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Haptoglobin and CD163 in Aspects of Disease

PhD thesis by

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Preface

This PhD thesis covers the main results obtained during my PhD in the period from February 2020 to April 2023 at the Department of Molecular Medicine, University of Southern Denmark. The work was conducted in the Moestrup-Graversen group at the Cancer and Inflammation Research Unit as a natural extension of my education as an MSc of Pharmaceutical Sciences at the University of Southern Denmark. The PhD project was part of the Center of Excellence ATLAS that aims to understand hepatic and adipose tissue plasticity in the context of metabolic diseases including non-alcoholic fatty liver disease.

During the course of my PhD, I learned that planning is very important, but adaption is even more important. Just one month into my PhD, the Covid-19 pandemic hit, and the lab was closed for several months. Luckily, Søren K. Moestrup encouraged me to write a review about CD163 and macrophage polarization and thereby made the best of the situation. In December 2021, life took a turn when surgical complications instantly carried a three-month sick leave, and all experiments were terminated. I'm beyond grateful for the indispensable help from lab technician Signe who undertook the full management of the animal studies during this time. Further, I sincerely appreciated the immense support I received from all my colleagues, friends, and family in that period.

I would like to thank my main supervisor Søren K. Moestrup for the opportunity to conduct my PhD under his wings. You have challenged, supported, and encouraged me during my scientific growth which I value highly. I have sincerely appreciated your guidance in the writing process of papers and learned a lot about scientific communication. A special thanks to my co-supervisor Jonas H. Graversen for daily supervision and always having an open door for discussions and sharing your practical knowledge. I am thankful for the excellent technical assistance from Signe M. Andersen and Malene B. Jørgensen, especially with the handling of mice. You have been a joy to work with and have been my rocks through the last three years. I would also like to express my thankfulness to the Hybridoma lab and the FACS core facility at the Department of Molecular Medicine. A special thanks to Lars Vitved, Søren Hansen, Anouk Benmamar-Badel, and Oriane Cédile for guiding me on the steep learning curve of flow cytometry, and to Maiken Henriksen for sharing your wide knowledge on ELISA establishment. In addition, I would like to thank all the former colleagues and students in the Moestrup-Graversen lab for the great collaborations and a wonderful work environment.

A sincere thanks to Kim Ravnskjær for guiding me on murine models of NAFLD and inviting me into your ingenious research group for two months. I am extremely grateful for the profound introduction to bioinformatics that I received from Frederik T. Larsen. I have highly valued being a part of ATLAS and appreciated working in a wide scientific field from basic to clinical science with many talented researchers. I want to highlight the collaboration with Charlotte Wernberg and Mette Lauridsen from Esbjerg Hospital who collected plasma and liver biopsies from obese patients and agreed to investigate CD163 further in these patients. I genuinely appreciated our collaboration. Also, a special thanks to pathologist Sönke Detlefsen for training me in liver histology and helping me quantify CD163.

Finally, to my family and friends, thank you for supporting me throughout the past three years. I wouldn't have completed this PhD without your important contribution to a healthy work-life balance through physical activity and dinners, and joyful plays with my beloved niece and nephews. A special thanks to my parents, who encouraged me throughout life to have a critical and curious approach to everything that has led me to the researcher I am today. Thank you for the scientific discussions, graphical assistance, and great statistical assistance. Last but certainly not least, my partner Mads. Thank you for your patience and support. I am blessed to have you by my side.

Maria K. Skytthe, Odense 2023

Table of content

List of publications

This PhD thesis is based on the following manuscripts:

Paper I:

Skytthe, MK., Sørensen, AL., Hennig, D., Sandberg, MB., Rasmussen, LM., Møller, HJ., Skjødt, K., Graversen, JH., Moestrup, SK. *Re-evaluation of the measurement of haptoglobin in human plasma samples.* Scand J of Clin Lab Invest. 2022 Oct;82(6):467-473. (https://doi.org/10.1080/00365513.2022.2122077)

Paper II:

Skytthe, MK., Sørensen, AL., Hennig, D., Sandberg, MB., Rasmussen, LM., Højrup, P., Møller, HJ., Skjødt, K., Moestrup, SK., Graversen, JH. *Haptoglobin-related protein in human plasma correlates to haptoglobin concentrations and phenotypes.* Scand J of Clin Lab Invest. 2022 Oct;82(6):461-466. (https://doi.org/10.1080/00365513.2022.2122076)

Paper III:

Skytthe, MK., Pedersen, FB., Wernberg, CW., Chandran, VI., Krag, A., Caterino, TD., Mandacaru, SC., Blagoev, B., Lauridsen, MM., Detlefsen, S., Graversen, JH., Moestrup, SK. *Obese Patients with Nonalcoholic Fatty Liver Disease Have an Increase in Soluble plasma CD163 and a Concurrent Decrease in Hepatic Expression of CD163.* Gastro Hep Advances. 2023 Mar. (https://doi.org/10.1016/j.gastha.2023.03.006).

Paper IV:

Skytthe, MK., Graversen, JH., Moestrup, SK. *CD163 expression protects against early development of hepatic steatosis in male western diet-fed mice.* 2023. Manuscript in preparation.

Additional contributions during the PhD not included in the thesis:

Skytthe, MK., Graversen, JH., Moestrup, SK. *Targeting of CD163^{+} Macrophages in Inflammatory and Malignant Diseases.* Review. Int J Mol Sci. 2020 Jul 31;21(15):5497. (https://doi.org/10.3390/ijms21155497)

Chandran, VI., Wernberg, CW., Lauridsen, MM., **Skytthe, MK**., Bendixen, SM., Larsen, FT., Hansen, CD., Grønkjær, LL., Siersbaek, MS., Caterino, TD., Detlefsen, S., Møller, HJ., Grøntved, L., Ravnskjaer, K., Moestrup, SK., Thiele, MS., Krag, A., Graversen, JH. *Circulating TREM2 as a noninvasive diagnostic biomarker for NASH in patients with elevated liver stiffness.* Hepatology. 2022 Jun 16. (https://doi.org/10.1002/hep.32620)

Chandran, VI., Wernberg, CW., Lauridsen, MM., **Skytthe, MK**., Moestrup, SK., Krag, A., Graversen, JH. *Use of soluble TREM2 as a non-invasive biomarker for non-alcoholic steatohepatitis (NASH).* 2022. Patent no. WO2022248363A1.

Abstract

Extracellular hemoglobin (Hb) is rapidly captured by haptoglobin (Hp) in the circulation to prevent oxidative stress and tissue damage. This tight complex is recognized and endocytosed by the macrophage-specific scavenger receptor CD163 leading to lysosomal degradation of the Hp-Hb complex and reuse of the components maintaining iron homeostasis. The aim of this thesis is to explore the proteins within this Hb scavenging system in relation to aspects of pathogenesis and their utility in diagnosis of intravascular hemolysis and non-alcoholic fatty liver disease (NAFLD).

During excessive hemolysis, Hp is virtually undetectable in plasma because the Hp-Hb complexes are degraded and the Hb scavenging capacity is exhausted. Thereby low Hp levels are used in the clinic as a biomarker of hemolysis. The clinical analytical methods rely on epitope-recognition which is challenged by the presence of Hp-related protein (Hpr) with the same epitopes and by complex structural differences between Hp phenotypes. Therefore, specific assays that differentiated between Hp and Hpr were generated, and phenotype-specific calibrators were used to overcome the oligomerization challenge. This uncovered a significant bias using unmatched calibrators especially in patients with the Hp1-1 phenotype (***Paper I***), but also revealed that the presence of Hpr has negligible influence on the measurements. Furthermore, the data showed that the concentration of Hpr correlated significantly with Hp phenotype and plasma concentrations. In fact, Hpr was also reduced during hemolysis concurrently with Hp (***Paper II***). Finally, the data provided evidence for a model explaining why Hp1-1 individuals generally have a lower Hp concentration compared to Hp2-1 and Hp2-2 individuals.

