Studies on the biological role of alpha-1-microglobulin

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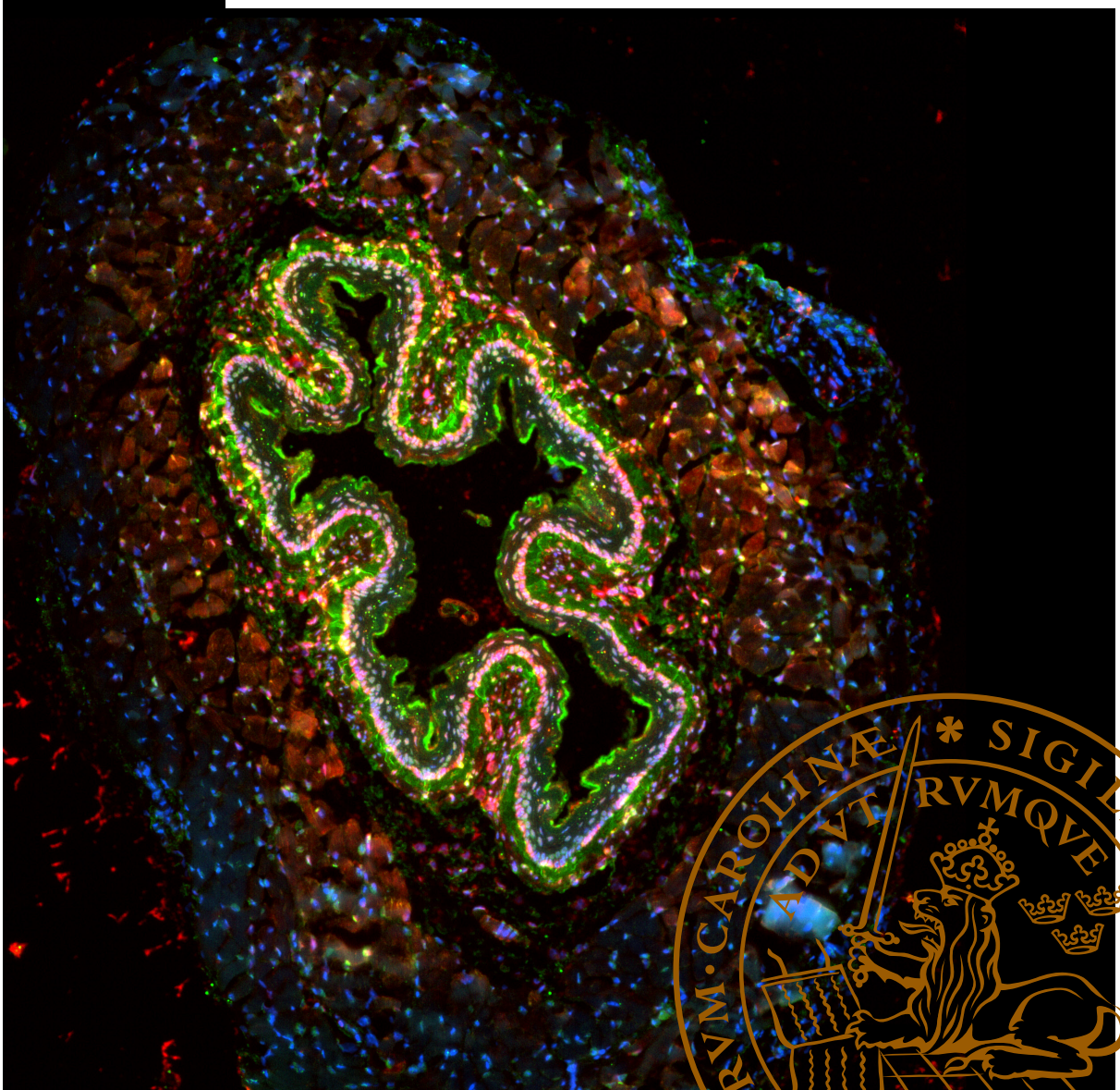

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Jesper Bergwik

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Faculty opponent
Prof. Michael Jonathan Davies
Department of Biomedical Sciences, Panum Institute, University of Copenhagen

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Abstract

α_1-microglobulin (A1M) is a ubiquitous plasma and tissue protein which has reductase and radical- and heme-binding properties. A1M is encoded by the α_1-microglobulin-bikunin precursor (AMBP) gene together with the proteinase inhibitor bikunin and the primary site of synthesis is in the liver. Several molecular mechanisms have been shown for A1M, and it has been found to be protective in vivo in animal models of oxidative stress-related diseases, but its biological role is not fully understood. The aim of this thesis was to deepen the knowledge about the different biological functions. These studies suggest several possible biological functions not previously described. Firstly, A1M was found to be necessary for the correct synthesis of bikunin, since the lack of A1M lead to misfolding and/or aggregation of bikunin. Secondly, A1M was found to provide red blood cell (RBC) stability and could to protect RBCs from hemolysis induced spontaneously or by osmosis, heme or radicals. Thirdly, A1M was found to bind to heparin both in vitro and in vivo, which may represent both a biological function of A1M as well as a biotechnological tool for purifying A1M from plasma. Lastly, a possible biological role of A1M as a protector against radicals formed during the illumination of riboflavin was shown where the A1M protein is cleaved upon reaction and parts of the riboflavin molecule are covalently attached to A1M. The results from these studies provide a deeper understanding of how A1M operates in the body and elucidates the biological mechanisms of A1M.

Key words: α_1-microglobulin, oxidative stress, ER-stress, red blood cells, heparan sulfate, riboflavin

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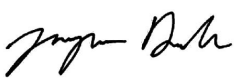
Signature  Date 2020-09-03

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Cover photo
Immunofluorescence microscopy image of a section from a mouse aorta with A1M in red, heparan sulfate in green and cell nuclei in blue, by Jesper Bergwik

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To my family and friends

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Acknowledgement

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List of original papers

I. **Knockout of the radical scavenger α_1-microglobulin in mice results in defective bikunin synthesis, endoplasmic reticulum stress and increased body weight.**
Bergwik J, Kristiansson A, Welinder C, Göransson O, Hansson SR, Gram M, Erlandsson L, Åkerström B.
Free Radic Biol Med. 2020 Feb 21:S0891-5849(19)32351-2.

II. **Human radical scavenger α_1-microglobulin protects against hemolysis in vitro and α_1-microglobulin knockout mice exhibit a macrocytic anemia phenotype.**
Kristiansson A, **Bergwik J**, Alattar AG, Flygare J, Gram M, Hansson SR, Olsson ML, Storry JR, Allhorn M, Åkerström B.
Free Radic Biol Med 2020 Feb 21:S0891-5849(19)32350-0.

III. **Binding of human α_1-microglobulin to heparin and heparan sulfate. Mapping of binding site, molecular and functional characterization, and co-localization *in vivo* and *in vitro*.**
Bergwik J, Kristiansson A, Larsson J, Ekström S, Åkerström B, Allhorn A.
Manuscript

IV. **α_1-microglobulin binds illuminated flavins and has a protective effect against sublethal riboflavin-induced damage in retinal epithelial cells.**
Bergwik J, Åkerström B.
Front Physiol. 2020 Apr 2;11:295.

Papers not included in thesis

The role of mitochondria, oxidative stress and radical-binding protein A1M in cultured porcine retina.
Åkerström B, Cederlund M, **Bergwik J,** Manouchehrian O, Arnér K, Taylor IH, Ghosh F, Taylor L.
Curr Eye Res. 2017 Jun;42(6):948-961.

Acute tissue reactions, inner segment pathology, and the effects of the antioxidant α_1-microglobulin in an in vitro model of retinal detachment.
Ghosh F, Åkerström B, **Bergwik J**, Abdshill H, Gefors L, Taylor L.
Exp Eye Res. 2018 Aug;173:13-23.

Abstract

α_1-microglobulin (A1M) is a ubiquitous plasma and tissue protein which has reductase and radical- and heme-binding properties. A1M is encoded by the α_1-microglobulin-bikunin precursor (AMBP) gene together with the proteinase inhibitor bikunin and the primary site of synthesis is in the liver. Several molecular mechanisms have been shown for A1M, and it has been found to be protective *in vivo* in animal models of oxidative stress-related diseases, but its biological role is not fully understood. The aim of this thesis was to deepen the knowledge about the different biological functions. These studies suggest several possible biological functions not previously described. Firstly, A1M was found to be necessary for the correct synthesis of bikunin, since the lack of A1M lead to misfolding and/or aggregation of bikunin. Secondly, A1M was found to provide red blood cell (RBC) stability and could to protect RBCs from hemolysis induced spontaneously or by osmosis, heme or radicals. Thirdly, A1M was found to bind to heparin both *in vitro* and *in vivo*, which may represent both a biological function of A1M as well as a biotechnological tool for purifying A1M from plasma. Lastly, a possible biological role of A1M as a protector against radicals formed during the illumination of riboflavin was shown where the A1M protein is cleaved upon reaction and parts of the riboflavin molecule are covalently attached to A1M. The results from these studies provide a deeper understanding of how A1M operates in the body and elucidates the biological mechanisms of A1M.

Populärvetenskaplig sammanfattning

Bakgrund

Fria radikaler är atomer eller molekyler som är reaktiva och därmed skadliga för kroppen. Oxidativ stress är ett tillstånd som uppstår när mängden fria radikaler i kroppen ökar och balansen mellan de fria radikalerna och kroppens egna antioxidanter rubbas. I kroppens normaltillstånd ser antioxidationsförsvaret till att de skadliga fria radikalerna motarbetas. På så sätt skyddas våra vävnader. Oxidativ stress kan uppstå i kroppen av flera olika anledningar. Det bildas exempelvis fria radikaler vid cigarrettrökning och även strålning från solen kan skapa fria radikaler i huden när den utsätts för sol. Denna avhandling fokuserar på den kroppsegna antioxidanten α_1-mikroglobulin (A1M), ett litet plasma- och vävnadsprotein som finns i däggdjur, fiskar, fåglar och reptiler. A1M bildas primärt i levern, varifrån det transporteras till kroppens alla vävnader via blodet. Ett flertal olika funktioner har påvisats hos A1M. Det kan bland annat oskadliggöra fria radikaler genom reduktion (motsatsen till oxidation) samt genom att binda radikalerna till sig och därmed neutralisera dem.

Syfte

Trots många års studier av A1M, i vilka flera olika molekylära funktioner har bevisats, har dess primära funktion i kroppen inte fastställts. Avhandlingens syfte är att fördjupa kunskapen om den biologiska funktionen hos A1M. Detta har åstadkommits med fyra olika studier.

Resultat och diskussion

Studie I

Genom att klippa bort den bit av arvsmassan som existerar för att tillverka A1M i möss, skapades en mus som saknar A1M. Detta gav oss möjligheten att studera vad som sker med mössen vid avsaknad av A1M. En intressant företeelse med A1M är att det bildas tillsammans med ett annat protein, nämligen bikunin. Denna gemensamma produktion av de två proteinerna har bevarats i samtliga djurarter genom evolutionen, men någon gemensam funktion efter att de lämnat levern har inte påvisats. Resultaten från studien visar att A1M är viktigt för att bikunin ska produceras

på ett korrekt sätt. Hypotesen är att A1M agerar som ett hjälpprotein. Studien visade även, något oväntat, att mössen som saknade A1M blev signifikant tyngre än de burkamrater som hade A1M i sina kroppar. Någon slutgiltig förklaring till viktökningen kunde dock inte fastställas men i studien påvisades att en viss ökning av fettinlagring i levern sker, vilket kan vara en bidragande faktor.

Studie II

De röda blodkropparnas huvudsakliga funktion är att transportera syre från våra lungor och ut till kroppens alla delar. Vid olika sjukdomstillstånd, till exempel vid malaria, går de röda blodkropparna sönder. Detta kallas för hemolys. Hemolys leder till att de röda blodkropparnas innehåll läcker ut, vilket gör att kroppen dels har svårare att transportera syre, dels till lokala skador vid området där hemolysen sker. De lokala skadorna sker till viss del på grund av bildandet av fria radikaler. De fria radikalerna som bildas vid hemolys kan även i sin tur skapa ytterligare hemolys genom att skada cellmembranet. I studie II konstaterades att A1M kan stabilisera de röda blodkropparna samt skydda dem från hemolys orsakad av radikaler, genom att reducera och/eller binda radikalerna till sig. Förmågan att skydda de röda blodkropparna är en biologisk funktion hos A1M som inte påvisats tidigare.

Studie III

I en studie publicerad av en annan forskargrupp år 2016 påvisades det att A1M är ett så kallat heparin-bindande protein. Heparin, och det snarlika heparansulfat (HS), är sockermolekyler som finns på de flesta cellytor i vår kropp. Flera olika proteiner har utvecklat en förmåga att binda till heparin och HS som en del av sin biologiska funktion. I studie III användes flera olika metoder för att studera den heparin-bindande förmågan hos A1M. Bindningen visades vara av elektrostatisk karaktär som därför kan brytas genom att salt adderas. Därutöver påvisades att A1M finns på samma platser som HS i flera olika organ i möss samt på ytan av mänskliga celler från blodkärl. Resultaten tyder även på att de molekylära funktionerna hos A1M är delvis nedsatta när A1M är bundet till heparin eller HS. Förmågan att binda till HS på cellytor, vilket möjliggör en ansamling av A1M vid cellytan, utgör troligtvis en viktig biologisk funktion hos A1M. Den heparin-bindande förmågan kan även utnyttjas för att rena A1M från blod.

Studie IV

Vitamin B2, även kallat riboflavin, är ett essentiellt vitamin som fyller flera viktiga funktioner i kroppen vid bland annat metabolismen. När riboflavin utsätts för ljus, i till exempel huden eller ögonen, bildas fria radikaler som i sin tur kan skada kroppens vävnader. I studie IV studerades interaktionen mellan A1M och riboflavin under belysning. Resultaten visade att A1M binder riboflavinet till sig, samtidigt som ena änden av A1M klyvs bort. Att en bit av A1M klyvs bort när det binder radikaler har påvisats tidigare och kan vara en generell mekanism för A1M vid

radikalbindning. Studien visade även att A1M kunde skydda mänskliga näthinneceller från riboflavinradikalerna. I huden och ögonen är både riboflavin och A1M närvarande, vilket gör att bindandet och oskadliggörandet av radikaler från belyst riboflavin är en trolig biologisk funktion hos A1M.

Slutsats

I den här avhandlingen har de olika molekylära funktionerna hos A1M satts i ett mer biologiskt perspektiv än i tidigare forskning. Framtida forskning kommer förhoppningsvis leda till ett mer slutgiltigt svar angående den sanna biologiska funktionen hos A1M och även till utvecklingen av A1M som ett läkemedel mot sjukdomar med oxidativ stress som en drivande faktor.

