atherosclerosis (Figure 28). In DBA and 5q-MDS, A1M could be used to battle the accumulation of heme and resulting toxicity. Hemolysis is a problem in blood transfusions, IVH and PE, and could be reduced by administration of A1M. Lastly, atherosclerotic plaques become more prone to rupture due to RBC extravasation and lysis, and therefore accelerate the pathology. The intraplaque ROS and hemolysis could be speculated to ameliorate with A1M administration. The heme- and radical-scavenging, reductase and antihemolytic abilities of A1M holds great potential to be developed into treatments for various diseases.

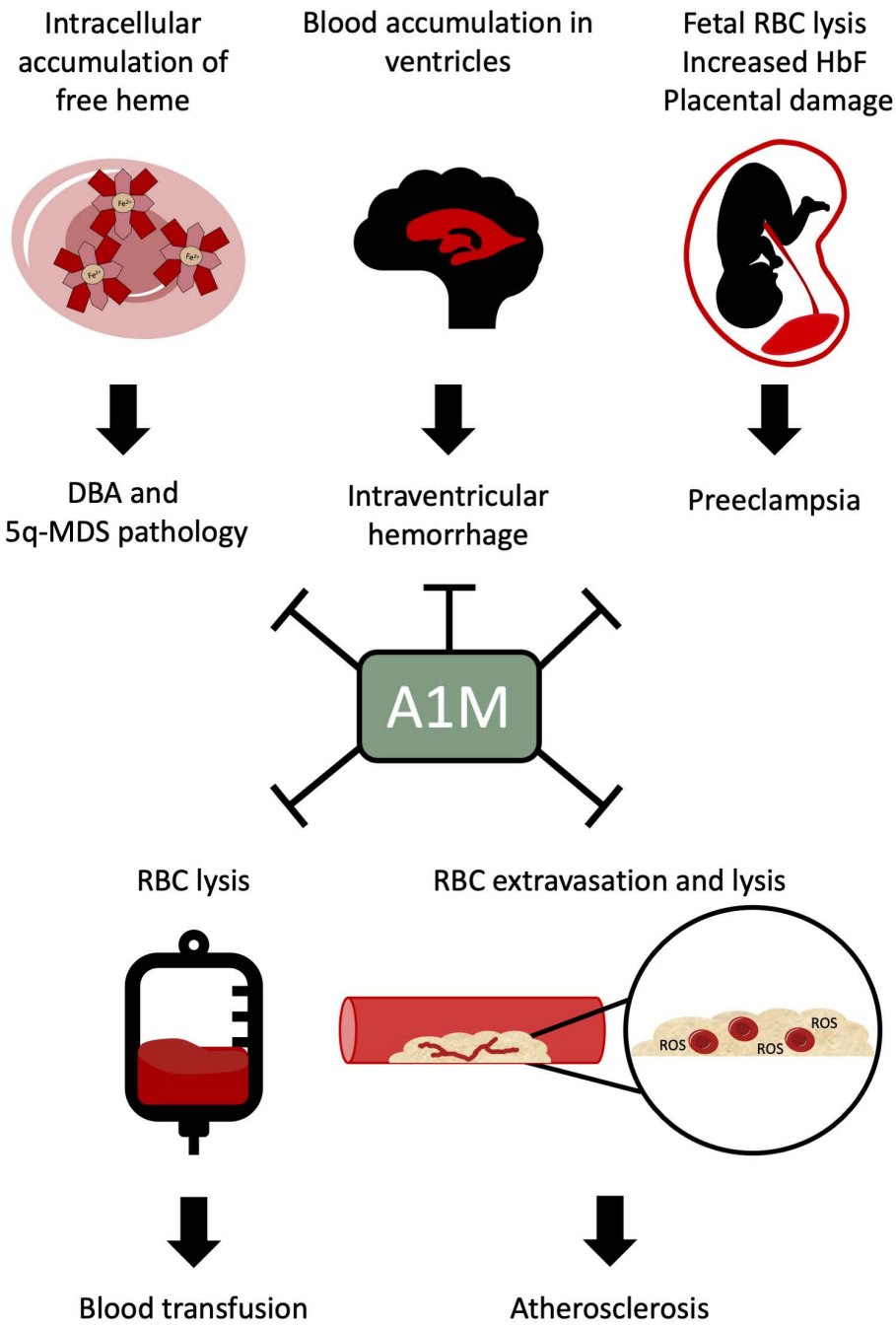

Figure 28. The anti-hemolytic and stabilizing effects of A1M could potentially be protective for RBCs in several aspects of DBA, 5q-MDS, blood transfusions (including storage), IVH, PE and atherosclerosis.

Conclusions

The overall aim of this thesis was to elucidate new therapeutic opportunities for the human antioxidant A1M, with the focus on kidney and erythrocyte pathology. The following conclusions could be drawn from the studies in this thesis:

- A1M protects kidney cells and erythrocytes in vitro from cell death.

- A1M acts as a kidney radioprotector during cancer radiotherapy in vivo without interfering with tumor treatment.

- A1M has a biological role in protein modifications and fat/appetite regulation.

- A1M possesses antihemolytic properties and a role in RBC homeostasis, which may be used clinically to treat erythropoietic and hemolytic conditions.

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