A soluble form of CD163 (sCD163) is released to the circulation during macrophage activation which has been associated with various inflammatory conditions such as non-alcoholic steatohepatitis (NASH), which is the progressive form of NAFLD. Increased plasma levels of sCD163 confirmed sCD163 as a potential biomarker of NASH in a unique cohort of obese patients before bariatric surgery. Matched liver biopsies revealed a concurrent reduction in CD163 expression in more severe NAFLD (***Paper III***). This negative association was further investigated in western diet-fed mice. As steatosis intensified the expression of CD163 decreased in the liver and CD163 deficiency exacerbated hepatic steatosis in early stages of steatosis suggesting a protective role of CD163 in fat deposition in the liver. Overall, this indicates a pathological importance of macrophages in the development of NAFLD that potentially can be used in therapy (***Paper IV***).

Resume

I blodkredsløbet binder haptoglobin (Hp) hurtigt ekstracellulært hæmoglobin (Hb) for at forhindre oxidativ stress og vævsskade. Dette stærke kompleks genkendes og optages af den makrofag-specifikke receptor CD163, hvorefter det nedbrydes, hvilket gør, at jern kan genbruges. Formålet med denne afhandling er at undersøge proteinerne i dette Hb scavenger-system i forhold til forskellige aspekter af sygdomsudvikling og deres anvendelighed i diagnosticering af intravaskulær hæmolyse og ikke-alkoholisk fedtleversygdom (NAFLD).

Ved betydelig hæmolyse kan Hp ikke måles i en blodprøve, da Hp-Hb komplekserne nedbrydes og opbruger derved kroppens Hb fangende kapacitet. Lave Hp plasma niveauer bruges derfor som en markør for hæmolyse i klinikken. Den analytiske metode der benyttes, afhænger af epitop genkendelse og er derfor følsom overfor tilstedeværelse af Hp-relateret protein (Hpr) og strukturelle forskelle mellem Hp fænotyperne. Derfor blev specifikke målemetoder udviklet der kan differentiere mellem Hp og Hpr og fænotype-specifikke standard benyttes for at overkomme udfordringerne med oligomerisering af Hp. Dette viste, at brug af en uparret standard medførte en signifikant bias især i patienter med Hp1-1 fænotype (***Paper I***) og, at tilstedeværelsen af Hpr var underordnet og påvirkede ikke målingerne signifikant. Derudover korrelerede koncentrationen af Hpr signifikant med Hp fænotype og plasmakoncentration. Hpr faldt også under hæmolyse, samtidig med at Hp faldt (***Paper II***). Endelig blev en forklaring på hvorfor man generelt ser en lavere Hp koncentration hos person med Hp1-1 fænotyper ift personer med Hp2-1 eller Hp2-2 præsenteret.

En opløselig form af CD163 (sCD163) frigives til cirkulationen i forbindelse med makrofag aktivering. Øget plasmakoncentration af sCD163 er associeret til en række inflammatoriske sygdomme blandt andet ikke-alkoholisk steatohepatitis (NASH), som er den progressive form af NAFLD. I en kohorte af overvægtige patienter bekræftede øget plasmakoncentration af sCD163 blev sCD163 som en mulig ny biomarkør for NASH. Derimod faldt udtrykket af CD163 i parrede lever biopsier i takt med sværere grad af NAFLD (***Paper III***). Denne negative association blev yderligere undersøgt i mus der blev fodret med en vestlig diæt. I takt med at mere fedt akkumulerede i leveren faldt det hepatiske udtryk af CD163. Derudover forværrede manglen af CD163 udviklingen af steatose i leveren i de tidlige stadier hvilket antyder en beskyttende effekt af CD163 i fedtdeponering. Dette indikerer samlet set at makrofager besidder en vigtig patologisk rolle i udviklingen af NAFLD, som potentiel kan udnyttes i behandling af sygdommen (***Paper IV***).

Abbreviations

ADAM17	Tumor necrosis factor α-converting enzyme
ADC	Antibody drug conjugate
ALT	Alanine transaminase
AST	Aspartate transaminase
AUROC	Area under the receiver operating characteristic
BMI	Body mass index
CCP	Complement control protein
$CD163^+$	CD163-positive
EMA	European Medicines Agency
FDA	U.S. Food and Drug Administration
Hb	Hemoglobin
HCC	Hepatocellular carcinoma
hCLS	Hepatic crown-like structure
Hp	Haptoglobin
Hpr	Haptoglobin-related protein
IL	Interleukin
KC	Kupffer cells
LAM	Lipid-associated macrophages
LPS	Lipopolysaccharide
LNP	Lipid nanoparticle
metHb	Methemoglobin
NAFLD	Non-alcoholic fatty liver disease*
NAS	NASH activity score
NASH	Non-alcoholic steatohepatitis
NO	Nitric oxide
RBC	Red blood cell
sCD163	Soluble CD163
SRCR	Scavenger receptor cysteine-rich
sTREM2	Soluble TREM2
TAM	Tumor-associated macrophages
TLF-1	Trypanosome lytic factor 1
TNF-α	Tumor necrosis factor alpha
TREM2	Triggering Receptor Expressed on Myeloid Cells 2

* In 2020 a new consensus nomenclature based on inclusion rather than exclusion was proposed [1]. Different new names are suggested including metabolic-associated fatty liver disease (MAFLD). However, in this thesis the former acronyms NAFLD and NASH will be used.

Introduction

Rapid scavenging of free hemoglobin (Hb) in the circulation is important to retain iron and prevent oxidative tissue damage by heme. Haptoglobin (Hp) is one of the Hb-scavenging proteins present in human circulation that averts its oxidative activity and facilitates CD163-mediated Hb clearance by macrophages. The aim of this thesis is to explore proteins in this scavenging system in relation to pathogenesis and their utility in diagnostics and therapy. The following sections will elaborate on the CD163-mediated Hp-Hb scavenging system and further expand on its use in context of diagnosis and treatment of diseases.

CD163-mediated scavenging of the haptoglobin-hemoglobin complex

Trivial hemolysis of aging red blood cells (RBCs) leading to release of free Hb to plasma is a physiologic phenomenon [2]. Hb is a tetrameric metalloprotein, which comprise two pairs of α- and β-subunits which each contain a heme group that coordinates an iron ion in a porphyrin ring essential for oxygen-transport within RBCs. Free Hb is deleterious due to its high redox potential where the ferrous iron (Fe^{2+}) in heme is prone to autooxidation and oxidation by physiological oxidants (such as hydrogen peroxide) to the ferric (Fe^{3+}) state generating highly reactive radicals and methemoglobin (metHb) [3, 4]. In addition, oxidation to the ferryl (Fe^{4+}) state results in the formation of oxyferrylHb and ferrylHb accompanied by globin radicals or superoxide and release of free iron ions, respectively [4]. Oxidized heme (hemin) is more avidly dissociated from metHb and oxyferrylHb in circulation and thereby contribute to the formation of toxic radicals through redox reactions as well as increasing membrane permeability by intercalating into lipid bilayers due to its amphiphilic nature [5-7]. Free radicals and hemin intercalation enables lipid peroxidation ultimately leading to ferroptosis [8, 9]. Furthermore, free heme induce a NF-κB mediated inflammatory response releasing cytokines and chemokines and enhance the expression of adhesion molecules. In addition, it activates the complement system and recruits and increases infiltration of neutrophiles [10]. Free Hb also affects vasculature by reacting with and limiting the availability of nitric oxide (NO) leading to vasoconstriction [6].

Multiple innate scavenging systems prevent the toxic effect of free Hb and heme (**Figure 1A**). The first line of defense against free Hb is facilitated by efficient Hp binding and CD163-mediated endocytosis of the Hp-Hb complex [11]. Hp is an abundant plasma protein that binds dimeric Hb with a very high affinity [12, 13]. The Hp-Hb complex formation protects Hb from oxidation and prevents

heme release, renal filtration, and NO consumption [14-16]. Furthermore, it facilitates rapid uptake by monocytes and macrophages via CD163-mediated endocytosis whereupon the Hp-Hb complex dissociates from CD163 and is degraded (**Figure 1B**) [11]. Heme is released to the cytosol and degraded by heme oxygenase 1 releasing ferrous iron (Fe^{2+}), carbon monoxide and biliverdin which is further reduced to bilirubin [17]. The free iron ions are bound by iron ligating proteins such as ferritin intracellularly and transferrin extracellularly. Meanwhile, CD163 is recycled to the cell surface [18]. Besides hindering Hb toxicity, this process also maintains iron homeostasis. Another important system is the hemopexin-CD91 heme-scavenging system that detoxifies free heme released by metHb mediating CD91-dependent endocytosis by hepatocytes and macrophages [19, 20]. To some degree this system should be regarded as a back-up system, when Hp is consumed and Hb releases heme in plasma (see below). This has been shown in mice with double knockout of the Hp and hemopexin genes [21]. In addition, haptoglobin-related protein (Hpr), a plasma protein 90% homologues to Hp, also binds Hb with high affinity [12]. However, this complex is not recognized by CD163, and its physiological clearance mechanism is currently unknown. Instead, Hpr takes part in the trypanosome lytic factor protein complex which is important in innate immunity against subtypes of *Trypanosoma brucei* infections [22].