Abbreviations

A1M	α_1-microglobulin
ROS	Reactive oxygen species
ETC	Electron transport chain
$O_2^{\bullet-}$	Superoxide anion
H_2O_2	Hydrogen peroxide
$OH^{\bullet}$	Hydroxyl radical
NO	Nitric oxide
$ONOO^-$	Peroxynitrite
ER	Endoplasmic reticulum
CYP	Cytochrome P450
HO	Heme oxygenase
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
^{1}Rib*	Singlet excited state riboflavin
^{3}Rib*	Triplet excited state riboflavin
SOD	Superoxide dismutase
CAT	Catalase
GPx	Glutathione peroxidase
GSH	Glutathione
Trx	Thioredoxin
Prx	Peroxiredoxin
GST	Glutathione S-transferase
NRF2	Nuclear factor 2-related factor 2
ARE	Antioxidant response element

KEAP1	Kelch-like ECH-associated protein 1
PTM	Posttranslational modification
UPR	Unfolded protein response
ERAD	ER associated protein degradation
BiP	Binding immunoglobulin protein
XBP1	X-box binding protein 1
CHOP	C/EBP homologous protein
RBC	Red blood cell
Hb	Hemoglobin
metHb	Methemoglobin
HbF	Fetal hemoglobin
GAG	Glycosaminoglycan
HS	Heparan sulfate
HSPG	Heparan sulfate proteoglycan
CS	Chondroitin sulfate
ECM	Extracellular matrix
HBPs	Heparin binding proteins
AMBP	Alpha-1-microglobulin-bikunin precursor
t-A1M	Truncated A1M
LDL	Low-density lipoprotein
IVH	Intraventricular hemorrhage
PE	Preeclampsia
PRRT	Peptide receptor radionuclide therapy

Introduction

Oxidative stress

Background

The human body is constantly exposed to external and internal stressors in the form of oxidizing and/or reducing substances and this represents a threat against the integrity, structure and functioning of our tissues and cells. The damaging nature of such agents results in a biological condition referred to as oxidative stress. This has been defined as a disturbance in the balance between the occurrence of reactive oxygen species/nitrogen species (ROS/RNS) and the inherent ability to counteract the oxidation through an antioxidative protective system.

Endogenous ROS

The main endogenous source of ROS is the electron transport chain (ETC) where, at the inner mitochondrial membrane, energy is generated as ATP. During this process, electron carriers NADH and $FADH_2$ transfer electrons via complexes I-IV to oxygen to generate ATP and H_2O. However, some electrons leak from the inner membrane and react with oxygen forming superoxide anions ($O_2^{\bullet-}$) [1]. The $O_2^{\bullet-}$ can further react with other molecules to form hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$). RNS can also be produced by $O_2^{\bullet-}$ through reaction with nitric oxide (NO) forming peroxynitrite ($ONOO^-$). Consecutive reactions can generate other RNS such as nitrogen dioxide ($^{\bullet}NO_2$) and nitrosoperoxycarbonate ($ONOOCO_2^-$).

Another significant source of ROS is the endoplasmic reticulum (ER). During protein folding, disulfide bonds are formed in an oxidative process which requires $O_2^{\bullet-}$ and approximately 25% of intracellular $O_2^{\bullet-}$ is produced within the ER [2]. This can be increased in cells which secrete large amounts of proteins and during ER-stress where the ER attempts to refold misfolded proteins.

Xenobiotics, which are chemical substances that are not normally present or produced in the body, can cause oxidative stress when they are broken down by enzymes. One of the most studied group of enzymes involved in the metabolism of xenobiotics is the cytochrome P450 (CYP) family. CYP enzymes are very versatile and are involved in a wide range of biochemical reactions often involving substrate

oxidation [3]. When CYP reacts with its substrate, ROS are produced in the form of H_2O_2 and $O_2^{\bullet}$.

Exogenous ROS

Ultraviolet light (UV) is divided into UV-A (long waves), UV-B (medium waves), and UV-C (short waves). UV makes up a portion of the sunlight, where UV-A and a small portion of UV-B reaches the surface of the earth. UV-C and most of UV-B are blocked by the ozone layer. The UV-B light reaching the skin can penetrate the epidermis to reach the dermis, where it can induce $OH^{\bullet}$ formation from H_2O_2. Additionally, absorption of UV-B by thymine or cytosine can cause direct damage to DNA. UV-A is less effective in causing DNA-damage since it has less photon energy. However, UV-A can penetrate the epidermis to a higher extent than UV-B, where it can induce formation of H_2O_2 and singlet oxygen ($^1O_2$).

If the energy of a photon is sufficiently high, it can displace an electron from a non-radical, leaving a radical cation ($X^{\bullet+}$) behind. UV-A and UV-B do not have enough energy to ionize, but γ-rays, X-rays, α-particles and β-particles can, and their radiation is therefore referred to as ionizing [4].

ROS-induced damage

Uncontrolled levels of ROS can lead to oxidation of various biological molecules including lipids, proteins and DNA. Hydroxyl radicals can cause lipid peroxidation of the plasma membrane in any cell or organelle with polyunsaturated fatty acid side chains. A carbon-centered radical ($C^{\bullet}$) is formed by abstraction of a hydrogen from the hydrocarbon sidechain. With oxygen present, a peroxyl radical ($-C-O-O^{\bullet}$) is formed, which is capable of abstracting a hydrogen atom from an adjacent fatty acid, propagating the reaction. High levels of lipid peroxidation lead to loss of membrane function and fluidity, and potentially activation of the apoptotic cascade [4].

Proteins are common targets for ROS due to their abundance in most systems within the human body. ROS-induced damage to proteins can result in backbone fragmentation, alterations in side-chain hydrophobicity, protein unfolding, conformational changes and aggregation via covalent cross-linking. These changes can alter the interaction between the protein and its biological partner or ligand, potentially obstructing the functionality of the protein [5]. Similar to the lipid peroxidation, with oxygen being present, peroxyl radicals and peroxides are formed, which can in turn oxidize nearby biomolecules and thereby propagate the reaction.

DNA modified through oxidation is abundant in several human tissues, especially in tumors [6]. To avoid accumulation of oxidatively modified DNA, many defense and repair mechanisms have evolved [7]. Despite these mechanisms, DNA mutations are unavoidable which results in cancer, inheritable diseases and ageing.

Excessive levels of ROS cause damage to all cellular constituents unless controlled. Conversely, at low to moderate concentrations, ROS act as important mediators in different signaling processes. Humans have evolved enzymes such as nitric oxide synthase (NOS), which generates NO, and NADPH oxidase producing $O_2^{\bullet-}$, to use as signaling molecules for several different biological functions [8].

Heme

The heme molecule and its toxicity play a central role in this work due to both its role in oxidative stress and the heme binding function of α_1-microglobulin (A1M). Its toxicity towards red blood cells and the protective effect of A1M are the main foci of paper II.

The heme molecule is a porphyrin complex with an iron atom chelated in its core (Figure 1). Heme is fundamental for aerobic organisms, due to is role in numerous different biological functions [9]. It is involved in oxygen transport, as part of hemoglobin and myoglobin and it functions as the active part of various heme-proteins [10]. Cytochrome c contains a heme group in its active site [11], where it is used for electron transport and energy generation. Cytochrome c has also been shown to play a role in the apoptosis cascade [12]. Another important heme protein is catalase which contains four heme groups, allowing it to react with H_2O_2 forming H_2O and O_2 [13]. The NO producing enzyme NOS, mentioned above, contains a heme molecule as a functional group [14]. The most abundant heme-protein is hemoglobin, which contains four subunits each carrying one heme group [15].

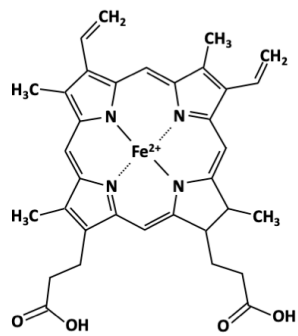

Figure 1. Molecular structure of heme with the iron atom in its ferric state (Fe^{2+})

The catabolism of heme in mammals results in biliverdin, CO and iron. Firstly, heme oxygenase (HO) converts heme, with the help of cytochrome P450 reductase, to biliverdin [16]. Biliverdin is further converted to bilirubin by biliverdin reductase, which requires NADPH as an electron donor [17]. Bilirubin, when bound to albumin, acts as an antioxidant [18]. When bound to albumin, bilirubin is excreted from the liver through the bile. Through bacterial decomposition, bilirubin is finally converted to stercobilin and excreted in the feces.

When bound to its protein, heme acts as a functional group. However, when heme is released from its protein, or if the heme containing protein is moved from its protected environment, e.g. extracellular hemoglobin, it can oxidize nearby bio-molecules causing damage to cells and tissues [9]. Accumulation of free labile heme and hemoglobin in large quantities occurs during pathological states with hemolysis

such as sickle cell disease, malaria, intraventricular hemorrhage [19] and pre-eclampsia [20]. Newly synthesized heme groups not yet incorporated into hemeproteins also adds to the labile heme pool. The redox active iron in the free heme group allows it to participate in the Fenton reaction, producing $OH^{\bullet}$, which leads to oxidative damage of lipid membranes, nucleic acids, and proteins. The release of free heme groups can also cause inflammation through activation of proinflammatory transcription factors [21]. The toxicity of free heme is further exacerbated by the hydrophobicity of heme, which enables it to intercalate into cell membranes, increasing the susceptibility of cells to oxidation mediated damage through the formation of lipid peroxides [22]. Heme has also been shown to act as a catalyst during the oxidation of low-density lipoprotein (LDL), which causes toxicity to the endothelium [23,24]. Finally, the free heme released from hemoglobin during hemolysis can damage the membranes on nearby RBCs, causing a feed-forward loop resulting in more hemolysis [25].

To defend the tissues against heme toxicity, the human body has developed several different systems (Figure 2). Cells exposed to heme, up-regulate the heme degrading protein HO and the iron storing protein ferritin. Studies on HO-1 deficient knockout mice showed very high concentrations of circulating heme [26]. The primary defense against circulating heme is hemopexin (Hpx) which is a plasma protein with high binding affinity for free heme [27]. When the free heme group is bound to Hpx it is transported to the liver where it is taken up by parenchymal cells and degraded by HO [28]. Another heme binding protein found in plasma is α_1-microglobulin, which can also bind heme intracellularly [29]. The primary source of circulating heme is from hemoglobin, which if not contained within the RBC can cause oxidative damage. To protect against hemoglobin induced damage, during for example hemolysis, the plasma protein haptoglobin (Hp) can bind Hb. The resulting Hb-Hp complex is then removed by macrophages through binding to CD163 [30].

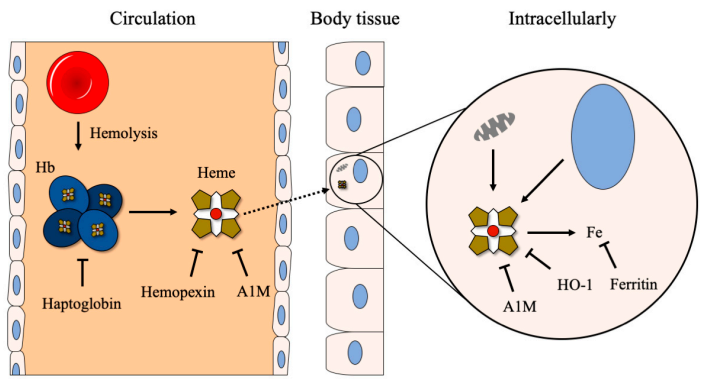

Figure 2. Overview of protective mechanisms against extracellular Hb, free heme and iron. In circulation, haptoglobin binds extracellular Hb. When the extracellular Hb is broken down into free heme this is bound by hemopexin and A1M. Intracellularly, free heme, released from heme proteins or newly synthesized, is bound by A1M or broken down by HO-1. The breakdown of the heme group by HO-1 results in free iron which is neutralized by ferritin.

Riboflavin

Another important molecule that plays a central role in this work is riboflavin. Its toxicity during illumination and its interaction with A1M are studied in paper IV.

Riboflavin (7,8-dimethyl-10-ribityl-isoalloxazine), more commonly known as vitamin B2, is a water-soluble essential vitamin found in milk, meat and leafy vegetables. Riboflavin is mostly found as a component of the prosthetic group flavin adenine dinucleotide (FAD) and flavin mononucleotide FMN, but can also be found in its free form [31]. Riboflavin, FAD and FMN can exist in different redox states; oxidized (quinone), one-electron reduced (semiquinone) and two-electron reduced (hydroquinone), enabling transportation of single electrons, hydrogen atoms and hydride ions. Both FAD and FMN are involved in several enzymatic reactions throughout metabolism.

When illuminated, riboflavin rapidly undergoes photo-degradation, generating ROS and thereby classifying it as a photosensitizer. During the photo-degradation, riboflavin is broken down into different molecules, where lumiflavin, lumichrome and formylmethylflavin represent the majority of products formed at physiological pH [31]. Photo-degradation generates a very fluorescent short-lived singlet excited state riboflavin (^{1}Rib*). Subsequently, ^{1}Rib*, through an intersystem crossing, is converted into the triplet excited state riboflavin ^{3}Rib*, which is more long-lived. ^{3}Rib* is a bi-radical and powerful oxidant, which can oxidize proteins, lipids and DNA [32]. The direct reaction between ^{3}Rib* and different biomolecules is termed the type I reaction [33]. The type I reaction generates $O_2^{\bullet-}$ which can further react with other molecules forming H_2O_2 and $OH^{\bullet}$ [34]. ^{3}Rib* can also transfer its excitation energy to O_2 forming the more unstable and reactive singlet oxygen ($^1O_2$). The latter reaction is termed the type II reaction [33]. The type I and II reactions are summarized in Figure 3.

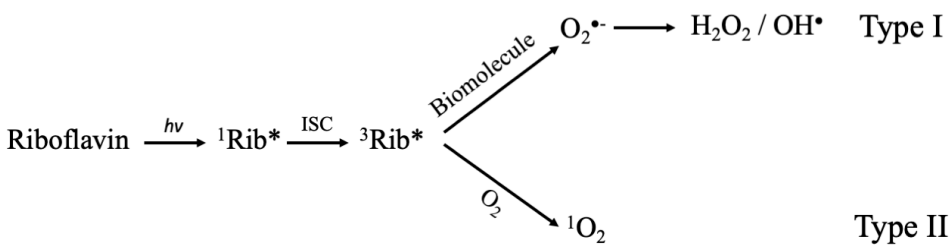

Figure 3. Photosensitization of riboflavin by the type I or type II mechanism.

Riboflavin (free form), FAD and FMN are all involved in metabolism and are therefore mostly found in organs with a high metabolic activity. However, they are also present in tissues that are exposed to light, such as skin and eyes [34]. This makes the light-exposed organs vulnerable to radicals formed from the photo-degradation of riboflavin. The riboflavin-generated radicals can react with proteins, DNA and lipids, which can cause cell death, mutations and potentially carcinogenesis [35]. Previous studies have shown that DNA mutations induced by UVA-light in fibroblasts are increased severalfold with riboflavin present as a photosensitizer [36]. Additionally, oxidative stress is involved in causing a wide range of eye diseases such as rhegmatogenous retinal detachment (RDD), age-related macular degeneration (AMD), glaucoma and cataracts [37-40].

Endogenous antioxidation defense

To protect cells and tissues against damage from oxidative stress, several different proteins and molecules with antioxidative properties have evolved. The anti-oxidation system can be divided into two different categories: enzymatic and non-enzymatic.

Enzymatic antioxidants

The antioxidant superoxide dismutase (SOD) catalyzes the dismutation of $O_2^{\bullet-}$ forming O_2 and H_2O_2 [41]. Three different SOD variants have been reported: soluble (SOD1), mitochondrial (SOD2) and extracellular (SOD3). SOD has been shown to be of primary importance for the prolongation of lifespan. Studies have revealed that mice lacking SOD1 [42] or SOD2 [43] suffer from immense oxidative stress, which leads to a severely reduced lifespan and a wide range of pathologies. H_2O_2 produced from the dismutation of $O_2^{\bullet-}$ is a reactive molecule that needs to be further processed. This is achieved by the enzymatic antioxidant catalase (CAT), which catalyzes the breakdown of H_2O_2 into H_2O and O_2 [13]. The breakdown of H_2O_2 can also be executed by another enzymatic antioxidant, glutathione peroxidase (GPx). GPx, together with glutathione (GSH) converts H_2O_2 into H_2O and glutathione disulfide (GSSG) [44]. The GSSG is reduced back to GSH by glutathione reductase (GR) with NADPH as an electron donor.

Thioredoxin (Trx) is an important enzymatic antioxidant which plays a role in several biological processes. Trx contains a CGPC motif, where the cysteine residues play a central role in breaking disulfide bonds in oxidized proteins [45]. In the process, a disulfide is formed between the cysteine residues in the CGPC motif, which is broken by thioredoxin reductase (TrxR) using NADPH.

