Dissertation

Liver disease alters high-density lipoprotein composition, metabolism and function

submitted by

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice”.

Parts of this thesis have been published in Trieb M, Horvath A, Birner-Gruenberger R, Spindelboeck W, Stadlbauer V, Taschler U, Curcic S, Stauber RE, Holzer M, Pasterk L, Heinemann A & Marsche G. “Liver disease alters high-density lipoprotein composition, metabolism and function.” BBA – Molecular and Cell Biology of Lipids (2016).

Graz, 17.11.2016 _____

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Summary

Liver disease is associated with an imbalanced inflammatory response and accompanied by an increased risk for infections and sepsis, but the underlying molecular mechanisms are poorly understood. The ability of high-density lipoprotein (HDL) to promote reverse cholesterol transport from lipid laden macrophages and other cells in the artery wall to the liver is considered to be the a major function of HDL. Recent insights have added other important HDL-mediated activities which are considered to contribute to HDLs protective properties, including anti-oxidative and anti-inflammatory activities. Therefore, HDL is emerging as a relevant player in innate and adaptive immunity and as a major endogenous inhibitor of inflammatory responses. It recently became evident that inflammatory disease can alter HDL composition thereby rendering it dysfunctional. Functional impairment of HDL might contribute to the excess mortality experienced by patients with liver disease, but the effect of cirrhosis on HDL metabolism and function remains elusive. Therefore, we sought to determine whether cirrhosis has an impact on HDL metabolism, composition and function. To get an integrated measure of HDL quantity and quality, we assessed several metrics of HDL function using serum HDL (serum containing HDL but depleted of low-density lipoprotein (LDL) and very low–density lipoprotein (VLDL)) from patients with compensated cirrhosis, patients with acutely decompensated cirrhosis and healthy controls. We observed a significant decrease in HDL-cholesterol levels and profoundly altered activities of enzymes involved in HDL metabolism in patients with cirrhosis, including phospholipid transfer protein, cholesteryl-ester transfer protein and lecithin-cholesterol acyltransferase. Native gel electrophoresis revealed a shift towards the large, cholesteryl rich HDL_2 subclass with a concurrent reduction in the amount of small HDL_3 particles. Furthermore, we identified several HDL-associated proteins to be altered in patients with cirrhosis. Interestingly, the detected changes in HDL-cholesterol levels, HDL protein composition and HDL particle distribution were strongly associated with metrics of HDL function. HDL of cirrhotic patients showed a significantly reduced capacity to inhibit nuclear factor-κB activation in lipopolysaccharide stimulated monocytes. This resulted in an increased production

of the pro-inflammatory cytokines tumor necrosis factor-α and interleukin-6. Furthermore, the ability of HDL to stimulate the activation of the endothelial nitric oxide synthase, as well as paraoxonase activity and endothelial regenerative and barrier promoting activities were significantly suppressed in cirrhotic patients. Of particular interest, cholesterol efflux, which was significantly reduced in patients with cirrhosis, appeared to be strongly associated with liver disease mortality.

In conclusion, HDL metabolism, composition and function are significantly altered in cirrhosis. Importantly, HDL cholesterol efflux capacity appeared to predict 1-year mortality independent of HDL-cholesterol levels. Our findings may be clinically relevant and improve our ability to identify cirrhotic patients at high risk.

Zusammenfassung

Bei akuten und chronischen Erkrankungen der Leber kann es zu unterschiedlichsten Störungen kommen. So haben Patienten mit chronisch entzündlicher Lebererkrankung ein erhöhtes Risiko für Infektionen und Entwicklung einer Sepsis. Über die molekularen Ursachen für diese Anfälligkeit für Infektionen ist jedoch wenig bekannt. Die Hauptfunktion von Lipoprotein hoher Dichte (HDL) ist seine zentrale Rolle im reversen Cholesterin Transport. Dabei wird überschüssiges Cholesterin aus Makrophagen und anderen Zellen der Gefäßwand zur Leber rücktransportiert. HDL besitzt aber auch wichtige anti-oxidative und anti-entzündliche Eigenschaften, daher wird HDL heutzutage auch als wichtiger Bestandteil der angeborenen und adaptiven Immunabwehr gesehen. Seit kurzem ist bekannt, dass sich die Zusammensetzung von HDL im Laufe einer entzündlichen Krankheit verändert. Dadurch kann HDL seine protektiven Eigenschaften verlieren und möglicherweise zu dem erhöhten Mortalitätsrisiko, welches Patienten mit Zirrhose haben, beitragen. Interessanterweise wurde die Auswirkung von Zirrhose auf die Funktionalität und Zusammensetzung von HDL noch nicht erforscht. Ziel meines Dissertationsprojektes war es zu untersuchen, ob Zirrhose den HDL Metabolismus, die Zusammensetzung der HDL Partikel und deren Funktion beeinträchtigt.