Under normal physiological circumstances the Hp-Hb scavenging system is sufficient. However, in connection to some pathological conditions severe intravascular hemolysis releases excessive amounts of free Hb that saturate the scavenging systems and cause inflammation and tissue damage [16]. Such conditions include sickle cell anemia, autoimmune hemolytic anemia, and infections such as malaria. Although abundant in plasma, the Hp pool is drained by the disproportionate amount of free Hb. Consequently, the plasma level of Hp is drastically reduced and is therefore used in the diagnosis of hemolytic anemia [23, 24]. This leads to accumulation of free Hb and heme in plasma which cause oxidative damage, NO depletion and systemic inflammation as described above (**Figure 1C**). The kidneys are particularly susceptible to Hb-induced organ dysfunction because they compose the primary clearance route of free Hb [3]. Moreover, the liver is prone to iron overload of hepatocytes and Kupffer cells (KC) during excessive intravascular hemolysis, which is associated with formation of fibrosis that may advance to cirrhosis and hepatocellular carcinoma (HCC) [25-28].

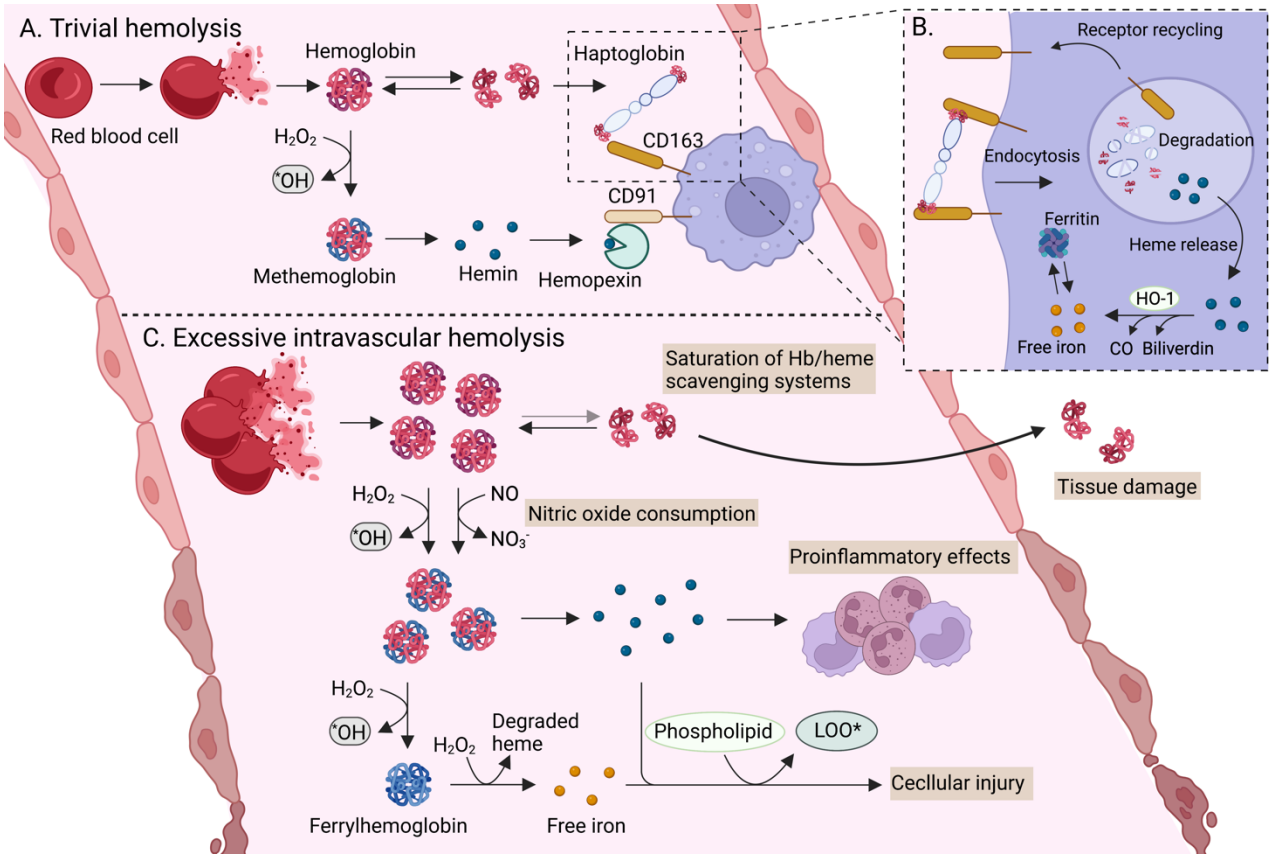

Figure 1: Mechanism of innate handling and toxicity of cell-free hemoglobin (Hb) under trivial and excessive intravascular hemolysis. A) Trivial hemolysis of red blood cells releases Hb which is dissociated into dimeric Hb or oxidized to methemoglobin that releases hemin. The innate Hb scavenging systems, haptoglobin-CD163 and hemopexin-CD91, capture free Hb or hemin avoiding Hb toxicity. B) Upon Hb binding, haptoglobin (Hp) is endocytosed by macrophages after CD163 recognition. Hp and Hb is degraded releasing heme while CD163 is recycled to the cell surface. Heme is further metabolized by heme oxygenase 1 in the cytosol liberating iron, carbon monoxide and biliverdin. C) Excessive intravascular hemolysis saturates the Hb scavenger systems causing production of reactive oxidative species, vasoconstriction through nitric oxide depletion, inflammation, and cellular injury in consequence of lipid peroxidation. This figure is inspired by [3] and created with BioRender.com.

Haptoglobin and haptoglobin-related protein

Hp is an abundant plasma protein with normal plasma concentrations in the range of 0.5-3 g/L [16]. Apart from being a marker of hemolysis, it is also an acute phase protein as the concentration increases in response to IL-6 and other cytokines [29, 30]. Hpr is less abundant than Hp with previously reported concentration of 30-50 mg/L [31, 32]. Exact measurement of Hpr concentration is complicated by high sequence homology and low presence of Hpr compared to Hp in human plasma.

Hp and Hpr are very similar with only 29 amino acids distinguishing them. Each protein is encoded by a single gene that encodes an α- and a β-chain that forms covalently joined heterodimers. The α chain comprises either one or two complement control protein (CCP) domains dependent on the genetic Hp allele (Hp1 or Hp2) in humans. Hence, three major phenotypes of Hp are present in humans – Hp1-1, Hp2-1, and Hp2-2. The CCP domains are responsible for oligomerization of the α,β-heterodimers through a beta-strand swap and a disulfide bond(s) in Hp and by a beta-strand swap alone in Hpr [13]. This enables formation of Hp dimers in individuals with Hp1-1 and Hp2-1 phenotype and multimers in individuals with the Hp2-1 and the Hp2-2 phenotype (**Figure 2A**). Hpr forms dimers because it only has one CCP domain in the α-chain like Hp1. The β-chain comprises an inactive serine protease that take part in the strong non-covalent binding of dimeric Hb in both Hp and Hpr [13]. Only the Hp-Hb complex is subsequently recognized by the scavenger receptor CD163 which is facilitated by the surface exposed loop extension, loop 3 in Hp [33] (**Figure 2B**). The simultaneous binding to several receptors increases functional affinity (i.e. avidity) which probably explains the less preferable binding of the Hp-Hb complex to the soluble form of CD163 compared to CD163 molecules displayed on the cell surface [34]. The absent binding of Hpr to CD163 is explained by two important amino acid substitutions in loop 3 (R252T and K262W). In addition, Val-259 is replaced by a cysteinylated cysteine that potentially hinders binding sterically [33].