Peroxiredoxin (Prx) is one of the most abundant proteins in RBCs apart from hemoglobin. Its primary targets are H_2O_2 and $ONOO^-$. Prx has a redox-active cysteine residue in its active site, which is oxidized to a sulfenic acid by the substrate

[46]. The sulfenic acid is reduced back to a free thiol by Trx, GSH or glutathione S-transferase (GST). GST is a detoxification enzyme with a wide range of functions. However, the main function of GST is to detoxify xenobiotics by catalyzing the conjugation of GSH to the xenobiotic, to prevent it from interacting with bio-molecules [47].

The expression of the above described enzymatic antioxidants, including A1M, is regulated by nuclear factor 2-related factor 2 (NRF2). Under non-stressed conditions, NRF2 is bound to Kelch-like ECH-associated protein 1 (KEAP1), which is ubiquitinated and degraded in the proteasome. During oxidative stress, NRF2 is released from KEAP1 and translocated to the nucleus. NRF2 induces the expression of antioxidants through binding to the antioxidant response element (ARE) present in the promotor region of the genes [48].

Non-enzymatic antioxidants

Non-enzymatic antioxidants are low molecular weight compounds, such as vitamin C and E, carotenoids, uric acid and GSH. Vitamin C, also known as ascorbic acid, is a water-soluble vitamin found in high doses in fruit and vegetables. Vitamin C can scavenge both $OH^{\bullet}$ and $O_2^{\bullet-}$ and it is one of the key antioxidants in the blood [49,50]. Vitamin E is a group of tocopherols and tocotrienols, which are fat-soluble antioxidants able to protect cell membranes against ROS. Vitamin E can donate a hydrogen atom to the radical to minimize the damaging effects [51]. The oxidized vitamin E can be recycled through reacting with a hydrogen donor, for example vitamin C [52].

Carotenoids are brightly colored fat-soluble pigments present in fruits and vegetables. The most studied of the carotenoids, which are also present in human tissues, are β-carotene and lycopene [53]. Carotenoids have radical scavenging abilities and can for example quench peroxyl radicals and thereby prevent propagation of the reaction.

Uric acid is a strong reducing agent that is found in the blood, where it together with vitamin C makes up most of the antioxidant capacity [54]. Uric acid has been shown to selectively bind $ONOO^-$ and inactivate it [55].

Finally, glutathione (GSH) is a tripeptide comprised of cysteine, glutamic acid and glycine. It is highly abundant in all cell compartments and it is one of the major soluble antioxidants. GSH can exist in its reduced (GSH) or oxidized form (GSSG) and the ratio between these two states is a common determinant of oxidative stress. GSH has several different antioxidant functions [56]. As described above, it can detoxify H_2O_2 together with GPx, followed by recycling to GSH through reduction by GR. It can also reduce vitamin C and E back to their active forms.

ER-stress

Protein folding and posttranslational modification

The biological function of a protein is dependent on its three dimensional (3D) native structure, which is encoded in the protein's amino acid sequence. Several different factors determine how a protein is folded [57], such as hydrogen bonds [58], van der Waals interactions, backbone angle preferences, electrostatic interactions, hydrophobic and hydrophilic interactions [59] and chain entropy. Another important part of the protein folding machinery are the molecular chaperones, which aid in the folding process [60]. Molecular chaperones bind to the folding protein and stabilize the otherwise unstable protein structures. The chaperones do not know how the protein is supposed to be folded, but operate to prevent improper folding conformations [61].

Oxidative protein folding

Several proteins depend on disulfide bonds for their folding and function and they are crucial to the structure and stability of the protein. Mispairing of cysteine residues is a common cause of misfolding. The formation of disulfides is a spontaneous process, but it is dependent on a redox reaction, making it very slow [62]. Protein disulfide isomerase (PDI) is a protein that can rearrange incorrect sulfides. In addition, PDI can catalyze disulfide formation and reduction [63]. For PDI to be able to catalyze the formation and rearrangement of disulfides it needs to be re-oxidized, which is carried out by ER oxidoreductin 1 (Ero1p) [64]. For the Ero1p to become oxidized again, it uses a flavin dependent reaction to transfer electrons to molecular oxygen [65]. This results in the generation of ROS, making it a source of oxidative stress in the cell.

Posttranslational modifications

After being translated, most proteins undergo a process called posttranslational modification (PTM), where the proteins are covalently modified. PTMs can be divided into two different categories, where the first category includes hydrolytic cleavage of one or more peptide bonds within the protein by proteases. The hormone insulin, for example, is translated as a single chain inactive prohormone which is then cleaved by a protease to generate the active two-chain form of insulin [66]. The second category is the covalent addition of one or more groups to the amino acids of the protein, such as glycosylation, acetylation or phosphorylation. This extends the chemical repertoire of the 20 amino acids by introducing new functional groups or by modifying existing ones [67].

Glycosylation, which is relevant to this work, is the enzymatic process where a glycan is attached to an amino acid sidechain. The list of biological effects due to

glycosylation of proteins is almost endless and more effects are added frequently [68]. For example, some proteins do not fold properly unless they are correctly glycosylated [69], and glycosylation also affects the solubility of the protein. Glycans also function as recognition markers, modulate immune responses and mediate interaction with pathogens. The process of glycosylation involves 13 types of monosaccharides which are attached to 8 types of amino acids and is performed by several different enzymes. This results in 5 classes of glycans being produced: N-linked (attached to a nitrogen of an asparagine or arginine side-chain), O-linked (attached to the hydroxyl oxygen of a serine, threonine, tyrosine, hydroxylysine or hydroxyproline side-chain), phosphoglycans (attached to the phosphate of a phosphoserine side-chain), C-linked glycans (attached to a tryptophan side-chain) and glypiation (addition of a GPI anchor linking the protein to a lipid through glycan linkages). Two glycans, that are of importance for this work, are the O-linked glycosaminoglycans (GAGs) heparan sulfate and chondroitin sulfate, which will be covered in more detail below.

ER-stress and the unfolded protein response

Most secreted and transmembrane proteins are folded within the endoplasmic reticulum (ER). Malfunctioning protein folding results in the accumulation of unfolded and misfolded proteins, leading to an overload state called ER-stress. To restore the cellular homeostasis, the unfolded protein response (UPR) is triggered [70], which leads to expansion of the ER membrane, where the additional space is used for increased protein folding machinery, such as chaperones [71]. Simultaneously, the influx of newly translated proteins progressing into the ER is decreased to handle the increased unfolded and misfolded protein load. To further combat the ER-stress, the ER-associated protein degradation (ERAD), which removes unfolded and misfolded proteins, is increased [72]. The ER-stress also leads to an up-regulation of antioxidants due to an increased production of ROS [73]. If the ER-stress is persistent, the activation of CHOP can lead to apoptosis [74].

UPR signaling

UPR signaling is mediated through three different transmembrane transducers: proteinase kinase RNA-like ER-kinase (PERK), activating transcription factor-6 (ATF6) and inositol-requiring enzyme-1α (IRE1α) [75-77]. Under normal unstressed conditions, binding immunoglobulin protein (BiP) forms a stable complex with PERK, IRE1α and ATF6. A build-up of unfolded or misfolded proteins leads to a reversible dissociation of the BiP-PERK/IRE1α/ATF6 complexes, initiating downstream signaling of the UPR [78]. The active PERK phosphorylates the eukaryotic initiation factor 2α (eIF2α), which leads to a general decrease in protein synthesis [77]. However, not all proteins are affected by the decrease in synthesis. This is due to certain regulatory sequences in the mRNA,

bypassing the translational stop. One of the mRNAs which has an increased translation in the presence of eIF2α is the transcription factor ATF4, which is involved in increasing the antioxidant response through NRF2 [79]. ATF4 also activates the apoptosis signaling protein C/EBP homologous protein (CHOP) [74]. After activation of IRE1α, it removes parts of the X-box binding protein 1 (XBP1) mRNA, forming the splice product XBP1s, which is a highly active transcription factor that increases the production of ER chaperones, enhancing ER biogenesis and induces ERAD [80]. Dissociation of BiP from AFT6 results in a translocation of ATF6 to the Golgi apparatus, where it is proteolytically cleaved by SP1 and SP2 into an active transcription factor (ATF_{NT}) [81]. This results in an increased expression XBP1 and chaperones. The signaling pathways are illustrated in Figure 4.

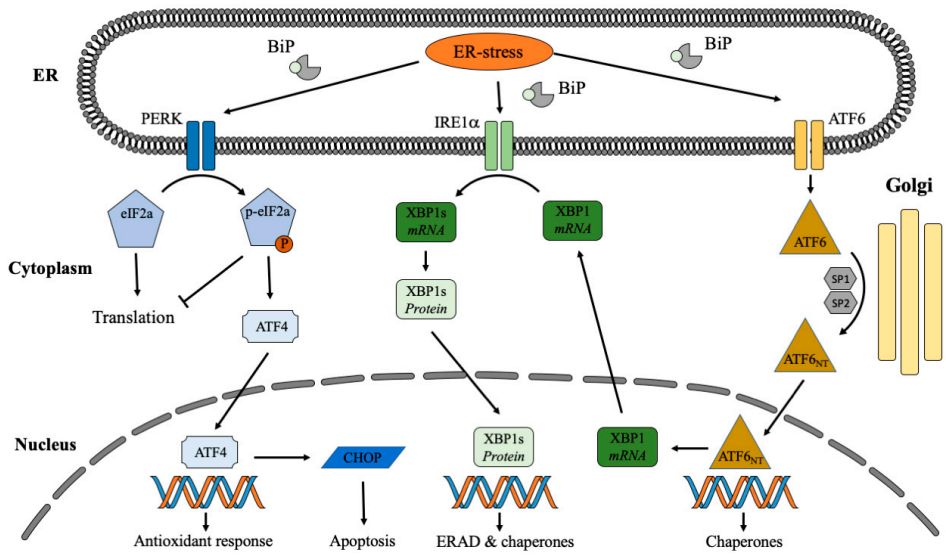

Figure 4. Signaling pathways during the unfolded protein response (UPR). Dissociation of BiP from PERK, IRE1α and ATF6 leads to activation of three downstream signaling pathways. PERK activation leads to an increase in expression of antioxidants and apoptosis signaling through CHOP. Activation of IRE1α generates the splicing product XBP1s, which activates the transcription of chaperones and induces ERAD. ATF6 activation leads to an increased transcription of chaperones and XBP1.

Red blood cells

Structure and function

The red blood cell (RBC) is the most common cell in the body, making up approximately 25% of total cells. Their primary function is to bind oxygen in the lungs and transport it to the different tissues of the body. The RBCs are produced in the bone marrow in a process called erythropoiesis where a hematopoietic stem cell undergoes a series of differentiations. In the first step, the hematopoietic stem cell becomes a proerythroblast, which then becomes an erythroblast, and in the final step, a reticulocyte is formed and released into the blood (Figure 5). The reticulocytes comprise roughly 1-2% of the circulating RBCs and after about 1-2 days they become mature RBCs. As the cells differentiate, several different characteristics change. The size of the cells is reduced, and the nucleus and DNA are removed. The cells also become redder in color as they start to produce hemoglobin (Hb). The mature RBCs have a life span of approximately 115 days [82] and are later broken down in the spleen.

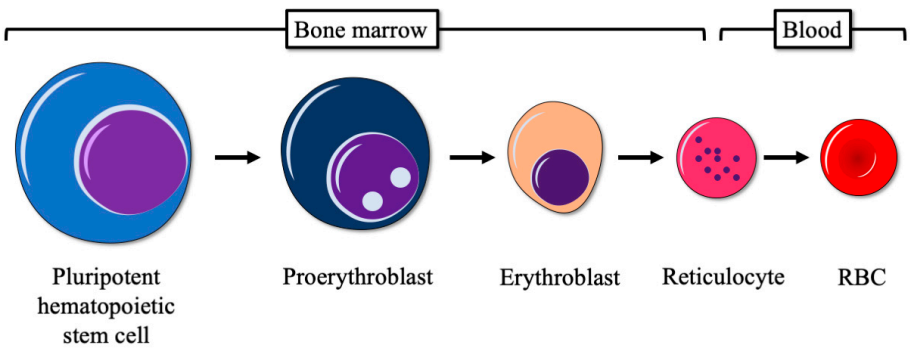

Figure 5. Erythropoiesis. A pluripotent hematopoietic stem cell differentiates into a proerythroblast which further differentiates into an erythroblast. The erythroblast is transformed into a reticulocyte and released into the blood stream. While in the blood stream, the reticulocyte matures into a red blood cell (RBC).

The RBCs are shaped like biconcave disks, which provides a larger surface area for gas exchange to occur. The space within the capillaries is very narrow, requiring the RBCs to fold in on themselves to be able to pass through. The extremely flexible nature of the RBCs is due to their cytoskeleton, which contains the flexible protein spectrin [83]. The RBCs do not have a nucleus or organelles, and due to the lack of mitochondria they have to rely on anaerobic glycolysis for ATP generation.

Hemoglobin

The lack of organelles in the RBC allows for additional space for oxygen carrying Hb molecules. The Hb molecule is composed of four polypeptide globin chains each containing a heme group with a chelated iron atom in the ferrous state (Fe^{2+}). In adult Hb (HbA), the Hb molecules are made up of two α-chains and two β-chains, whereas in the fetal Hb (HbF), the β-chains are replaced by γ-chains. The γ-chains provides the HbF with a higher oxygen affinity than HbA, facilitating the oxygen transfer from the maternal circulation to the fetal circulation in the placenta [84].

Oxidative stress in the RBC

During the lifetime of an RBC, it is continuously exposed to oxidants, which if not removed leads to impaired flexibility of the RBCs, making it more difficult for them to move through the narrow capillaries. To avoid oxidative stress, the RBCs contain an array of antioxidants and reducing enzymes, including SOD, CAT and Prx [85-87], as well as the non-enzymatic antioxidants vitamin C and vitamin E. For Hb to be functional, the iron has to be kept in its ferrous state (Fe^{2+}), whereas the ferric state (Fe^{3+}), called metHb, severely weakens the oxygen binding capacity. Hb is continuously being auto-oxidized, which leads to the production of metHb, $O_2^{\bullet-}$ and H_2O_2 [88]. To avoid auto-oxidation of Hb, and the accumulation of metHb and ROS, the enzyme glucose-6-phosphate dehydrogenase (G6PDH) supplies reducing energy to maintain the level of NADPH. NADPH in turn keep the levels of reduced GSH and GPx high enough to keep the iron in Hb in its ferrous state. Studies on patients with mutations in their G6PDH gene, which impairs the function of the G6PDH protein, have shown an increase in the levels of free radicals causing membrane disruption [89].

In the circulation, ROS are being released from neutrophils and macrophages which can cause damage to the RBCs. Studies have shown that RBCs are particularly susceptible to peroxides formed in the lipid membrane, which contains high levels of unsaturated fatty acids [90]. Some of the ROS are also internalized by the RBCs and are neutralized by the intracellular antioxidant defense. Membrane disruption in RBCs leads to hemolysis, resulting in extracellular Hb in the blood. The extracellular Hb can be further broken-down releasing free heme groups. Both the free heme groups and the extracellular Hb causes oxidative stress, creating a feed-forward loop resulting in more hemolysis [25]. This is covered more in detail above.

Heparin and heparan sulfate

Structure

Heparin and heparan sulfate (HS) are members of the glycosaminoglycan (GAG) family of carbohydrates, together with chondroitin sulfate (CS), dermatan sulfate (DS), hyaluronic acid (HA) and keratan sulfate (KS).

Heparin and HS are both linear disaccharide polymers composed of alternating units of α-D-glucosamine (GlcN) and either α-L-iduronic acid (IdoA) or β-D-glucuronic acid (GlcA). The disaccharides are linked together by (1→4) glyosidic linkages. The GlcN subunits in heparin are almost always sulfated. The GlcN subunits of HS can either be N-acetylated (GlcNAc) or N-sulfated (GlcNS6S) and these make up regions of GlcNS6S (NS domains), GlcNAc (NA-domains) or both types (NS/NA domains) (Figure 6). GlcNS6S disaccharides can be further modified by glucuronyl C5-epimerization and O-sulfation at positions 2, 3 or 6, which gives theses domains diverse properties [91-93].

HS is primarily found as part of heparan sulfate proteoglycans (HSPG) and the most common HSPG families are the syndecans and glypicans. The syndecans are transmembrane proteins that bind components of the extracellular matrix (ECM) to endothelial cells [94]. The name syndecans comes from the Greek word “syndein”, translated to “bind together”. Glypicans are directly linked to membrane phospholipids and this glycosyl phosphatidyl inositol (GPI) linkage is the reason for their name [95]. HSPGs are often found in the ECM or on cell surfaces. Membrane bound syndecans can be enzymatically shed by several different proteinases, releasing the ectodomain of the syndecans [96]. Shedding of the ectodomain is an important mechanism regulating paracrine and autocrine signaling. The shedding process is increased during pathophysiological events such as wound healing.

Heparin can be seen as a more sulfated, tissue specific, HS variant which is only found within mast cells. Heparin is primarily known as an anticoagulation pharmaceutical and it is one of the most used anticoagulants in the world. Heparin exerts its anticoagulant activity through binding of antithrombin III, facilitating the subsequent inhibition of thrombin and activated factor X, blocking the coagulation cascade [97].

Physiological role and protein binding

HS in various forms play an important role in a multitude of biological functions, including: inflammation [98], anticoagulation, wound healing, receptor- and co-receptor functions, binding of proteins including growth factors and cytokines [99], as well as axonal development and guidance [100]. HS regulates theses biological

processes through interactions with a wide range of proteins, known as heparin-binding proteins (HBPs) [101]. The protein binding properties is dependent on both sequence specificity and/or electrostatic interactions. Arginine and lysine, which are both positively charged amino acids, are important contributors during binding to the negatively charged HS molecule [102]. The multi-functional nature of HS is due to the large variations in sulfation and acetylation of the disaccharides constituting the molecule, and this large diversity of HS molecules has been titled the heparanome [103].

Protein binding sequences and heparin binding domains

The specificity of the binding is contributed to by both protein binding sequences in the HS chain and the presence of heparin-binding domains in the sequences of the HBPs. The interaction between HS and proteins is dependent on sulfate groups, where the NS or NA/NS function as protein binding motifs on the HS chain. The first protein binding site that was characterized was to antithrombin III [97], where the binding site was found to be a pentasaccharide with a rare modification of the sugar backbone. However, the majority of binding sites are dependent on more common modifications, arranged in different patterns. The length of the saccharide binding sequence has been shown to vary a lot between different interactions, where some proteins require a binding sequence of more than 20 saccharides.

Characterization of general heparin binding domains on HBPs was first attempted in the late 80s, where the heparin binding domains of vitronectin, apoE, apoB-100 and PF-4 were used to find 21 novel HBPs [104]. Since then, several heparin binding domains have been determined and found to be enriched with basic amino acids. Recently, Manissorn et al characterized HBPs in urine using applied affinity purification-mass spectrometry [105]. This resulted in the discovery of numerous new HBPs and amongst these was the primary protein of this work, A1M.

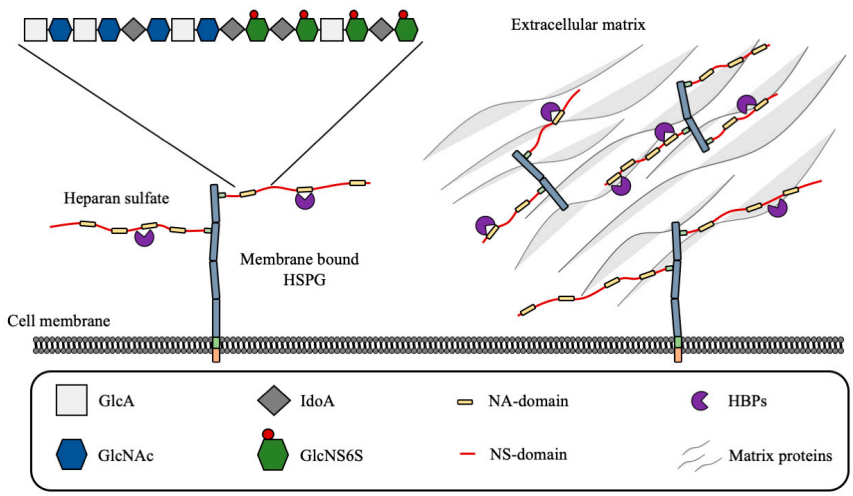

Figure 6. Structure and physiological functions of heparan sulfate (HS). HS is made up of NA- and NS-domains, which make up binding sites for heparin binding proteins (HBPs). HS can be attached to transmembrane proteins or make up parts of the extracellular matrix.

α_1-microglobulin

A1M is a small protein found both intra- and extracellularly in most vertebrate tissues. It has been described as a housekeeping protein due to its heme-binding capacity, reducing abilities and radical trapping properties.

Structure

Human A1M consists of a peptide chain with 183 amino acids [106] and it has a molecular weight of 26 kDa [107]. A1M is glycosylated in three different positions, two sialylated complex type, biantennary and triantennary carbohydrates attached to Asn17 and Asn 96, and one O-linked oligosaccharide attached to Thr5 [108]. A1M has been well conserved during evolution, with homologues found and sequenced from other mammals, amphibians, fish and birds [109-112]. A1M is a member of the lipocalin protein family, which is a group consisting of 40-50 proteins present in all branches of life, such as bacteria, fungi, plants and animals [113], and 12 human lipocalins have been described. All lipocalins share a similar tertiary structure with a single polypeptide containing 150-190 amino acids, which forms a β-barrel consisting of eight antiparallel β-sheets with one open end and one closed end. Most lipocalins contain a binding site for small hydrophobic compounds within the β-barrel [114]. The crystal structure of A1M (Figure 7) shows the typical lipocalin fold with a β-barrel with four loops at the open end [115]. A1M has an important side chain, Cys34, which is located on loop 1 at the proximity of the open end of the β-barrel. Cys34 is conserved in all species, can participate in one-electron oxidation and reduction reactions, and is involved in reductase activities and the binding and neutralization of target compounds [116]. When isolating A1M from urine, A1M carries covalent modifications on Lys69, Lys92, Lys118, Lys130 and Cys34, and these chromophores have been suggested to contribute to the charge heterogeneity and yellow-brown color of the protein [117,118].

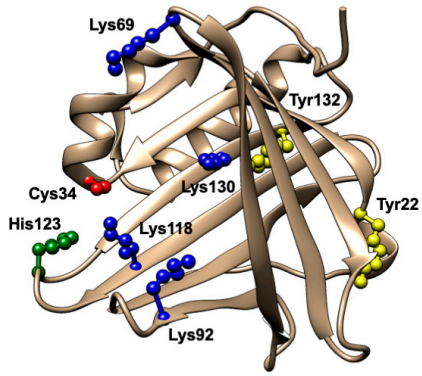

Figure 7. 3D-strucutre of A1M. Amino acids important for molecular mechanisms are shown in blue (Lysine), yellow (Tyrosine), green (Histidine) and red (Cysteine) with their side chains visible.

The AMBP gene

A1M is encoded by the alpha-1-microglobulin-bikunin precursor gene (AMBP) which also encodes bikunin [119,120]. Bikunin is a Kunitz-type plasma proteinase inhibitor and a structural component of the extracellular matrix [121,122]. The AMBP gene has 10 exons where exons 1-6 encode A1M and exons 7-10 encode bikunin (see Figure 8). After transcription of the AMBP gene and translation of the resulting mRNA, the AMBP precursor protein is formed with A1M and bikunin linked together by a tripeptide [123]. The AMBP precursor is then folded in the ER, followed by transportation to the Golgi, where a chondroitin sulfate chain is attached to the N-terminal of the bikunin. After leaving the Golgi, heavy chains (HC) HC1, HC2 and HC3 are covalently bound to the chondroitin sulfate chain [124]. The attachment of the HC later results in the formation of inter-α-trypsin inhibitor (IαI), which consists of bikunin, HC1 and HC2, or pre-α-inhibitor (PαI), which consists of bikunin and HC3 [125]. Finally, the AMBP precursor is proteolytically cleaved between A1M and bikunin, and the IαI, PαI and A1M are secreted separately from the cell [126]. No functional or physical connection between A1M and bikunin or its complexes has been found and the reason for co-synthesis is unknown. However, A1M has recently been shown to be important during synthesis and posttranslational modification of bikunin [127]. Similar to other enzymatic antioxidants, the expression of A1M has been shown to be regulated through the KEAP1/NRF2 signaling system [128,129].

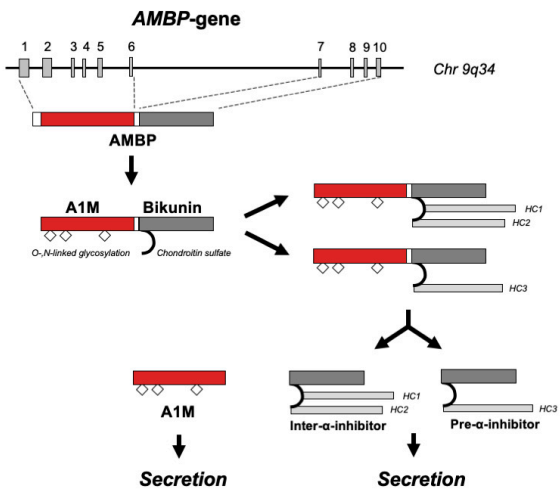

Figure 8. Structure of the AMBP gene and posttranslational modifications of A1M and bikunin. The AMBP gene contains 10 exons, where exon 1-6 encode A1M and exon 7-10 encode bikunin. After translation, A1M is O- and N-linked glycosylated and a chondroitin sulfate chain is attached to bikunin. Heavy chains (HC) are attached to the chondroitin sulfate chain, forming inter-α-inhibitor and pre-α-inhibitor. The two proteins are then cleaved and secreted separately. Figure from [127].

Cloning of the AMBP gene in humans [130] and mice [131] mapped the gene to the lipocalin gene cluster at the 9q32-33 region in man [130] and to chromosome 4 in mice [132]. Between exon 6 and 7, there is a large intron containing retroposons and other repeated sequences, which suggests that this a recombinatorial hot spot [131]. This might have provided a foundation for the fusion between an ancestral bikunin gene and an ancestral A1M gene. The genetic construction of the AMBP gene has been shown to be conserved in all species where A1M has been studied.

Synthesis, distribution and catabolism

The primary site for A1M synthesis is the liver [133], but it is also expressed at a lower rate in peripheral organs. From the liver, it is secreted to the blood where it is found in either its free form, which has the Cys34 in a free functional state, or bound to IgA with a reduction resistant disulfide bond including Cys34 [134]. Minor complexes have also been found with albumin and prothrombin [135]. Complexed forms of A1M are present in all studied species, but with different complex-partners, indicating evolutionary conservation of complex formation ability. The plasma concentration of A1M is approximately 20-50 mg/ml [136], with men having marginally higher levels than women [137]. The main sites of A1M localization are in the liver, blood plasma and kidneys [138]. A1M and its complex forms are quickly equilibrated between the intra- and extravascular compartments with a half-life in blood of approximately 2-3 min [139].

The presence of A1M mRNA, other than in the liver, has been identified in most other cell types and the A1M protein has been detected in the perivascular connective tissue of most organs [140-142], where it is often colocalized with elastin and collagen. *In vitro* studies have shown that A1M can bind directly to collagen [142]. During pregnancy, A1M is located at the interface between the maternal blood and the fetal tissue in the placenta [143].

A1M has also been shown to bind to the surface of a wide variety of cells, including keratinocytes, blood cell lines, lymphocytes and neutrophils [142,144,145]. The binding of A1M to the cell surface is specific for A1M, saturable and sensitive to trypsin, indicating the presence of an A1M receptor. However, no specific receptor has been identified yet.

A1M is found intracellularly, where it partly is located to the mitochondrial respiratory chain bound to Complex I [146]. Uptake and mitochondrial localization of exogenously added A1M *in vitro* has been shown in keratinocytes, blood cells and liver cells, and RBCs have also been shown to contain cellular A1M [25]. Mutated A1M, which lacks Cys34, showed a lower cellular uptake indicating an important role of Cys34 in the uptake mechanism [147]. The functional role of A1M intracellularly has not been established, but it has been shown that its presence lowers the redox charge and increases the levels of free protein thiol groups in the

cytosol [144]. It was also speculated that mitochondrial A1M is involved in maintaining mitochondrial energy delivery during apoptosis [146]. During normal mitochondrial respiration, $O_2^{\bullet-}$ is leaking from Complex I and III. A1M has therefore been hypothesized to act as a radical scavenger, counteracting and eliminating the leaking $O_2^{\bullet-}$, and thereby preventing oxidative damage to nearby molecules. The uptake of A1M in RBCs was shown to play a minor role in protection against hemolysis, suggesting a non-hemolysis related function in the cytosol of RBCs [25].

The final destination for A1M is the kidneys, passing through the glomerular membranes to the primary urine, where the large majority is reabsorbed by the proximal tubular cells and catabolized [148]. Notable levels of A1M are secreted in the urine, making it a sensitive and clinically used indicator of tubular renal damage [107]. The life cycle of A1M is illustrated in Figure 9.

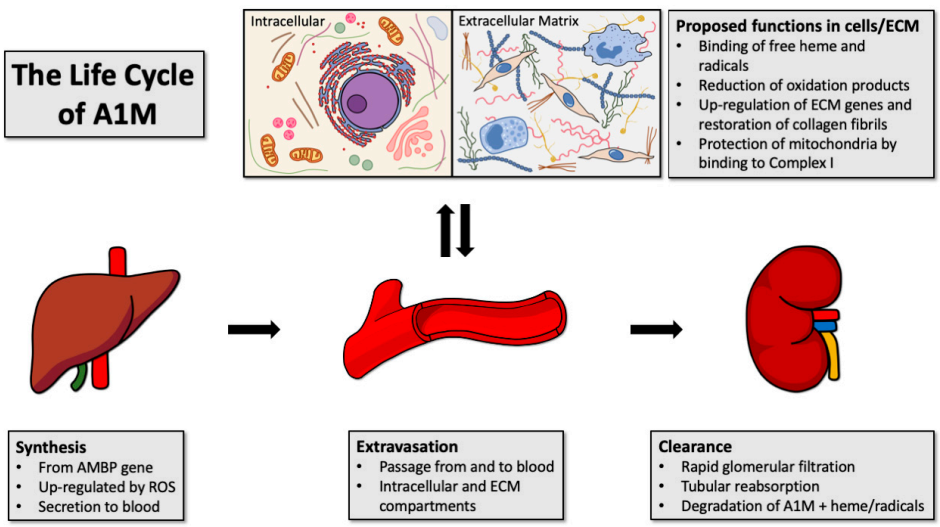

Figure 9. The life cycle of A1M. A1M is primarily synthesized in the liver where it is secreted into the blood. It passes through the vessel wall into the extracellular matrix and/or is taken up intracellularly. After contributing to tissue housekeeping by radical- and heme-binding, mitochondrial protection and tissue repair, it is transported to the kidneys where it is filtrated in the glomeruli. Finally, it is reabsorbed in the tubuli and broken down together with heme and/or radicals.

Molecular mechanisms

A1M is a protein with several different molecular mechanisms and protective functions, which have been studied both *in vivo* and *in vitro*. The mechanisms and functions can be divided into two different categories; immunoregulation and anti-oxidation, where that latter is of primary focus in this work.