The N-terminal signal peptide is retained in Hpr but not in Hp, and this mediates assembly of Hpr into the trypanosome lytic factor 1 (TLF-1) [35, 36]. In complex with Hb, Hpr functions as a ligand for the *T. brucei* Hp-Hb receptor [22, 37]. The parasite is unable to produce heme and relies on scavenging it from Hb complexes, but the receptor is unable to distinguish between Hp-Hb and Hpr-Hb, also when it is a part of the TLF-1 complex [22]. The trypanolytic activity of TLF-1 is subsequently mediated by apolipoprotein L-I which forms anionic pores in the lysosomal membrane of the parasite [38-40]. This system gives rise to the innate immunity against *T. b. brucei*, however, the sub-species *T. b. gambiense* and *T. b. rhodiesuense* are resistant and can thus cause African sleeping sickness in primates [40, 41].

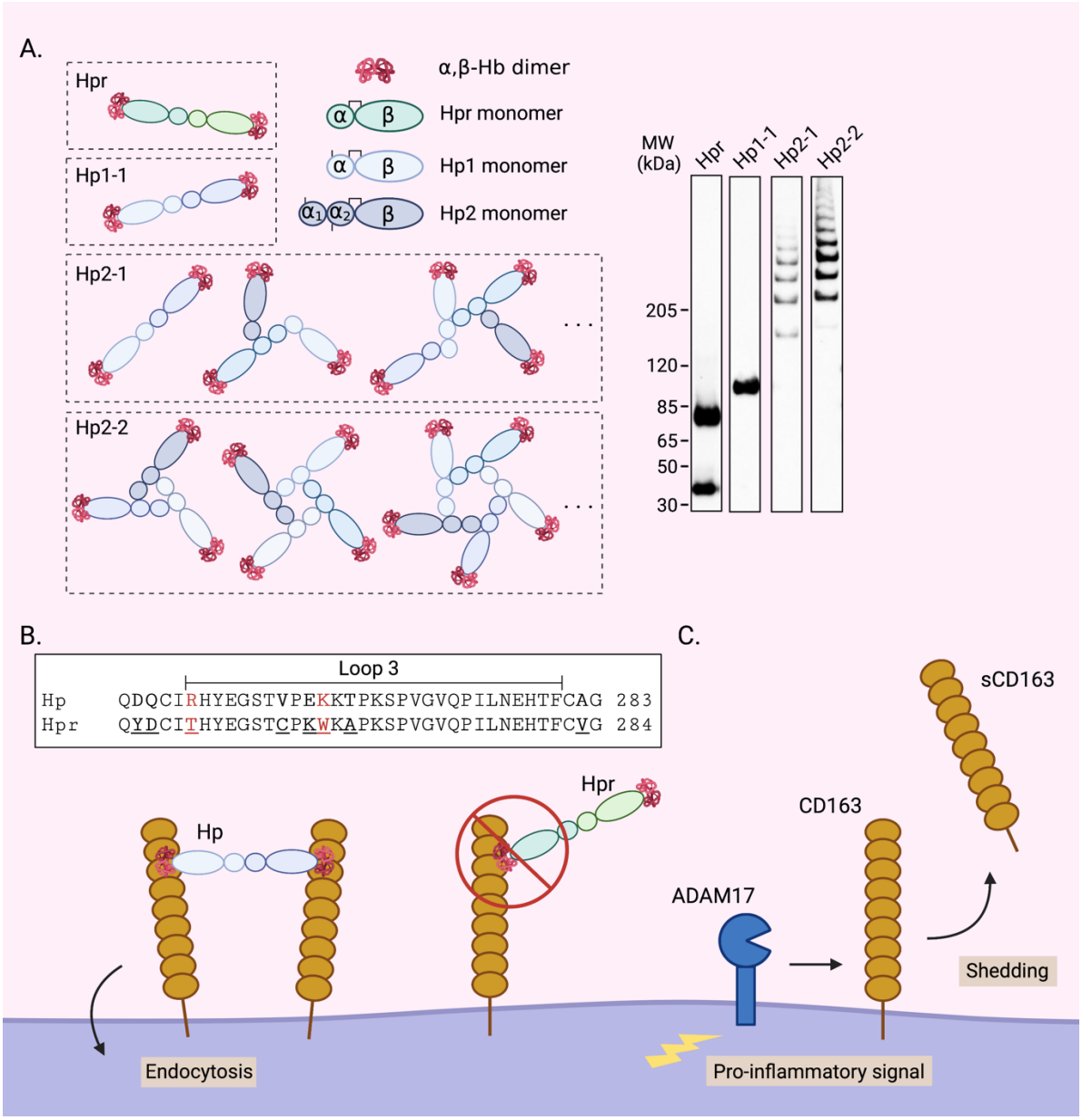

Figure 2: Structure of haptoglobins and CD163 scavenging and release. A) Two major Hp gene variants (Hp1 and Hp2) which encode one or two α-domains that give rise to three structurally different phenotypes through beta-strand swap between two independent CCP domains forming a fusion domain. Thus, Hp1-1 forms dimers (~100 kDa) and Hp2-2 forms circular oligomers (>180 kDa) whereas Hp2-1 forms dimers and linear oligomers. Structurally Hpr resembles Hp1-1 dimers. The α and β chains are linked covalently in both Hp and Hpr. However, only Hp oligomers are connected covalently between the α,β-monomers. In western blotting, this results in a single band of Hp1-1 dimers at 100 kDa while Hpr displays a band at 45 kDa reflecting the α,β-monomers (recombinant Hpr is not completely processed into α- and β-chain in the recombinant expression system resulting in dimeric Hpr in the presented western blot). B) CD163 binds Hp-Hb complexes, but not Hpr-Hb, in a calcium dependent manner and mediates endocytosis of the complex. Hpr-Hb is not recognized due to two essential amino acid replacements in loop 3 compared to Hp. C) In response to proinflammatory stimuli, ADAM17 cleaves CD163 from the cell-surface releasing soluble CD163 (sCD163) to the circulation. Created with BioRender.com.

CD163

CD163, the endocytic Hp-Hb scavenger receptor, belongs to the scavenger receptor cysteine-rich (SRCR) protein family and is exclusively expressed by monocytes (low expression) and macrophages (high expression). Structurally, it is comprised of nine extracellular SRCR domains, a transmembrane region and a short cytoplasmic tail [42]. Binding of the Hp-Hb complex is calcium-dependent and relies on the calcium-coordinated acidic triads in SRCR domain 2 (Asp-185, Asp-186 and Glu-252) and domain 3 (Asp-292, Asp-293 and Glu-359) interacting with basic amino acids (Arg-252 and Lys-262) in loop 3 of Hp [43]. Individually, Hb and Hp show weak or no binding to CD163 in humans [11, 33], whereas in mice CD163-mediated clearance of Hb is independent of Hp [44]. Besides Hp-Hb, CD163 also binds to tumor necrosis factor-like weak inducer of apoptosis (TWEAK) [45] and haptoglobin-bound HMGB1 [46]. CD163-expressing macrophages are associated with an anti-inflammatory function and CD163 is used as a marker of M2-like macrophages although not all M2-like macrophages express it [47]. *In vitro*, CD163 expression is upregulated by glucocorticoids, IL-6, IL-10, and heme/Hb and downregulated by lipopolysaccharide (LPS), TNF-α, INF-γ and GM-CSF. The promotive effect of glucocorticoids has been verified in humans [48-50] and several glucocorticoid receptor binding sites are located in the promoter region of the CD163 gene [51].

Inflammation induces shedding of CD163 and release of soluble CD163 (sCD163) to the circulation [52]. The membrane-bound metalloproteinase tumor necrosis factor α-converting enzyme (TACE/ADAM17) recognizes an essential palindromic sequence (Arg-Ser-Ser-Arg) in the juxta membrane region and rapidly cleaves the ectodomain (domain 1-9) as it does for TNF-α (**Figure 2C**) [53, 54]. Murine CD163 lacks this palindrome that hinders ADAM17-mediated release of sCD163 [54]. Relatively high levels of sCD163 (0.9-4.0 mg/L) are seen in healthy individuals as sCD163 continuously is shedded at a certain extent [52]. Owing to the increased shedding during inflammation, sCD163 serves as a long-circulating surrogate marker of inflammation as sCD163 remains elevated 24 hours after LPS challenge in humans. In contrast, TNF-α which also is released by ADAM17 cleavage, is cleared within 3 hours [53]. The physiological role of sCD163 is still unclear but Hp-Hb scavenging is unlikely due to poor binding [34].

CD163 in inflammatory diseases

In general, macrophages are involved in regulation of inflammation by releasing cytokines, chemokines, and growth factors to the microenvironment as well as clearing debris and facilitating tissue repair. In the early stages of inflammation, infection and tissue injury, macrophages recruit and activate hematopoietic and non-hematopoietic cells to mediate a proinflammatory response. At later

stages, the macrophages mediate an anti-inflammatory response to restore tissue homeostasis [55, 56]. This is possible because macrophages are highly plastic and heterogenous cells that comprise a spectrum of phenotypes beyond the traditional dichotomous classification of M1 (proinflammatory) and M2 (anti-inflammatory) macrophages, observed after *in vitro* maturation of macrophages[57, 58].

Chronic inflammation triggers imbalances and changes in macrophages that are seen in a range of diseases. For instance, adipose tissue macrophages shift from a M2-like to a M1-like phenotype in obesity contemporary with an increased infiltration of monocyte-derived M1-like macrophages [59]. The significant accumulation of proinflammatory macrophages surrounds dying adipocytes following hypertrophy-induced hypoxia and secrete proinflammatory cytokines (TNF-α, IL1β, IL-6) that induce systemic insulin resistance and thereby the metabolic syndrome [60]. Obesity, and inflammation in general, also influences iron homeostasis by increasing hepcidin levels [61] and thereby withhold iron within macrophages which promotes M1-like macrophage polarization [62]. Stainable hepatic iron stores, especially in macrophages, are associated with advanced histological features of non-alcoholic fatty liver disease (NAFLD) including fibrosis [63, 64]. In addition, iron accumulation increases production of reactive oxygen species, induce lipid peroxidation and even KC apoptosis [65].

Both CD163-positive ($CD163^+$) macrophages and levels of sCD163 in the circulation increase in several infectious disease, acute and chronic non-infectious inflammation as well as malignant diseases [66]. For instance, $CD163^+$ macrophages accumulate in the synovial membrane of affected joints in rheumatoid arthritis [67, 68] and sCD163 levels increase in sera and in particular in the synovial fluid [69, 70]. In hemophagocytic lymphohistiocytosis, which is characterized by excessive and uncontrolled macrophage activation, peripheral blood mononuclear cells as well as splenic macrophages highly express CD163 and serum levels of sCD163 are significantly increased [71, 72]. In malignant diseases, specialized tumor-associated macrophages (TAMs) form part of the microenvironment promoting tumor growth and metastasis [73, 74]. TAMs often express CD163 that exhibit a pro-tumorigenic phenotype which consequently is associated with poor prognosis, metastasis, and reduced overall survival [66]. The regulation of CD163 expression during inflammation may suggest that CD163 plays a yet unknown role in the inflammatory response beyond Hb scavenging. Indeed, a handful of animal studies using CD163 deficient ($CD163^{-/-}$) mice further supports this. For instance, CD163-deficiency increases the inflammatory response and mortality following LPS exposure [75], increases plaque formation in atherosclerosis [76] and exacerbates collagen-induced arthritis [77]. CD163-deficency has also shown beneficial effects such as delayed

injuries after intracerebral hemorrhage [78] and abrogated tumor growth and extended survival in murine sarcoma [79].

The use of sCD163 as a biomarker has been intensively studied in liver diseases due to the liver containing the largest pool of CD163 positive macrophages (Kupffer cells). In acute and chronic liver diseases plasma levels of sCD163 are increased in relation to disease severity [80-82]. In hepatitis B and C viral infection [83, 84], primary sclerosing cholangitis [85] and NAFLD [86-89] concentrations of sCD163 are moderately increased and associated with increasing hepatic inflammation and fibrosis. The degree of hepatic fibrosis is the main predictor of poor outcome in liver diseases [90, 91] and is the result of prolonged wound-healing where immune cells and injured hepatocytes activate hepatic stellate cells (myofibroblast-like cells) that produce and deposit extracellular matrix in the parenchyma [92]. Fibrosis can advance to cirrhosis which is eventually lethal and involves complications including variceal bleeding and ascites driven by portal hypertension. Elevated plasma concentration of sCD163 has been shown to predict cirrhosis-associated complications [93-95]. In addition, sCD163 levels are more dramatically increased (up to 10-fold) in acute liver injuries such as alcoholic hepatitis, acute liver failure and acute-on-chronic liver failure. Consequently, sCD163 has been suggested as an independent predictor of mortality [96-98]. The origin of sCD163 is not known with certainty but liver macrophages are likely the source [99] because higher concentrations of sCD163 were measured in the hepatic vein than in the portal vein in patients with cirrhosis and NAFLD [88, 100].

Non-alcoholic fatty liver disease

The steadily increasing incidence of the obesity pandemic carries a range of comorbidities including NAFLD which is the leading cause of chronic liver disease worldwide with an estimated prevalence exceeding 25% in European adults [101]. NAFLD is defined as >5% hepatic steatosis in a liver biopsy from patients without excess alcohol intake as NAFLD is indistinguishable from alcohol- or drug-induced liver damages histologically [102]. NAFLD involves a spectrum of diseases from simple steatosis to non-alcoholic steatohepatitis (NASH) that may involve hepatic fibrosis and evolve to cirrhosis and hepatocellular carcinoma (**Figure 3**) [103]. Not all NAFLD patients progress to NASH with advanced fibrosis (Kleiner fibrosis score ≥F3) [104] which is associated with hepatic decompensation and mortality [90, 91]. However, even simple steatosis induces liver dysfunctions [105].

Under normal conditions, the liver is involved in coordination of metabolism of nutrients and toxins among many other specific functions. For instance, glucose and free fatty acids

are processed to triglycerides that are packed in very-low density lipoproteins (VLDL) and delivered to adipocytes for storage [106]. However, under continuous intake of a high-energy diet and limited physical activity, adipose tissue capacity is diminished whereupon free fatty acids and triglycerides are stored in the liver and skeletal muscles and contribute to multi-organ insulin resistance [107]. Multiple factors, including dietary (e.g. high fat, fructose and/or cholesterol intake), gut microbial and genetic factors induce NAFLD. Although the pathophysiology is not fully understood the current understanding is described by a 'multiple parallel-hit' model [108].

Persistent hepatic steatosis may further involve infiltration of immune cells and hepatic lipotoxicity that triggers diagnosis of NASH defined as joint presence of hepatic steatosis, lobular inflammation, and hepatocellular ballooning with or without fibrosis [109]. Similar metabolic and histological features can be induced in rodent models of NASH using modified diets, chemicals and/or genetic modifications. Obesogenic diets, high in fat, cholesterol and fructose, are frequently used to induce NAFLD as they mimic the full spectrum of human NAFLD in wild-type mice although the models are often time-consuming and requires a relatively high sample size due to a high inter-individual variability [110].

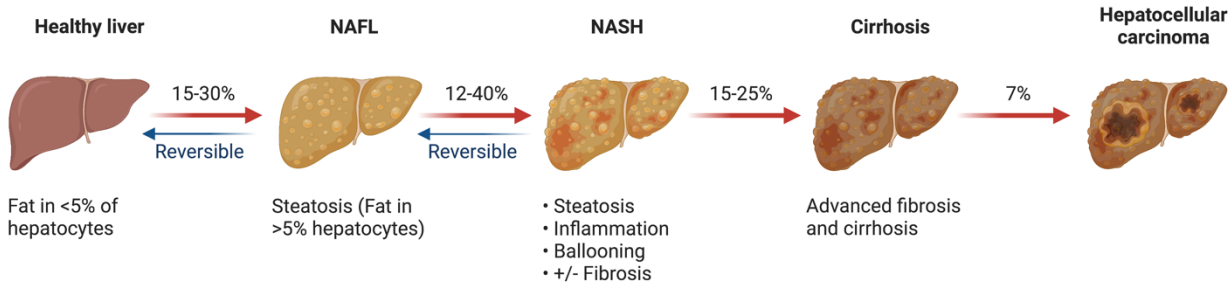

Figure 3: Progression of non-alcoholic fatty liver disease (NAFLD). The disease comprises a spectrum from simple steatosis to severe non-alcoholic steatohepatitis (NASH) involving inflammation and damage of hepatocytes (ballooning). Over time fibrosis can advance to cirrhosis and lead to hepatocellular carcinoma. Created with BioRender.com.