Reduction

A1M has been shown to possess enzymatic reductase/dehydrogenase properties with several different organic and inorganic substrates (Figure 10A). A1M could reduce the heme proteins metHb and cytochrome c, as well as free iron and the synthetic molecule nitro blue tetrazolium (NBT)[149]. In the presence of ascorbate, NADH or NADPH, a catalytic reductase effect was seen. The reductase and dehydrogenase ability of A1M was found to be dependent on Cys34 and the three residues Lys92, 118 and 130, which are all close to each other at the open end of the A1M barrel. It was speculated that the three lysyl residues create a positively charged microenvironment around the Cys34, which lowers the pK_a of the free thiol, favoring its oxidation. Also, A1M could, in a Cys34 dependent reaction, reduce the synthetic radical ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) [116].

Reduction of physiological substrates, both intra- and extracellular, has been shown for A1M. Using the erythroid cell line K562, A1M was found to decrease the amount of oxidized molecules in the cytosol, more specifically reduce the thiol groups of cytosolic proteins [144]. Additionally, the amount of carbonyl groups on oxidized collagen was significantly reduced by A1M with the reaction entirely dependent on Cys34 but only partially dependent on Lys92, 118 and 130 [142,147]. More recently, the role of A1M in knee injuries, where bleeding, subsequent inflammation and oxidative stress was studied. A correlation was found between increased A1M concentrations and a decrease in carbonyl groups [150]. However, the chemical mechanism of action during the reduction of carbonyl groups by A1M has not been shown in detail yet.

Radical scavenging

The ability of A1M to bind and neutralize radicals has been studied using the synthetic radical ABTS [116]. The reaction between A1M and ABTS has been shown to involve a Cys34-catalyzed reduction, resulting in covalent trapping of the radical metabolites to different amino acid side chains on A1M. During the reaction, A1M reduces 5-6 ABTS molecules and, in a semi catalytic mechanism, covalently traps 3 additional ABTS radicals by attaching them to amino acid side chains, where ABTS-adducts on tyrosyl side chains Tyr22 and Tyr132 were identified (Figure 10B). Additionally, the side chains of Lys69, 92, 118 and 130 of A1M have been shown to be covalently modified *in vivo* [118,151], possibly as a result of radical trapping. This is supported by a report showing that urine A1M from hemodialysis

patient carried the lysyl modification 3-OH-kynurenine, which is a tryptophan metabolite known to form free radicals [152]. The lysyl side chains Lys92, 118 and 130 may therefore play an important role in both the lowering of the pK_a of Cys34 and as electron donors during radical trapping. Cys34 has also been found to be modified by unidentified oxidation products *in vivo*, potentially contributing to the brown color of the protein [153].

A1M has been described as a "radical sink" due to the fact that after reacting with a radical, both the radicals and A1M become electron neutral and thereby do not cause any further oxidative stress. A1M purified from urine is heavily modified and brown colored. This is not seen in other proteins isolated from urine, such as albumin, which suggest that the radical scavenging is not a non-specific protein modification but an A1M-specific reaction.

Heme binding and degradation

A1M binds heme and the apparent dissociation constant is approximately 10^{-6} [154,155]. The ability of A1M to bind heme has been conserved through evolution and has been found to take place in plasma from man, rat, mouse cow, guinea pig, plaice and chicken [155]. When human recombinant A1M binds heme, a trimeric A1M/heme complex is formed, where each A1M molecule binds two heme groups, i.e. $[(heme)_2(A1M)]_3$ [156]. The crystal structure of A1M has revealed a potential heme-binding site, which is similar to those seen in the CYP family, located at loop 1 (Cys34-Pro35) [115]. A possible covalent binding has also been shown, which remained after boiling in SDS [154,155]. More recently, A1M was confirmed to have two heme binding sites, where one of them showed a higher heme binding affinity [157]. The two heme binding sites were suggested by molecular simulation to be located in the lipocalin pocket, in close proximity to Lys92, 118 and 130, and more superficially, between loop 1 and 4 with involvement of Cys34 and His123 [158] (Figure 10C).

A processed form of A1M with heme degrading abilities, called t-A1M (t=truncated), was shown to be generated when full length A1M reacted with lysed RBCs or purified Hb [154]. The degradation of heme by t-A1M results in heterogenous chromophores being bound to A1M. Formation of t-A1M has been shown to occur *in vivo* and has been found in the skin, urine and placenta [154,159-161].

A1M also interacts with the heme-containing enzyme myeloperoxidase (MPO), which is a protein that catalyzes the production of free radicals and hypochlorite after being released by neutrophils during inflammation due to a bacterial infection [162]. When exposed to MPO, A1M was proteolytically cleaved, forming t-A1M, which contains iron and heme-degradation products. Additionally, A1M inhibited MPO- and H_2O_2-induced oxidation of LDL.

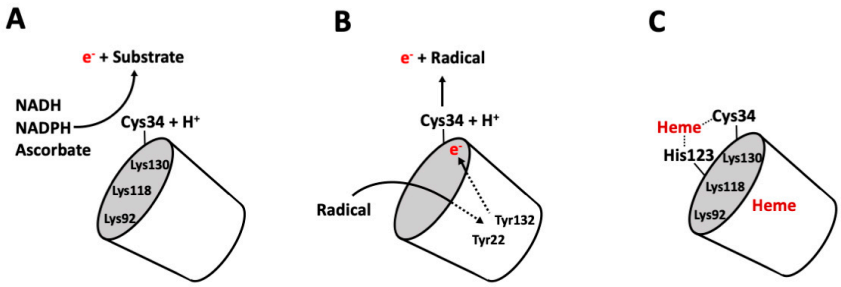

Figure 10. Molecular mechanisms of A1M. (A) Reductase properties. The pKa of Cys34 is lowered by Lys92, 118 and 130. Electrons transferred from Cys34 can reduce various substrates. Electron-donating cofactors NADH, NADPH and ascorbate can regenerate the thiolyl radical, which makes the reduction faster and catalytic. (B) Radical trapping mechanism based on reaction with the synthetical radical ABTS. Similar to (A), the Cys34 transfers an electron to a substrate, which in this case is a radical. Without the presence of electron-donating cofactors, the resulting thiolyl radical is regenerated by intramolecular reactions with Tyr22 and 132, producing tyrosyl radicals. The tyrosyl radicals then react with another radical substrate, creating a stable Tyr-radical adduct. (C) Heme-binding properties. Two heme-binding sites have been found. The first is located within the lipocalin pocket, in close proximity to Lys92, 118 and 130. The second binding site is located more superficially and involves Cys34 and His123.

Regulation of AMBP expression

As described above, the AMBP gene is, similar to many other enzymatic anti-oxidants, regulated by the KEAP1/NRF2 system [128,129]. The up-regulation of A1M, both mRNA and protein, after exposure to oxidative stress conditions with H_2O_2, $OH^{\bullet}$, Hb and heme, has been shown to occur in human cell lines, primary skin keratinocytes and skin and retina explants [142,163,164]. Up-regulation of A1M during oxidative stress is also supported by *in vivo* data. Using samples from pre-eclampsia patients, a correlation was found between the concentration of A1M in plasma and placenta tissue and Hb, as well as between A1M and the oxidative stress markers protein carbonyls groups and the plasma peroxidation capacity [161].

Protective functions

The molecular mechanisms described above makes A1M a potential protector against oxidative damage to cells and tissues. A1M can function as a radical sink, which removes oxidants and free radicals from the tissues, including Hb, free heme and iron. After binding and/or neutralizing the radicals, A1M is transported to the kidneys where it is degraded and/or excreted in the urine. The protective functions of A1M in cell, organ and animal models are presented below.

Cell models

Several studies have shown *in vitro* protective effects of A1M in cell cultures exposed to Hb, heme and free radicals. Exogenously added A1M prevented intra-cellular oxidation and up-regulation of HO-1 in primary keratinocytes and the

erythroid cell line K562, after exposure to heme or H_2O_2 and $OH^{\bullet}$ generated by the Fenton reaction [142,165]. Additionally, silencing of the endogenous A1M expression by addition of siRNA resulted in an increased cytosol oxidation [165]. When exposing the K562 cells to 200 μM heme, more than 50% of the cells were lysed. Addition of 2 μM of A1M reduced the proportion of lysed cells to 15%. In a more recent study, the lysis of human (adult and fetal) and murine RBCs by spontaneous, osmotic, heme and radical-induced hemolysis was significantly reduced through addition of A1M [25]. The protection by A1M against heme-induced hemolysis was shown to be more potent than that of hemopexin, vitamin C and Trolox (vitamin E). Moreover, addition of A1M to K562 cells, keratinocytes and liver cells exposed to heme and H_2O_2 inhibited mitochondrial swelling and reversed the severely abrogated ATP production of the mitochondria [146]. Furthermore, induction of ER-stress by heme in aortic smooth muscle cells was shown to be completely inhibited when adding A1M to the cell medium [166]. Induction of oxidative stress in cell cultures of retinal epithelial cells through illumination together with the photosensitizer riboflavin, could be partly inhibited by addition of A1M, shown by a decreased expression of HO-1 and the cell cycle regulator p21 [167]. Finally, A1M showed a protective effect against heme induced damage to primary kidney cells (RPTEC) and cells from the kidney cell line HK2. Treatment with A1M resulted in a decreased expression of stress-related genes, preservation of the mitochondrial respiration and reduced activation of the Nrf2-pathway [168].

Alpha particle radiation of cells causes damage not only to the irradiated cells, but also to the adjacent bystander cells, which is thought to be caused by oxidants and free radicals. Addition of A1M inhibited the propagation of cell death induced by radiation of cultured liver cells [147,169]. The cells were irradiated with a low dose of alpha particles in a small restricted area. Addition of A1M to the cell cultures prior to radiation showed a decreased number of dead cells (50% reduction) in the directly hit cells and by 100% in the bystander cells. It also completely inhibited the irradiation induced apoptosis, formation of carbonyl groups and up-regulation of HO-1, p21 and p53.

Organ models

Using skin explants, exogenously added A1M was shown to inhibit heme-induced damage, up-regulation of antioxidation and cell cycle regulating genes and the formation of carbonyl groups [142]. A1M was found to be localized ubiquitously in the dermal and epidermal layers of the skin, and the expression of A1M was also upregulated when exposing the skin explants to heme or $OH^{\bullet}$ generated by the Fenton reaction. To mimic damages to the placenta during preeclampsia, *ex vivo* perfusion with Hb in the fetal circulation was used. This lead to a significant increase in perfusion pressure, leakage of Hb from fetal to maternal circulation, morphological damage and up-regulation of genes connected to oxidative stress, immune response and apoptosis [170]. Addition of A1M to the maternal circulation,

mimicking a potential clinical setting, abrogated the Hb leakage, morphological damage and up-regulation of stress genes.

The studies on skin explants and placenta also gave knowledge on the ability of A1M to protect the ECM against oxidative stress. Destruction of collagen fibrils, through addition of Hb, heme or Fenton reaction-generated $OH^{\bullet}$, could be completely inhibited by adding A1M [142]. Also, addition of A1M after the destruction had taken place repaired the collagen fibrils. The placentas infused with A1M showed a significant increase in expression of ECM genes, and the amount of collagen fibrils observed was increased drastically [170].

Animal models

In a study where rats were continuously infused with HbF, A1M was shown to prevent kidney damage [171]. The infusion of HbF resulted in an apparent increased glomerular permeability, which was almost completely reversed when treated with A1M. In another study, the effect of infused HbF in pregnant rabbits with or without co-administration of A1M, was studied [172]. The rabbits developed proteinuria and had an increased glomerular permeability, where both were ameliorated by co-administration of A1M. Transmission electron microscopy analysis of the kidneys and placenta showed both intra- and extracellular damage. Co-administration of A1M resulted in significantly reduced structural and cellular changes. In a more recent study, the effect of A1M treatment against damage induced by extracellular Hb during intraventricular hemorrhage (IVH) in preterm infants was studied in a rabbit pup model [173]. By injection of A1M into the periventricular white matter regions after induction of IVH, a decrease in structural tissue and mitochondrial damage and a reduced expression of proinflammatory genes was seen. This suggests a potential role for A1M as a neuroprotective treatment against IVH in preterm infants.

Preeclampsia (PE) is associated with oxidative stress where extracellular Hb has been identified as a plausible causative factor [174]. PE is a serious pregnancy-related condition that causes both maternal and fetal morbidity and mortality. PE complicates approximately 2-8% of pregnancies and it is the leading cause of maternal death in low income countries [175]. The potential use of A1M as a treatment against PE was investigated using a pregnant ewe model, where starvation-induced hemolysis resulted in the release of extracellular Hb, heme and free radicals [176]. All starvation-induced PE-like symptoms, e.g. increased levels of the hemolysis marker bilirubin, structural damage to the placenta and kidneys and increased glomerular permeability, were all ameliorated by infusion of A1M.

The therapeutic potential of A1M against PE was further studied using the Stork-head box 1 (STOX1) PE mouse model, where pregnant females develop severe and early onset manifestations, similar to those seen in human PE, e.g. gestational hypertension, proteinuria and organ alterations [177]. Infusion of A1M was shown

to significantly reduce the gestational hypertension, the levels of hypoxia and nitrative stress in the placenta and reduce the cellular damage in both the kidney and the placenta.

In a mouse model, A1M was evaluated as a therapeutic agent against oxidative stress in the kidneys as a result of treating metastatic neuroendocrine tumors with peptide receptor radionuclide therapy (PRRT). PRRT uses radiolabeled peptides that target cancer cells and eradicates them through irradiation damage. However, the peptides are also retained in the kidneys due to glomerular filtration, causing irradiation damage in this organ. In a first study, on the pharmacokinetics and biodistribution of A1M and radiolabeled peptides in mice, it was shown that both molecules are localized to the kidney cortex [178]. Immunohistochemistry and fluorescence microscopy revealed a co-localization in the epithelial cells in the proximal tubules. In a second study, the mice where treated with PRRT, which resulted in DNA double-strand breaks in the renal cortex, up-regulation of apoptosis and stress related genes, proteinuria, kidney lesions and increased animal deaths, all of which were suppressed by coadministration of A1M [179]. In a third study, the effect of coadministration of A1M during PRRT treatment on tumor killing was evaluated in tumor bearing mice. The results from the treatment with and without A1M showed no statistical significant difference between the groups, demonstrating that A1M does not affect tumor killing [180].

The potential effect of A1M treatment against kidney damage during glycerol-induced rhabdomyolysis was evaluated using a mouse model [181]. Glycerol injections into the thigh muscle ruptures the muscle, releasing myoglobin, heme, radicals and other tissue components. A mild dose (2 ml/kg) did not affect creatinine/blood urea nitrogen (BUN) levels but a massive increase in the stress related genes HO-1 and HSP70 was seen. Injections of A1M inhibited or reversed the up-regulation of both stress genes.

Table 1. Summary of animal studies performed to evaluate the therapeutic potential of A1M.

Species	Pathology	Treatment	Effect of treatment	Reference
Rat	Infusion of HbF Increased glomerular permeability	A1M i.v.	Restored glomerular permeability	Sverrisson et al. 2014
Rabbit	PE-like symptoms after HbF infusion Proteinuria, increased glomerular filtration Structural damage to placenta and kidneys	A1M i.v.	Reversed proteinuria and structural changes to placenta and kidneys Restored glomerular permeability	Nääv et al. 2015
Rabbit	IVH induced by glycerol injection Structural tissue and mitochondrial damage Expression of proinflammatory genes	A1M ventricular injection	Decreased structural tissue and mitochondrial damage Reduced expression of proinflammatory genes	Romantsik et al. 2019
Sheep	PE-like symptoms after starvation induced hemolysis Structural damage to placenta and kidneys Increased glomerular permeability	A1M i.v.	Decreased hemolysis and structural damage to placenta and kidneys Restored glomerular permeability	Wester-Rosenlöf et al. 2014
Mouse	PE-like symptoms (STOX1 transgenic) Gestational hypertension, proteinuria and organ alterations	A1M i.v.	Decreased gestational hypertension. Lowered hypoxia and nitrative stress in placenta Reduced cellular damage to placenta and kidneys	Erlandsson et al. 2019
Mouse	PRRT treatment induced kidney damage DNA damage, upregulation of apoptotic- and stress-related genes, proteinuria, kidney lesions and death	A1M i.v.	Decreased DNA damage and upregulation of apoptotic- and stress-related genes Reduced proteinuria and kidney lesions Less animal deaths	Kristiansson et al. 2019
Mouse	Rhabdomyolysis induced mild AKI Up-regulation of stress genes HO-1 and Hsp70 in kidneys	A1M i.v.	Reversed up-regulation of stress genes	Åkerström et al. 2019

Clinical use of A1M

Therapeutic potential

Oxidative stress is associated with several different diseases and conditions where oxidation of cells and tissues causes a buildup of reactive free radicals. The state of oxidative stress can lead to tissue damage and subsequent development of disease. The use of drugs with antioxidative properties against diseases caused by oxidative stress or for prophylactic purposes has been tried in the past decades but with no successful outcome [182].

The ability of A1M to reduce oxidized molecules, bind free heme groups and to scavenge free radicals, makes it a good candidate for treatment in conditions where oxidants, radicals, Hb and/or heme play an important role in the pathology of the disease. *In vivo* data has shown, as described above, that A1M is an effective treatment against experimental PE in mice, rats and sheep. It has also been shown that A1M can reduce side effects from PRRT treatment of neuroendocrine tumors in mice, by reducing the damage to the kidneys. Additionally, it was shown to reduce rhabdomyolysis induced kidney damage in mice. Finally, a study on rabbit pups has shown promising results for A1M as a treatment against extracellular Hb toxicity in IVH in preterm infants.

A1M has several advantages over some other much studied antioxidants and radical scavengers, such as vitamin E and C. Firstly, the molar capacity of A1M in binding/buffering of radicals is higher than vitamin C and E (1:10 vs. 1:1). Secondly, A1M occurs naturally in the body and possesses a metabolic route which ensures a controlled delivery and clearance. Thirdly, when the radicals are bound to A1M, it remains electroneutral and does not cause any further oxidative stress to the surrounding tissues.

Even though the therapeutic opportunities for A1M are plentiful, more research is needed before it can be clinically used.

A1M as a biomarker in PE

Recently, several reports have focused on predicting PE early during pregnancy or to avoid or reduce later complications. Algorithms have primarily been based on placental biomarkers such as soluble FMS-like tyrosine kinase 1 (s-FLT-1), placental growth factor (PLGF) and pregnancy-associated plasma protein A (PAPP-A) [183]. More recently, an alternative model was suggested, which is based on the levels of HbF and A1M [184]. The model had a prediction rate of 69% at a false positive rate of 5%. A notable advantage of this model was that it performed equally well without uterine artery Doppler pulsatility index, making it more suitable for low income countries. Subsequent studies have verified the increase of plasma A1M and HbF during early pregnancy, in women later developing PE [185,186].

Additionally, a study investigating the plasma levels of extracellular HbF and the heme scavenging proteins A1M, Hpx and Hp in women with PE at term, showed similar results [187]. Women with PE had increased plasma levels of A1M and HbF, and a decreased level of Hpx and Hp, suggesting a potential role of HbF and the heme scavenging proteins as biomarkers of PE. The plasma levels of A1M have also been found to be higher in the late second trimester in women with severe PE compared to non-severe [188], suggesting a possible use for A1M as a biomarker of severe PE.

Recombinant A1M

rA1M

The A1M used in these studies is a recombinant protein expressed as inclusion bodies in E.coli [189]. Briefly, E.coli cells were transformed with a plasmid containing the human A1M gene, with the addition of a polyhistidine-tag in the N-terminal. The protein expression was induced using IPTG and after harvesting the cells, the inclusion bodies, i.e. protein precipitates, were carefully washed repeatedly, dissolved in 6M guanidine-HCl and applied on a Ni-NTA column. The bound proteins were eluted with imidazole, and the A1M containing fractions were mixed with water and reduced glutathione and then dripped into a refolding buffer containing oxidized glutathione. The samples were then dialyzed twice and further purified by size-exclusion chromatography. UV circular dichroism and radio immunoassay were used to determine that the A1M was correctly folded. Recombinant A1M (rA1M) has been shown to be fully functional in comparison with endogenous A1M [116,149].

C34S

Several different rA1M variants have been produced where amino acids shown to be important for the molecular mechanisms of A1M have been substituted. One of them, which has been used in this work is C34S, where the cysteine in position 34 has been replaced by a serine. Studies using C34S have revealed an important role of Cys34 in the protection against irradiation damage and in cellular uptake [147] and in reduction of radicals [116] and carbonyl groups on oxidized collagen [142].

A1M-035

Studies using rA1M has shown that A1M can protect against Hb, heme and ROS induced damage to cells and tissues both *in vitro* and *in vivo*. However, rA1M, which lacks glycosylation, has a reduced solubility and stability when compared to A1M purified from plasma or urine. Due to its rather poor solubility and stability it is difficult to obtain highly concentrated solutions and to store it for longer times in physiological buffers. This limits its use as a drug for human use. To address this problem, rA1M variants with different amino acid substitutions were screened for physiochemical and enzymatic properties, which resulted in an rA1M variant called A1M-035 [181]. An important difference between rA1M and endogenous A1M is the presence of two N-glycans attached to amino acids N17 and N96 in endogenous A1M. The rationale behind the design of A1M-035 was to add a negative charge to N17 and N96 by replacing them with the negatively charged amino acid aspartic acid (D). Additionally, R66 was substituted with a histidine, which is present in all rodent A1M homologues. A1M-035 was shown to have improved stability and solubility while having an intact heme-binding, reductase, antioxidation and cell protecting ability. This makes A1M-035 a more suitable candidate for future drug development.

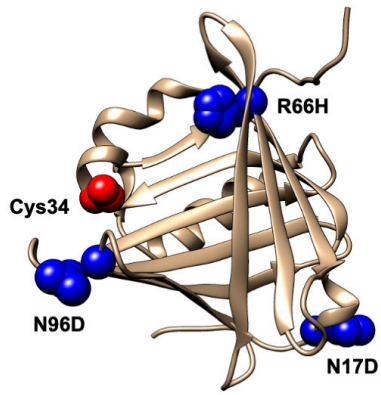

Figure 11. 3D-structure of 035-A1M. Amino acid substitutions R66H, N96D and N17D are shown with their sidechains in blue. Cys34 is displayed with its sidechain in red.

Aim

The overall aim of this thesis was to further investigate the possible biological roles of A1M. The more specific aims were to:

1. Characterize the A1M knockout mice (A1M-KO) and thereby gain additional knowledge about *in vivo* functions of A1M and the co-expression of A1M and bikunin.
2. Study the potential biological role of A1M as a protector of red blood cells *in vitro* and *in vivo.*
3. Investigate the interaction between A1M and heparin/heparan sulfate *in vitro* and *in vivo* and its potential use during purification of A1M from human plasma.
4. Analyze the interaction between riboflavin and A1M during illumination and the protective effect of A1M against riboflavin induced damage to retinal epithelial cells.

Results and discussion

A1M is needed during bikunin synthesis

Reasons behind A1M and bikunin co-expression from the AMBP gene has never been established and no common functions of the two proteins have been found after their secretion from the liver. Two proteins sharing the same gene, yet having no common function, is to our knowledge a very rare phenomenon. However, the AMBP gene is conserved in all species found to express A1M, indicating that there must be a biological explanation to why the two proteins are expressed together. Recently, we developed an A1M knockout mouse (A1M-KO) with a selective deletion of the A1M exons (exons 2-6), resulting in complete loss of A1M while maintaining the expression of bikunin. This new mouse model gave us a novel tool to use when studying the biological reason behind the co-expression of the two proteins. In the A1M-KO mice, the deletion of the A1M exons was achieved using Cre-Lox recombination, where two loxP sites were inserted into the introns after exon 1 and 6. The localization of the loxP sites and the difference between the WT and KO alleles are shown in Figure 12.

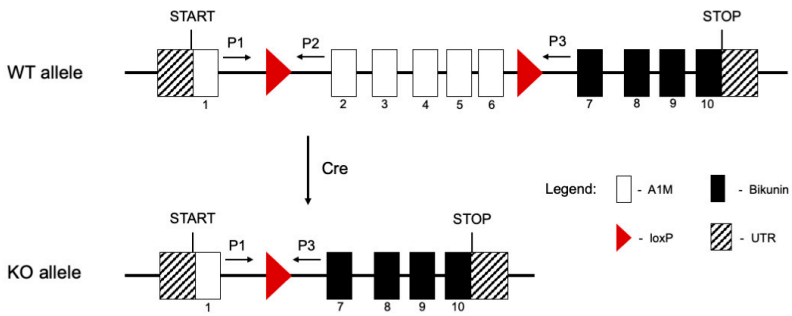

Figure 12. Graphical overview of the removal of the A1M exons in the A1M-KO mice. Exons 1-6 (white boxes) represent A1M and exons 7-10 (black boxes) represents bikunin. The exons were removed through cre deletion and P1, P2 and P3 represent the primers used for genotyping of the mice. Figure from [127].

The A1M-KO mice had no detectable levels of A1M in plasma (Figure 13A, upper panel) and no A1M mRNA (Figure 13B) was detected in the liver. The mRNA levels of bikunin in the liver were found to be slightly increased compared to the wildtype mice (Figure 13C), whereas decreased levels of the bikunin complexes IαI

and PαI were seen in plasma compared to the wildtype mice (Figure 13A, lower panel). A possible explanation for the discrepancy between the mRNA and protein levels include the chaperone function of A1M during the folding and subsequent attachment of the heavy chains to bikunin, and therefore, less bikunin is folded correctly and secreted in mice lacking A1M. Western blot analysis of liver homogenates showed the presence of two new bands exclusively found in the KO mice (Figure 13D). These new bands presumably represent free uncomplexed bikunin-forms, further supporting the requirement of A1M being present during the posttranslational handling of bikunin. In addition, one of the bands carried the signal peptide of the AMBP precursor, suggesting incomplete cleavage of the signal peptide. Furthermore, the KO mice showed signs of hepatic ER-stress, measured by the mRNA levels of the UPR mediators XBP1s and CHOP (Figure 13E and F), possibly partly due to the misfolded bikunin accumulating over time, resulting in activation of the UPR.

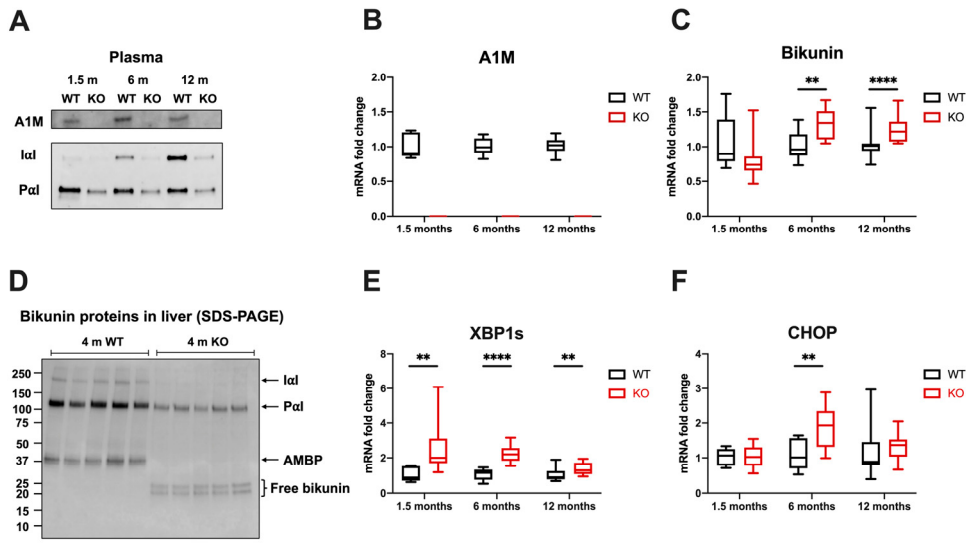

Figure 13. (A) SDS-PAGE followed by Western blot analysis of plasma using antibodies directed at A1M (upper panel) and bikunin (lower panel) from KO and WT mice at age 1.5, 6 and 12 months. (B,C,E,F) RT-PCR analysis of A1M, bikunin, XBP1s and CHOP gene expression in liver from KO and WT mice aged 1.5, 6 and 12 months. The results are shown as boxplots with medians, 25th and 75th percentiles (box) and min to max (whiskers). Animals per group: 1.5 m: WT n = 5 KO n = 10, 6 m: WT n = 10 KO n = 9, 12 m: WT n = 15 KO n = 14. Statistical testing of differences between groups was performed with Mann-Whitney U tests. **p < 0.01, ****p < 0.0001. (D) SDS-PAGE (without β-mercaptoethanol), followed by western blot analysis using antibodies directed at bikunin on liver homogenates from 4-month-old mice. Figure adapted from [127].

A possible chaperone mechanism could be to keep the free thiol groups of bikunin reduced to ensure that proper disulfide bond formation occurs. Bikunin contains six disulfide bonds, making it sensitive to incorrect disulfide bond formations, which would result in misfolding of the protein. Another potential function of A1M could

be to facilitate the addition of the CS and the heavy chains to bikunin. In paper III, we revealed a novel function of A1M as a heparin and HS binding protein. Considering the similarity of CS and heparin/HS, A1M could potentially bind to CS to facilitate the attachment to bikunin as well as assisting during the subsequent complex formation with the heavy chains.

These data suggest that A1M is a requirement for optimal production of bikunin in the liver, which partly explains the reason for the co-synthesis of A1M and bikunin. Studies on bikunin knockout mice, which lack the expression of bikunin but retain preserved A1M expression, found no evidence of reduced levels of A1M in the plasma [190], suggesting that bikunin is not a requirement for the production of A1M.

Protection against oxidative stress in different cell types

Red blood cells

The ability of A1M to protect against oxidative stress induced damage has been studied in a wide range of different cell types. In paper II, we hypothesized that A1M could protect RBCs against spontaneous, heme-, radical- (Fenton reaction), or osmotic-induced hemolysis. The extent of hemolysis was assessed by measuring the amount of lactate dehydrogenase (LDH) and hemoglobin in the medium after exposure to different stressors. The results showed a statistically significant protective effect of A1M against spontaneous hemolysis after 1 and 3 h (Figure 14A and B). The protective effect of A1M was also found to be significantly increased compared to the lipocalin orosomucoid, indicating a specificity of A1M. Moreover, A1M was also shown to protect against heme- and Fenton-induced hemolysis after 3 and 22 h (Figure 14C-F). Finally, the degree of hemolysis after exposure to osmotic stress was significantly reduced when adding A1M (Figure 14G and H).