KC are important operators in and regulators of hepatic lipid metabolism [111] and possess an important role in the development and progression of NAFLD [112, 113]. Early animal studies have demonstrated their importance in NAFLD where macrophage depletion protected against the development of hepatic steatosis although it did not lead to regression of NAFLD [114-118]. Recent advances in single-cell transcriptomics have revealed that dynamic changes occur in the macrophage population in NAFLD [119]. In the healthy liver, KC are one of the main non-parenchymal cell types in the liver, but mouse studies have indicated that resident KC populations are reduced and replaced by monocyte-derived macrophages as NAFLD progresses [120-123]. All the mentioned studies

identify bona fide KC as *Clec4F*$^+$, *Timd4*$^+$ and *Cd163*$^+$. After development of diet-induced NAFLD, the KC pool was replaced by monocyte-derived KC (*Clec4F*$^+$, *Timd4*$^-$, *Cd163*$^-$) as well as lipid-associated macrophages (LAMs), resembling the *Trem2*$^+$ LAMs identified in adipose tissue in obesity [124]. In the liver LAMs locate to areas of hepatocellular damage, inflammation and fibrosis [125], and form part of the hepatic crown-like structures (hCLS) also seen in adipose tissue [121]. Whether LAMs are pathogenic or protective is still unclear but a reduced number of LAMs in hCLS correlates with fibrosis [121] and *Trem2* knockout impairs tissue repair and induce weight gain [124-126] suggesting a protective role of LAMs. Replacement of KC has not yet been verified in humans because a specific marker of bona fide KC is still missing [119].

CD163 in NAFLD

CD163$^+$ macrophages surround fat-laden hepatocytes and form cellular aggregates in NASH but not in healthy livers [86, 88, 127]. Nevertheless, the association with disease severity is ambiguous. Additionally, the intrahepatic distribution of CD163$^+$ macrophages is altered as it increases in the portal tract but tend to decrease in lobular regions of NASH biopsies compared to healthy controls [128]. In children with NAFLD, De Vito et al. found a significant association between the number of CD163$^+$ cells and disease severity [129], however, in another study of a larger child cohort no association was found [130]. At the transcriptional level *CD163* mRNA does not change significantly in obese patients with and without NASH [88] and in mouse studies *Cd163* mRNA is reduced as NAFLD progresses [123, 131-133]. More specifically, resident KC express more *Cd163* compared to monocyte-derived KC [122].

Elevated plasma levels of sCD163 indicate macrophage activation in patients with advanced metabolic syndrome due to obesity [134], insulin resistance [135, 136], type 2 diabetes [137] and NAFLD [86-89, 127]. In patients with biopsy-proven NAFLD, higher sCD163 levels are associated with NASH diagnosis and high NASH activity scores (NAS) [86-89], which is an unweighted score of steatosis, lobular inflammation, and hepatocellular ballooning ranging from 0 to 8 [109]. In addition, sCD163 is associated with the degree of steatosis and severity of hepatic fibrosis [86-88, 127] and remains associated with NAS and fibrosis after adjusting for age, gender, BMI, presence of diabetes, hypertension, and dyslipidemia [88]. Further, an elevated sCD163 level is a risk factor for clinical progression of NAFLD [89]. The presence of advanced fibrosis predicts mortality in NAFLD [90, 91] but because the disease usually is asymptomatic and requires a liver biopsy for diagnosis, some patients already have fibrosis at the time of diagnosis. Therefore, non-invasive biomarkers are essential to identify patients at risk of advanced disease early. As sCD163 increases

with NAFLD severity and is easy to measure in plasma it has been suggested as a potential biomarker in various inflammatory diseases including NAFLD [86, 113, 138]. Another recently suggested non-invasive biomarker include soluble triggering receptor expressed on myeloid cells 2 (sTREM2) [125, 139], however, this biomarker still needs thorough validation. On the other hand, transient elastography by FibroScan has gradually been introduced in clinical practice to assess liver fibrosis to identify patients eligible for a liver biopsy, though, this requires expensive equipment only available at specialized units [140]. Alternatively, different composite biomarkers have been introduced to evaluate fibrosis in patients with NAFLD (e.g. FIB-4) [141, 142].

Treatment of NAFLD

Lifestyle modifications are first line treatment of NAFLD. Sustained weight reduction of ≥10% is associated with NASH resolution and fibrosis regression [143], however, only few succeed in and sustain such a significant weight loss within a year [144]. Instead, some obese patients are eligible for bariatric surgery which also significantly improves NAFLD alongside the major weight loss [145]. A very recent randomized controlled trial even suggests that bariatric surgery is superior to lifestyle intervention in NASH treatment in obese patients as 57% versus 16% experienced histologically resolution of NASH without worsening of fibrosis at 1 year follow-up [146]. Lifestyle and surgical interventions reduce plasma concentrations of sCD163 [88, 134, 147-149]. There are currently no approved pharmaceutical treatments for NASH. Recently, FDA and EMA approved the GLP-1 analog semaglutide (Wegovy®), originally approved for type 2 diabetes (Ozempic®), indicated for chronic weight management in adults with obesity in addition to a healthy lifestyle [150, 151]. In addition to significant weight loss [152], semaglutide induce NASH resolution without worsening of fibrosis in a 72-week double-blinded phase 2 trial involving patients with biopsy-proven NASH and fibrosis (F1-F3) [153]. However, in patients with NASH-related compensated cirrhosis (F4), 48 weeks of semaglutide did not improve fibrosis significantly compared to placebo [154]. Other promising drugs in NASH management include FXR agonists (e.g. obeticholic acid), PPAR agonists (e.g. elafibranor) and thyroid receptor agonist (e.g. Resmetriom) [155].

CD163 has also been suggested as a potential entry site for NASH treatment. CD163 is an optimal target of antibody-based drug-delivering systems because it is an extracellularly available endocytic and recycling receptor solely expressed by monocytes/macrophages that is upregulated in sites of inflammation [66, 156]. Targeting a 50-fold lower dose of dexamethasone directly to $CD163^+$ macrophages using an antibody drug-conjugate significantly reduced hepatic steatosis, inflammation, and hepatocellular ballooning as well as fibrosis in a fructose-induced NASH model in rats, while an

equivalent dose of free dexamethasone or control antibody-drug conjugate did not show similar effects [157]. This therapeutic strategy has been shown to be advantageous because the serious adverse effects of dexamethasone (e.g. weight gain, insulin resistance, dyslipidemia, immunosuppression, osteoporosis [158]) are evaded. This has been evidenced in mice exposed to endotoxin where an equipotent dose of the antibody conjugated dexamethasone prevented decreased serum cortisol, and thymus and spleen weight reductions as seen in animals treated with high dose free dexamethasone [159]. The utility of CD163-targeting is not limited to NAFLD but has also been shown beneficial in animal models of Parkinson's disease [160], ischemia/reperfusion injury [161], and in malignant diseases [162, 163] where depletion of $CD163^+$ TAMs promote tumor regression and limit metastatic spread.

Scope of the thesis

Multiple studies have highlighted the utility of the Hp and CD163 in clinical and pre-clinical settings to diagnose and treat hemolytic and inflammatory diseases. The principal aim of this thesis was to further explore the applicability of Hp, Hpr and CD163 in diagnosis of intravascular hemolysis and NAFLD.

Excessive intravascular hemolysis essentially depletes Hp in the circulation which is used clinically in the diagnosis. But the measurement is complicated by presence of Hpr and structurally different oligomers among Hp phenotypes. To address this, an in-house ELISA was established based on a Hp-specific monoclonal antibody raised against the unique loop 3 of Hp to investigate the influence of Hp phenotype and to compare the method with immunoturbidimetry used in the clinic.