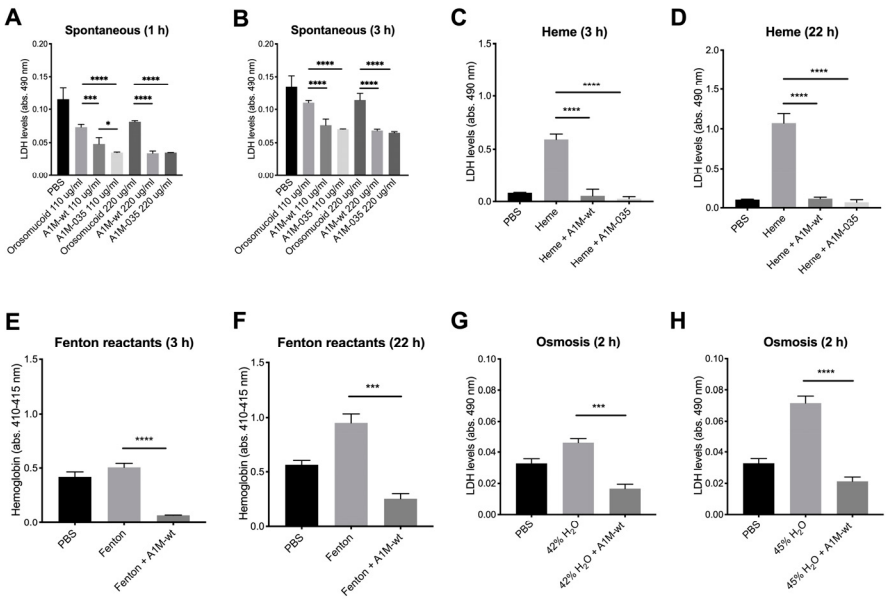

Figure 14. Protection of RBCs by A1M against spontaneous-, heme- and osmotic-induced hemolysis. (A,B) Hemolysis, determined as LDH release from the cells, after incubation of washed RBCs (n = 3) with orosomucoid (110 or 220 µg/ml), A1M-wt (110 or 220 µg/ml) or A1M-035 (110 or 220 µg/ml) for 1 h (A) or 3 h (B). (C,D) Hemolysis, determined as LDH release from the cells, after addition of heme (30 µM) to washed RBCs with A1M-wt (220 µg/ml) or A1M-035 (220 µg/ml) after 3 h (C) and 22 h (D) of incubation. (E,F) Hemolysis, determined as Hb release from the cells, after addition of Fenton reactants to washed RBCs with A1M-wt (220 µg/ml) or A1M-035 (220 µg/ml) after 3 h (E) and 22 h (F) of incubation. (G,H) Hemolysis, determined as LDH release from the cells, after addition of 42% H_2O (A) or 45% H_2O to washed RBCs with or without A1M-wt (220 µg/ml). Values are shown as mean ± SD and the differencse between groups were calculated using one-way ANOVA with post hoc Sidak (A-D) or unpaired two-tailed t-test (E-H). ***p < 0.001, ****p < 0.0001. Figure adapted from [25].

To further study the protective role of A1M against hemolysis, comparisons were made with the heme binding protein hemopexin (Hpx) and the antioxidant compounds Trolox (vitamin E) and ascorbic acid (vitamin C). The results showed a superior protective effect of A1M compared to hemopexin against spontaneously induced (Figure 15A), heme-induced (Figure 15B) and osmosis-induced hemolysis (Figure 15C). Addition of Trolox or ascorbic acid showed no protection against spontaneously induced hemolysis, whereas A1M significantly reduced the amount of hemolysis (Figure 15D).

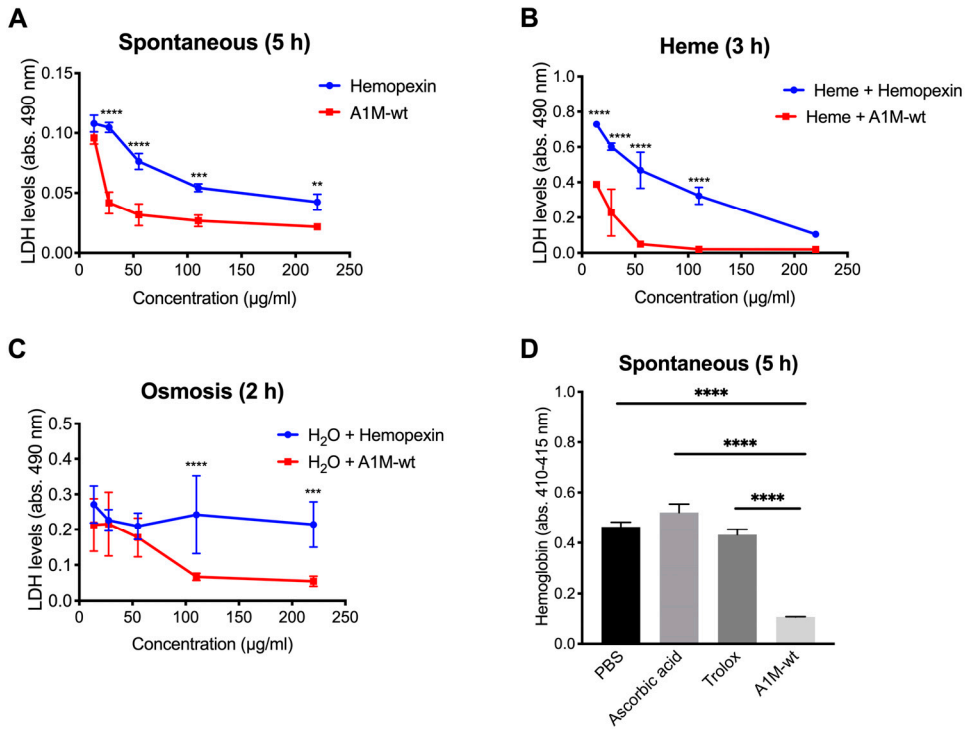

Figure 15. Comparison of protection against hemolysis between A1M, Hpx, Trolox and ascorbic acid. (A-C) Hemolysis, determined as LDH release from the cells, after addition increasing concentrations of A1M-wt or Hpx to washed RBCs exposed to spontaneous stress for 5 h (A), heme-induced stress (30 µM heme) for 3 h (B) or osmotic stress (42% H_2O) for 2 h (C). (D) Hemolysis, determined as Hb release from the cells, after incubation of washed RBCs with ascorbic acid (220 µg/ml), Trolox (220 µg/ml) or A1M (220 µg/ml) for 5 h. Values are shown as mean ± SD and the differencse between groups were calculated using one-way ANOVA with post hoc Sidak. ***p < 0.001, ****p < 0.0001. Figure from [25].

During hemolysis, Hb leaks from its protective intracellular environment into the circulation. Here, it undergoes autooxidation, generating radicals that cause damage to the membranes of nearby RBCs. Additionally, Hb is broken down, releasing its heme groups, causing further damage to the membranes of the nearby RBCs. The

generation of extracellular Hb and free heme groups creates a feed-forward loop resulting in even more hemolysis. The mechanism behind the protective effect of A1M could be due to the neutralization of the free heme groups and radicals produced by the extracellular Hb and thereby stopping the feed-forward loop. The superior protective effect of A1M when compared to hemopexin indicates the involvement of more than the neutralization of free heme groups. However, the reducing effect alone, which was examined using Trolox and ascorbic acid, did not provide any protection. It may be speculated that A1M with is combination of heme-binding, reduction and radical binding, has evolved a unique RBC protective function.

Retinal epithelial cells

In paper IV we examined the protective role of A1M against light-induced damage to retinal epithelial cells. The light-induced damage was generated by adding the photosensitizer riboflavin to the cell media followed by illumination. This produces riboflavin radicals, which can both directly oxidize cell components and generate more harmful ROS, which thereby exposes the cells to oxidative stress. Addition of A1M to the cell media prior to illumination resulted in a statistically significantly reduced expression of the cell cycle regulator p21 (Figure 16A). Reduced expression of the oxidative stress marker HO-1 (HMOX1in the Figure) failed to reach significance, but a trend was seen where increasing concentrations of A1M reduced the expression (Figure 16B).

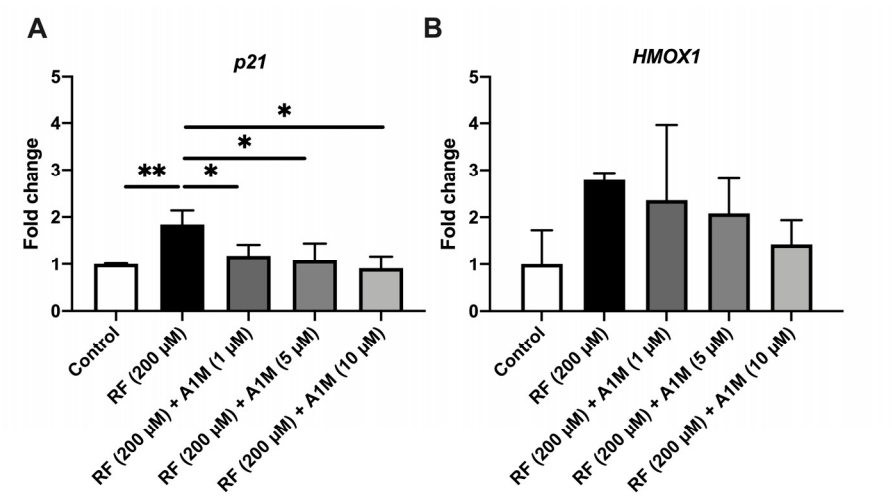

Figure 16. RT-PCR analysis of ARPE19 cells. (A,B) Relative mRNA levels of p21 and HMOX1 in ARPE19 cells after addition of riboflavin and A1M follwed by illumination. Values are shown as fold change ($2^{-\Delta\Delta Ct}$). Statistical comparisons of differences between samples were performed using one-way ANOVA with post hoc Sidak. *p < 0.05, **p < 0.01. Figure from [167].

These results suggest a protective effect of A1M against riboflavin radicals, which can be seen as a decrease in expression of stress response genes. The ability of A1M to protect against riboflavin induced damage could potentially take place in light-exposed parts of the body, such as in the skin and eyes. Both A1M and riboflavin is present in the skin and eyes, making the interaction plausible.

These results, from paper II and IV, give further knowledge into the cell protecting abilities of A1M. Two new cell types, RBCs and retinal epithelial cells, have been added to the long list of the cell types that A1M can protect against oxidative stress. Paradoxically, this might make it even more challenging to establish the main biological target cell of A1M. Contrarily, the rather promiscuous ways of A1M, regarding both its binding targets and its cell type interactions, might be due to the fact that there is no main biological target cell and that the protection is of a more general nature. However, more research on the topic is needed to reach a final conclusion.

A1M binds to heparin and heparan sulfate and can be purified from plasma using heparin Sepharose

Recently, heparin binding proteins were detected in urine using affinity chromatography and mass spectrometry [105], which included the detection of AMBP. However, since AMBP is not found in urine, it could not be determined whether it was A1M or bikunin that was detected. The authors found three heparin binding sites within the AMBP sequence, suggested to play an important role in the binding of AMBP to heparin. In paper III we further investigated the molecular interactions between A1M and heparin/heparan sulfate.

We showed that it is possible to purify A1M from human plasma using heparin affinity chromatography and size exclusion chromatography. A flowchart with the required steps is shown in Figure 19. The starting material, 400 ml human plasma diluted with 0.2M NaCl-buffer, is applied to a column with heparin Sepharose, which separates A1M from plasma proteins with no or weak affinity to heparin (Figure 19A and D). Since several other heparin binding proteins are eluted with A1M (Figure 19D), the sample is further purified using size exclusion chromatography (Figure 19B). A more purified A1M fraction is obtained, shown by SDS-PAGE and Western blotting analysis (Figure 19D). To achieve a completely pure A1M fraction, an additional size exclusion step is performed (Figure 19C).

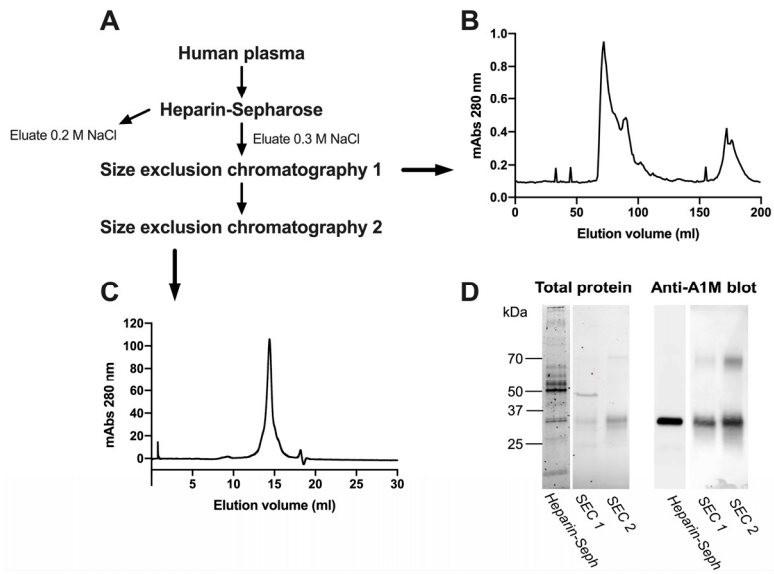

Figure 19. Purification of A1M from plasma using heparin-Sepharose and size exclusion chromatography. (A) Flowchart with the steps required for purification of A1M from plasma. (B) UV-absorbance (280 nm) of fractions eluted from the first size exclusion chromatography (SEC 1). (C) UV-absorbance (280 nm) of fractions eluted from the second size exclusion chromatography step (SEC 2). (D) Coomassie total protein stain (left panel) of heparin sepharose, SEC 1 and SEC 2. Western blot of heparin sepharose, SEC 1 and SEC 2 with rabbit anti A1M antibodies (right panel).

The nature of the interaction was further studied and found to be predominantly driven by electrostatic interactions. The binding strength was shown to be dependent on the degree of sulfation of the heparan sulfate and was suppressed by high ionic strength. The binding was further examined using hydrogen deuterium exchange mass spectrometry (HDX-MS). The HDX-MS showed two regions, amino acids 29-44 and 162-180, with reduced deuterium uptake, indicating potential binding areas. These were found to be located on the outside of the barrel shaped A1M molecule (Figure 20). In the paper described above, where the authors detected AMBP as a heparin binding protein in urine, three different heparin binding motifs were found in the AMBP molecule. Interestingly, none of these coincide with the binding regions found with HDX-MS.

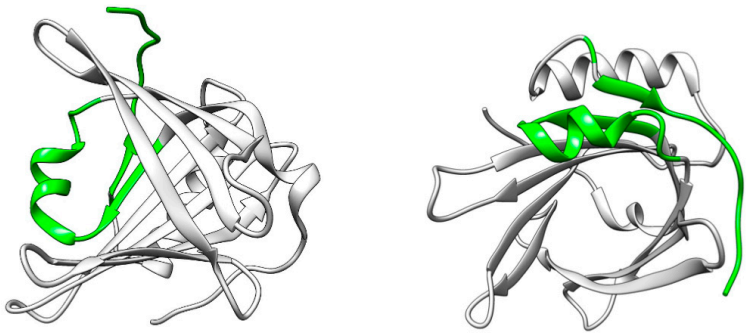

Figure 20. Front and sideview of a 3D model of A1M with potential heparin binding areas highlighted in green. The potential heparin binding areas, amino acids 29-44 and 162-180, are based on reduced deuterium uptake during hydrogen deuterium exchange mass spectrometry (HDX-MS).

Purification of A1M from plasma in large quantities is technically difficult. Affinity chromatography using specific antibodies is costly, the heterogenous charge of A1M makes ion exchange chromatography and electrophoretic methods less efficient, and the low concentration in plasma results in low yields. The heparin binding function, which is described in paper III, could represent a useful and cost-effective tool for purifying A1M from plasma. The method has several advantages compared to the methods currently used. For example, the elution of A1M from heparin is performed with NaCl, which is mild and keeps the protein functional. Purification from plasma might also be a more attractive alternative to recombinant A1M produced in E.coli for clinical drug use, since the plasma A1M is glycosylated and therefore more stable. Additionally, plasma A1M does not contain a His-tag, making it less likely to cause antigenic reactions in the body.