In contrast to Hp, Hpr is not influenced by hemolysis according to previous semi-quantitative studies. The low abundance of Hpr and high sequence homology between Hp and Hpr complicates specific measurement of Hpr in human plasma. So far only a few studies have measured the plasma concentration of Hpr. Therefore, Hpr was measured using an in-house developed Hpr-specific ELISA and compared to the Hp levels and phenotype.

To improve the diagnosis and treatment of NASH, the association between disease severity and sCD163 as well as hepatic CD163 expression at protein and mRNA levels in paired samples covering liver healthy to NASH in a cohort of 61 patients with obesity were investigated. To better understand the role of CD163 in development of NAFLD, we also investigated the hepatic expression of CD163 in a mouse model that mimic human NAFLD and explored the influence of CD163-deficiency in early development of hepatic steatosis.

Essential results and discussion

The proteins of the Hb scavenger system are clinically relevant, this is exemplified by decreased plasma concentration of Hp during intravascular hemolysis and increased release of sCD163 in inflammatory diseases. The scope of the thesis covers a wide area reflecting different aspects of Hp, Hpr and CD163 utility and biology. Therefore, this section highlights the main results and set them in context of current knowledge and future perspectives. The work has been assembled into four individual papers (***Paper I-IV*** of which ***Paper I-III*** are published), and a section on further results demonstrating antibody-mediated targeting of $CD163^+$ macrophages in mice (***Appendix I***).

In ***Paper I*** we revealed how the current clinical method for measurement of Hp is independent of Hpr but influenced by the phenotype of the calibrator that may result in substantial bias in a cohort of 112 anonymized patients. Using the same cohort, ***Paper II*** demonstrates for the first time how Hpr is reduced concurrently with Hp during hemolysis, and Hpr may be applicable as a novel diagnostic tool enabled by the established ELISA. On the other hand, ***Paper III*** discloses that sCD163 is significantly increased in NASH and identifies NASH patients with an AUROC of 0.78. However, hepatic CD163 expression is concurrently decreased in liver biopsies from obese patients with NASH indicating an inverse association with sCD163 in plasma. Finally, ***Paper IV*** shows that hepatic CD163 correlates with hepatic steatosis in mice and is suggested to possess a protective role in early development of hepatic steatosis as CD163-deficient mice are more sensitive to western diet (WD).

Hp measurement is influenced by the Hp phenotype of the calibrator rather than presence of Hpr

In a cohort of 112 anonymized patient samples, the median plasma concentration of Hp and Hpr were determined to be 1.1 g/L (range: 5.7) and 3.4 mg/L (range: 10.2), respectively, using two independent assays that permit specific measurement of Hp (***Paper I***) and Hpr (***Paper II***) in human plasma. Specific measurement of Hp did not deviate significantly from the Hp concentration determined by immunoturbidimetry in the clinic. Thereby, the unspecific binding of Hpr in immunoturbidimetry did not influence the measurement of Hp. In addition, Hpr comprises a very small part of the total Hp/Hpr pool in normal human plasma and is therefore also negligible in the quantification of Hp in the clinic even during hemolysis where Hpr constitutes 12% because Hp is depleted (Hp <0.1g/L). However, it might become more important in case Hpr is increased in disease or in individuals frequently exposed

to *Trypanosoma brucei* or other parasites. For instance, Gabonese children possess a 5-fold higher plasma concentration of Hpr compared to Caucasian children (280 mg/L vs. 49 mg/L) [32].

On the contrary, Hp measurement particularly relies on the phenotype of the calibrator. Using a calibrator with an unmatched phenotype in the ELISA entails a significant underestimation or overestimation of Hp in individuals with Hp1-1 or Hp2-2 phenotype, respectively. This phenomenon has previously been explained as the consequence of different immunoreactivity of the Hp phenotypes [164]. In a solid phase immunoassay like ELISA, high-order oligomers of Hp enable amplification of detection by increasing the number of available epitopes per captured protein. Therefore, less Hp2-2 is necessary to elicit the same signal of Hp1-1 in an ELISA which, consequently, overestimates Hp2-2 when Hp1-1 is used as a calibrator. In our ELISA, the discrepancy in immunoreactivity is limited by detecting Hp with a polyclonal antibody by which sterically hindrance might influence the binding of the oligomeric Hp2-1 and Hp2-2.

To obtain the true Hp level in human plasma the use of a phenotype-matched calibrator in immunoassays is preferred. Although optimal, it is not feasible to perform phenotyping in the clinic, where fast diagnosis is critical. Further, because the Hp concentration determined by the current immunoturbidimetric assay only deviates slightly (±37%) from the true Hp concentration it is not clinically relevant to further complicate the current assay. However, clinicians should be aware that Hp is overestimated in patients with Hp1-1 phenotype, which amounts to 14% in Europeans [165], and therefore are at risk of being overlooked in the diagnosis of hemolysis. In this case, other markers of hemolysis such as reduced Hb, reticulocytosis, increased lactate dehydrogenases, and bilirubin probably complete the full clinical picture and confine the risk of overlooking the diagnosis [166].

Hpr is reduced concurrently with Hp during hemolysis

Levels of Hpr and Hp correlate significantly in human plasma in accordance with a previous study [32]. In addition, Hpr levels is significantly lower in individuals with the Hp2-2 phenotype. Hp levels are also significantly lower in Hp2-2 subjects [164, 167, 168] which might be a consequence of faster transcription of *Hp1* compared to *Hp2* as shown *in vitro* [164] and/or increased consumption of Hp2-2 at subsaturated Hb levels during trivial hemolysis (***Paper I***). Although each Hp genotype has been linked to a range of diseases, there is no general indication that one allele is associated with a higher risk of disease compared to the other [16].

It has not previously been demonstrated that the Hpr concentration is significantly lower in individuals with a concurrent low Hp plasma concentration (Hp <0.1g/L). Instead, Hpr was

believed to be unaffected by hemolysis as indicated by western blot analysis [12, 31] which makes sense in the context of the current knowledge of exclusive CD163-mediated clearance of Hp-Hb [33]. However, our data underline that a yet unknown clearance mechanism of Hpr-Hb exists that might be unable to distinguish between Hp-Hb and Hpr-Hb resulting in the reduced Hpr plasma concentration during hemolysis.

Although Hpr is reduced in hemolytic patients, Hp is more markedly reduced and therefore still superior in identifying hemolysis. Instead, Hpr might serve as a biomarker of other diseases where the newly established Hpr-specific ELISA is a cost-effective technique to verify its applicability and validity in larger cohorts. Proteomic analysis of serum has revealed increased expression of Hpr in pediatric patients with pneumonia especially in mycoplasma pneumonia compared to bacterial pneumonia [169]. In addition, elevated expression of Hpr has also been demonstrated in the metabolic syndrome [170] and after initiation of hemodialysis [171], while reduced expression of Hpr was found in patients with infectious endocarditis [172] and idiopathic pulmonary fibrosis [173] using proteomic analysis of serum. In addition, earlier studies have revealed that the Hpr gene is expressed by malignant cell lines [174], and the presence of epitopes of an alternative Hpr, also designated p21, was associated with several malignancies [175-177]. However, subsequent peptide sequencing identified the protein as fatty acid synthase and thus not an alternative Hpr [178, 179]. Nevertheless, a more recent study using mass spectroscopy indicates that Hpr might be increased in serum from breast cancer patients [180]. Therefore, the association between Hpr and malignancies should be revisited which the new Hpr-specific ELISA enables.

More severe NAFLD is associated with increased sCD163 but decreased hepatic CD163 expression in patients with obesity

Soluble CD163 has been suggested as a potential biomarker of NASH [86, 113, 138]. Currently, a robust non-invasive biomarker is lacking, and liver biopsies are needed to diagnose NAFLD making it highly underdiagnosed [181]. In a cohort of obese patients, measurement of sCD163 was able to identify patients with biopsy-proven NASH with an AUROC of 0.78 (CI95%: 0.65-0.92) (***Paper III***). Similarly, previous studies have shown that sCD163 predicts NASH with an AUROC of 0.65-0.70 in larger cohorts of obese patients undergoing bariatric surgery [87, 88]. AUROC values below 0.8 are not convincing since they make the risk of false outcomes likely. Furthermore, although significantly increased in NASH, sCD163 is not specific for NAFLD and is not increased multifold as observed in acute liver injuries [82]. Therefore, sCD163 as a single biomarker of NASH is not optimal. Instead, sCD163 might be useful as a component in a composite biomarker that reflects

different features of the disease. For instance, combining it with aspartate transaminase (AST), reflecting liver injury, the AUROC increased to 0.82 (CI95%:0.69-0.94). Parallel to this study, we found that sTREM2 is highly increased in human plasma from patients with NAS ≥ 4 [139] reflecting the high infiltration of LAMs in NASH [125, 182]. Alone sTREM2 is superior to sCD163 in identifying NASH (AUROC of 0.91 (CI95%: 0.83-0.98)) and combining the two plasma proteins does not improve the diagnostic accuracy in obese patients. With a proteo-transcriptomic approach, Govaere and colleagues recently identified a novel non-invasive composite biomarker that enables the identification of at-risk NASH patients with advanced fibrosis [183]. This composite marker includes sTREM2 and is significantly superior to current markers (e.g. FIB-4). The role of sCD163 as a biomarker of NASH still needs further validation, although other biomarkers currently seem more convincing in identifying NASH and at-risk NASH.

The increased plasma concentration of sCD163 has previously been suggested to originate from liver macrophages [88]. However, we demonstrate that elevated plasma concentration of sCD163 is not accompanied by increased hepatic expression of CD163 in patients with NASH but rather a significantly decreased expression. This may be a result of downregulated transcription, KC depletion during NAFLD development, and/or increased shedding by ADAM17. Hepatic transcription of *CD163* was reduced in patients with NASH compared to liver-healthy patients which is in accordance with previous mouse studies [123, 131-133]. Likewise, *SLC40A1* (ferroportin-1) is downregulated in NAFLD [184] while hepcidin serum levels are increased [185] mediating iron accumulation in the liver which is associated with NAFLD [63]. Iron overload is toxic and may cause ferroptosis of hepatocytes and macrophages. In mice, KC are reported to die as NAFLD develops [120-123]. Thereby, the CD163 expression might simply decrease because the KC are high CD163 expressors while the acquired monocyte-derived macrophages are not. However, KC apoptosis still needs to be proven in human NAFLD [119]. Finally, increased shedding of CD163 from the cell surface might also contribute to the decreased cellular expression of CD163 in the liver [52]. ADAM17 activity is difficult to evaluate *in vivo*, but the increased levels of sTREM2 and TNF-α in plasma indirectly indicate elevated ADAM17 activity, albeit it does not address hepatic origin. Collectively, a combined effect of the above-mentioned causes may lead to the decreased hepatic CD163 expression, which is underlined by a recent publication indicating a reduced number of $CD163^+$ cells and reduced expression level on macrophages in NAFLD [186].

Compared to previous studies ***Paper III*** stands out by thoroughly measuring the hepatic expression of CD163 at the protein and mRNA level that unanimously indicate a reduction in NASH. This is in accordance with previous studies of the transcriptional changes in NAFLD in humans and

mice [121, 123, 131-133, 182, 187] and the protein changes in human NAFLD using mass spectroscopy [188] although not significant in the latter study. Despite reduced hepatic expression of CD163, aggregates of $CD163^+$ macrophages appear in liver tissue from NASH patients [86, 88, 127]. These aggregates might correspond to hepatic crown-like structures (hCLS) that surround and engulf lipid-laden hepatocytes [189]. In the liver specimens from the obese patients in ***Paper III***, the number of $CD163^+$ hCLS were counted and were significantly elevated in NASH patients (0.1 vs. 0.8 hCLS/mm^2, $p=0.0006$). The hCLS has been linked to fibrogenesis and are located close to fibrosis [189-191]. Thus, the CD163 expressing macrophages present in hCLS in NASH might be involved in tidying up the tissue and inducing tissue regeneration as expected of M2-like macrophages. However, prolonged fibrogenesis will eventually lead to cirrhosis.

CD163 protects the liver from early development of steatosis in male mice

The hepatic CD163 expression distinctly correlated negatively with steatosis grade in mice fed with the WD as they became more diseased (***Paper IV***) which is consistent with the human study (***Paper III***). In both mice and humans, the hepatic expression of CD163 was generally decreased in areas of steatosis which usually initiate near the central vein (zone 3) in NAFLD [192]. Previous studies have quantitatively shown regional differences in humans with NAFLD with increased expression of CD163 in the portal region but decreased expression in the rest of the tissue [99, 128].

Somehow CD163 expression seems to protect the liver against the development of hepatic steatosis as CD163-deficient mice produce more advanced steatosis compared to wildtype littermates after just eight weeks of WD (***Paper IV***). This beneficial effect might be achieved through the direct scavenging of proinflammatory ligands by CD163, or by a phenotypic change in hepatic macrophages whereby CD163 expression reflects this shift. Presumably multiple facts interplay, and further causes might exist.

Scavenger receptors usually have multiple ligands [193], it is therefore plausible that CD163 also binds other proinflammatory mediators directly (beyond the Hp-Hb complex) limiting their inflammatory and lipogenic effects. For instance, CD163-mediated binding of TWEAK may hinder Fn14-mediated intracellular signaling through the proinflammatory NF-κB pathway and thereby limit the shift towards the M1-like phenotype [194]. Further, in a mouse model of atherosclerosis, which has been suggested as the cardiovascular equivalent to NAFLD [195], CD163-deficiency causes more unstable plaques and exogenous TWEAK further aggravates the atherosclerotic lesion [76]. In addition, administration of sCD163 hinders the TWEAK-induced aggravation suggesting that sCD163 might possess a protective and immunoregulatory role in human

NAFLD and other inflammatory diseases. Other yet unknown ligands of CD163 may also exist and contribute to the effect.

Alternatively, lack of CD163 might alter the balance between pro- and anti-inflammatory macrophages and their paracrine signaling in the tissue promoting hepatic steatosis. Previous studies have revealed that excess free fatty acids induce the release of proinflammatory cytokines including TNF-α which influence lipid metabolism in hepatocytes resulting in early development of steatosis [114, 196-198]. In addition, TNF-α mediates a KC-dependent response to leptin in hepatocytes [199]. Hence, inflammatory signals drive fatty acid synthesis, while inhibition of these drive fatty acid oxidation [111]. NAFLD is associated with a proinflammatory macrophage phenotype and even before NASH is histologically visible M2-like markers are transcriptionally reduced in high-fat-fed mice after just 4 weeks [200]. Hence, the shift towards the proinflammatory microenvironment might favor the development of hepatic steatosis and disease progression. Therefore, manipulation of the M1/M2 ratio seems like an attractive therapeutic strategy as several studies have indicated [157, 201, 202]. It remains unclear whether the M1-like and M2-like macrophages represent distinct macrophage populations, or a single population skewed in one direction [111, 203]. Furthermore, the situation *in vivo* is probably very complex and not limited to the dichotomy of the *in vitro* based M1/M2 nomenclature.

Put together, early changes in the macrophage population might contribute to the development of NAFLD in parallel with lipid overload and insulin resistance as described by the 'multiple parallel-hit' model of NAFLD [108]. Future studies are needed to clarify the causality between $CD163^+$ macrophages and the early development of hepatic steatosis and later stages of NAFLD.

Concluding remarks

In this PhD thesis the importance of Hp, Hpr, and CD163 is highlighted in the context of disease development and their utility in clinical biochemistry. Plasma levels of the Hb scavenging proteins are influenced by diseases and are already used as diagnostic tools to identify diseases, however, further applications might be discovered expanding their beneficial clinical use. Little is currently known about the diagnostic utility of Hpr. Now the novel Hpr-specific ELISA enables investigation of Hpr in cohorts of patients with, for example, cancer or infectious diseases. Studying CD163 in the context of NAFLD uncovered the early importance of macrophages in hepatic steatosis even before NASH is histologically present. Further understanding of the role of macrophage's generally, and more specifically, CD163-expressing macrophages, in the pathogenesis of NAFLD is needed. It is important to understand the disease progression including the role of macrophages to explore potential therapeutic and diagnostic strategies, as neither pharmaceutical drugs are currently approved, nor good diagnostic non-invasive biomarkers are available that can identify at-risk patients.

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