A1M binds to cell surfaces and is internalized

In paper III we studied the binding interaction between A1M and heparin/heparan sulfate (HS). A1M was found to bind to both heparin and HS and the binding was shown to be of electrostatic nature. HS is present on the surface of most cells in the body, making it a plausible binding target for A1M. Immunohistochemistry with antibodies directed at A1M and HS on endothelial cells revealed a co-localization (Figure 17A). The mean Pearson's correlation coefficient was calculated to 0.87, suggesting a strong correlation. When treating the endothelial cells with heparinase, which cleaves off the surface bound HS, prior to adding the antibodies, most of the A1M signal was lost (Figure 17B). Additionally, when using immunohistochemistry to analyze the co-localization of A1M and HS *in vivo* in mice, co-localization was shown in parts of the kidney, skin, lung and aorta. The binding seen *in vivo* further supports a physiological function of the interaction.

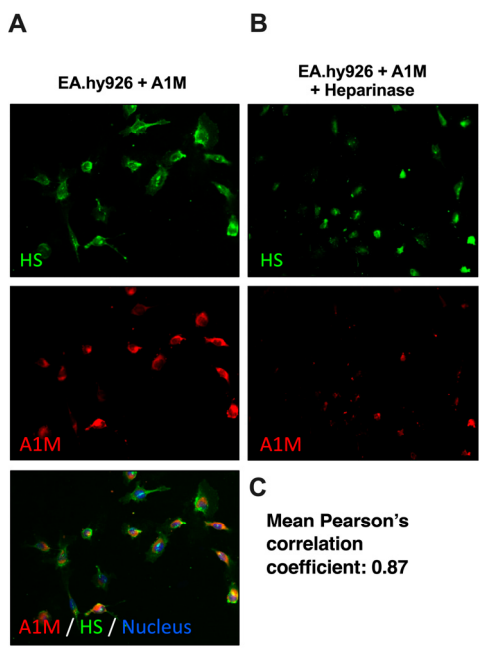

Figure 17. Fluorescence staining of endothelial cells (EA.hy926). (A) Heparan sulfate staining (green, upper panel), A1M staining (red, middle panel) and merged picture with DAPI nucleus staining (bottom panel). (B) Heparinase treatment (heparinase I and III) followed by staining with anti heparan sulfate (green, upper panel) and anti A1M (red, lower panel). (C) Pearson's correlation coefficient of the view in the merged picture.

In paper II, we used anti-A1M antibodies, with gold-labeled secondary antibodies, to detect A1M in RBCs. Using sections from a full-term placenta with RBCs, A1M was detected on both the cell surface and in the cytosol (Figure 18A), which suggests the presence of A1M in RBCs *in vivo*. Additionally, washed RBCs were incubated with A1M for 0, 3 and 7 days, fixated, sectioned and stained with anti-A1M and gold-labeled secondary antibodies. The results showed the presence of A1M both intracellularly and on the surface at all timepoints (Figure 18B).

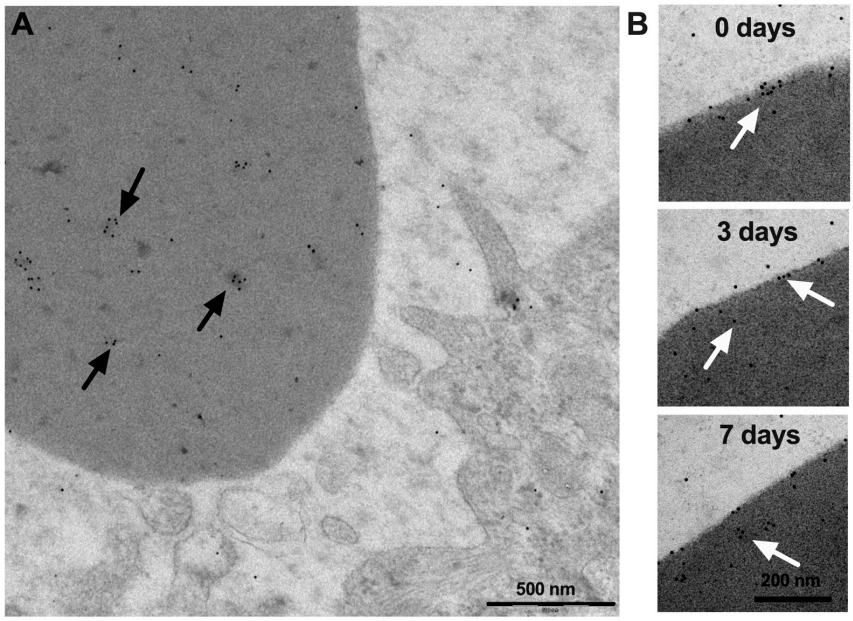

Figure 18. In vivo detection and in vitro uptake of A1M in red blood cells (RBCs). (A) Section of a full term placenta with RBCs, stained with anti A1M and gold labeled secondary antibodies. Scalebar = 500 nm. (B) Washed RBCs incubated with A1M-035 for 0, 3 or 7 days followed by fixation, embedding, sectioning and staining with anti A1M and gold labeled secondary antibodies. Scalebar = 200 nm. Figure adapted from [25].

In both paper II and III we have shown the presence of A1M both intracellularly and on the cell surface of RBCs and endothelial cells. HS has been shown to be present on the surface of RBCs [191] and endothelial cells [192]. The presence of HS on the surface on both cell types could represent a potential binding site for A1M, from where it is further internalized through an unknown mechanism. Cellular uptake of A1M has previously been shown in liver cells [169], keratinocytes [142] and blood cells [144] and the uptake was found to be increased during apoptosis and irradiation. However, the uptake mechanism has not been determined. Another possible consequence of the binding to HS on the surface could be that the surface bound A1M has the potential to protect the cells against oxidants close to the plasma membrane, thereby preventing oxidative stress and potential hemolysis. The plasma membrane is sensitive to oxidation, and hydrophobic molecules such as heme can intercalate into the cell membrane making it more susceptible to lipid peroxidation. Reduction of oxidants and/or binding of heme molecules by A1M at the cell surface could possibly represent a biological function of A1M *in vivo*.

New insights on molecular mechanisms and physiological functions of A1M

Radical-induced cleavage of A1M

As described above, riboflavin, or vitamin B2, can generate ROS when illuminated and thereby cause oxidative stress. In paper IV we studied the interaction between A1M and illuminated riboflavin. The results revealed a binding of riboflavin to A1M and a cleavage of the A1M molecule in the N-terminal region (Figure 21A). The cleavage was also seen when A1M reacted with flavin adenine dinucleotide (FAD), which has a riboflavin molecule as its functional group (Figure 21B). No cleavage was seen when the incubation took place in darkness, which indicates that the cleavage reaction mainly occurs during illumination. Cleavage of A1M has been shown previously when it reacts with the synthetic radical ABTS [116]. When reacting with ABTS, an N-terminal peptide is cleaved off, indicating a similar reaction as with riboflavin. This could potentially represent a general radical interacting mechanism where A1M is cleaved when interacting with the radicals. The mechanistic and biological function of the cleavage reaction is, however, not known and needs to be studied further.

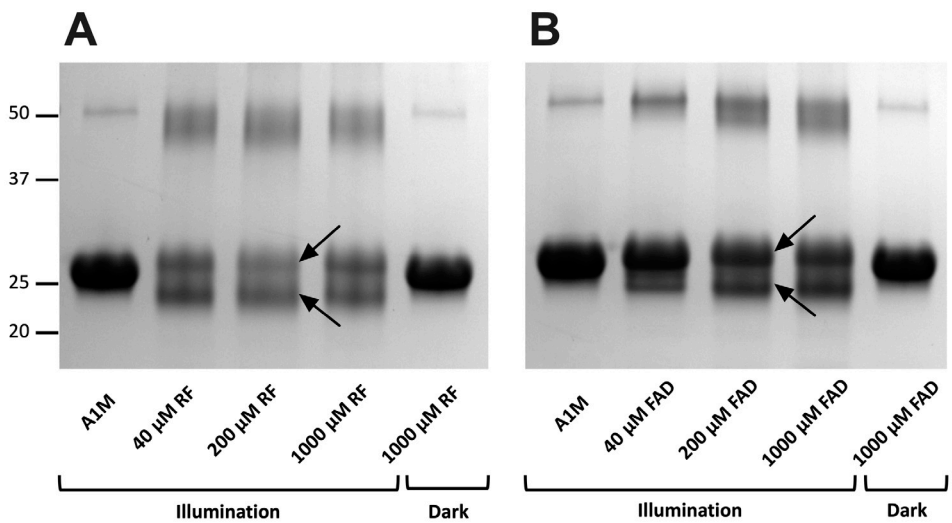

Figure 21. SDS-PAGE stained with Coomassie after incubation with (A) riboflavin and (B) flavin adenine dinucleotide (FAD). The samples were illuminated with a fluorescent lamp (1200 lumen) at a distance of 0.2 m or kept in darkness for 1 h. The upper arrows indicate the full length modified A1M and the lower arrows indicate the cleavage product. Figure from [167].

A1M regulates ER-stress and fat metabolism *in vivo*

When studying the A1M-KO mice, in paper I, some unexpected physiological changes were seen in the mice. The lack of A1M resulted in a statistically significant increase in body weight in both male and female mice (Figure 22A). Additionally, the mice showed a tendency towards increased fat accumulation in the liver (Figure 22B and C). A trend was also seen in the weight of the liver, spleen and heart, where the organs of the KO mice weighed slightly more. The underlying mechanism behind controlling the body weight of an organism is very complex and to a large extent unknown. A potential partial explanation to the increased body weight could be a general increase in organ weight, since the KO mice displayed a trend towards increased weight of the liver, spleen and heart (Figure 22D-F). The increased liver weight could be explained by an increased production of lipids due to the presence of ER-stress. ER-stress has been found to be a key contributor to the development of the metabolic syndrome, which includes increased body weight [193,194]. It could be speculated that the lack of A1M leads to ER-stress which subsequently contributes to the development of the metabolic syndrome.

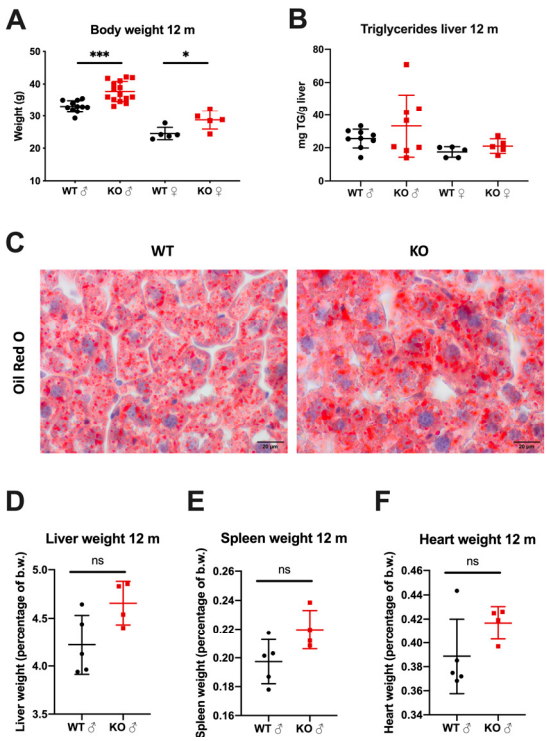

Figure 22. Analysis of body weight, liver fat and organ weights of A1M-KO and WT mice. (A) Body weight of 12-month-old KO and WT mice. Statistical comparisons between groups were performed with Mann-Whitney U tests. *p < 0.05, ***p < 0.001. (B) Triglyceride content in the livers of male and female mice at the age of 12 months. (C) Representative sections of livers from male KO and WT mice at the age of 12 months, stained with Oil Red O. Scale bars = 20 µm). (D,E,F) Weight of liver, spleen and heart as percentage of body weight from 12-month-old male KO and WT mice. Figure from [127].

A1M regulates blood cell homeostasis

Blood analysis of whole blood from A1M-KO and WT mice showed tendencies towards a lower RBC count in the KO mice, and the mean corpuscular volume (MCV), mean corpuscular Hb (MCH) and red cell distribution width (RDW) were all statistically significantly increased in the KO mice (Figure 23). No differences were found in Hb, reticulocyte percentage and hematocrit. These changes in blood values indicate a steady state macrocytic anemia phenotype. The difference in size and width distribution could be due to a disturbance in the erythrocyte development or due to an increased number of immature RBCs as a response to counteract the lower RBC count. The lack of A1M in the KO mice could potentially lower the protection against hemolysis, which would explain the lower RBC count. This further strengthens the potential physiological role of A1M as a protector of RBCs *in vivo*.

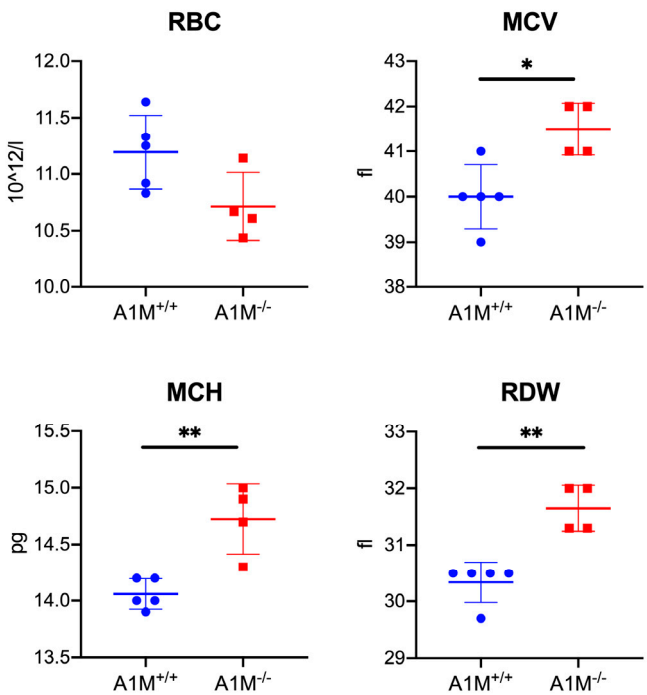

Figure 23. Morphology of RBCs in A1M-KO mice. Samples of whole blood from 12-month-old KO (n = 4) and WT (n = 5) mice were analyzed using a blood analyzer (Abacus Vet5 Diatron). The plots show RBC count, mean corpuscular volume (MCV), mean corpuscular Hb (MCH) and red cell distribution width (RDWs). Figure adapted from [25].

Conclusions

The central aim of this thesis was to gain new knowledge about the different potential biological roles of A1M. The four studies included in the thesis have provided new perspectives on the topic.

In paper I, a previously unknown role of A1M as a chaperone during bikunin synthesis was discovered. The chaperone function of A1M might also partially explain the co-expression of A1M and bikunin. The results in paper I also touched on a new field in the A1M research, namely obesity and the metabolic syndrome. More research is needed, but hopefully, follow up studies on the A1M-KO mice could perhaps lead to a novel method in the battle against human obesity.

Paper II revealed a new role of A1M as a protector of RBCs against various forms of stress. Additionally, the presence of A1M intracellularly and on the RBC membrane was shown *in vivo*. When comparing the protective effect of A1M against other antioxidants or the heme binding protein hemopexin, A1M displayed superior protection, indicating a unique protective function of A1M. The results suggest that protection of RBCs in the blood stream is a plausible biological role of A1M.

In paper III, the newly discovered ability of A1M to bind to heparin was studied in more detail. The heparin binding ability possibly represents a biological anchoring mechanism used by A1M to extravasate from the blood and/or bind to the surface of cells to protect them against oxidants or for internalization of A1M. Additionally, the heparin binding ability represents a useful tool for plasma purification of A1M

Paper IV showed a new role of A1M as a protector against radicals induced through the illumination of riboflavin. The presence of both riboflavin and A1M in light exposed parts of the body suggests that the interaction is biologically relevant.

Altogether, these studies provide additional information surrounding the multi-faceted functions of A1M simultaneously placing them in a biologically relevant context. Further research will hopefully lead to a more definitive answer to the question regarding the biological role of A1M and finally result in the development of A1M as a pharmaceutical.

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