Für unsere Untersuchungen der HDL Funktionalität wurde so genanntes „Serum HDL“ von Patienten mit kompensierter Zirrhose, von Patienten mit dekompensierter Zirrhose und von gesunden Kontrollen verwendet. Dabei handelt es sich um Serum, das HDL enthält, von welchem jedoch die Lipoproteine niedriger Dichte (LDL und VLDL) entfernt wurden. Wir stellten fest, dass Patienten mit Zirrhose signifikant reduziertes HDL-Cholesterin aufwiesen. Des Weiteren waren die Aktivitäten von Serumenzymen, welche in der Reifung und im Abbau der HDL Partikel eine wichtige Rolle spielen, stark verändert. So zeigten Analysen mittels nativer Gel Elektrophorese, dass Patienten mit Zirrhose eine veränderte HDL Partikel Verteilung aufweisen. Zirrhotiker haben deutlich verringerte HDL_3- aber erhöhte HDL_2-Spiegel. Aber nicht nur die Struktur der HDL Partikel ist bei Zirrhotikern verändert, auch die Proteinzusammensetzung weist deutliche Unterschiede auf. So zeigte sich zum Beispiel, dass am HDL von Zirrhotikern die

Apolipoproteine A-I, A-II, C-I, C-II, C-III und E signifikant reduziert waren, wobei hingegen die HDL Partikel mit den akuten Phase Proteine Serum Amyloid A1 und A2 angereichert waren.

Wir konnten nachweisen, dass die Änderungen der HDL Menge (HDL-Cholesterin), der HDL Zusammensetzung und der HDL Größenverteilung mit unterschiedlichen HDL Funktionen eng verknüpft waren. Wir stellten fest, dass HDL von Zirrhotikern eine deutlich reduzierte anti-entzündliche Wirkung aufweist. Normalerweise kann HDL die Expression des bei Entzündungen entscheidenden Transkriptionsfaktor Nuclear Factor-κB in Monozyten unterdrücken. Diese Eigenschaft von HDL war aber bei Zirrhotikern deutlich eingeschränkt. Dies wiederum resultierte in einer erhöhten Produktion der pro-inflammatorischen Zytokinen tumor necrosis factor-α und Interleukin-6 von Monozyten. Interessanterweise zeigte sich auch, dass bei Lebererkrankungen Endothel-protektive Eigenschaften von HDL, wie die Aktivierung der Aktivität der endothelialen Stickstoffmonoxid Synthase und Stabilisierung der endothelialen Barrierefunktion beeinträchtigt sind. Von besonderem Interesse war unsere Beobachtung, dass das signifikant reduzierte Cholesterin Efflux Potential von Serum HDL von Patienten mit Lebererkrankung sehr stark mit der Mortalität assoziiert war.

Zusammenfassend konnten wir zeigen, dass sowohl der HDL-Metabolismus, wie auch die Zusammensetzung und Funktionalität von HDL bei Zirrhose signifikant gestört sind. Interessanterweise scheint das HDL Cholesterin-Efflux Potential negativ mit der 1-Jahres Mortalität bei Zirrhotikern zu assoziieren. Unsere Befunde könnten daher von klinischer Relevanz sein.

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List of abbreviations

ABCA1	ATP-binding cassette, sub-family A, member 1
ABCG1	ATP-binding cassette, sub-family G, member 1
apo	apolipoprotein
ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CETP	cholesteryl ester transfer protein
CM	chylomicron
EL	endothelial lipase
eNOS	endothelial nitric oxide synthase
FBS	fetal bovine serum
HDL	high-density lipoprotein
HL	hepatic lipase
IDL	intermediate-density lipoprotein
IL	interleukin
KBr	potassium bromide
kDa	kilo Dalton
LCAT	lecithin cholesterol acyltransferase
LC-MS/MS	liquid chromatography – tandem mass spectrometry
LDL	low-density lipoprotein
LPL	lipoprotein lipase
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein 1
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
PDVF	polyvinylidene difluoride
PI-3K	phosphatidylinositol-3-kinases,
PLTP	phospholipid transfer protein
PON	paraoxonase
rHDL	reconstituted high-density lipoprotein
S1P	sphingosine-1-phosphate
SAA	serum amyloid A
Ser	serine
SR-BI	scavenger receptor, class B, type 1
Thr	threonine
TNF-α	tumor necrosis factor α
VLDL	very low-density lipoprotein

1. Introduction

1.1. The liver

The liver is the second largest organ in the body and carries out a variety of functions including immunological, vascular, secretory and excretory functions. In addition, it is also a key player in the metabolic control of the body by being critically involved in protein, glucose and fat metabolism (1). Briefly, the liver synthesizes non-essential amino acids and builds up most of the plasma proteins including albumin, clotting factors and apolipoprotein (apo) A-I. Additionally, it removes toxic ammonia from the body by the hepatocyte-mediated conversion of ammonia into urea which is then excreted through the kidneys. The liver plays a prominent role in regulating blood glucose levels. After a meal, excess glucose is removed from the blood by a rapid uptake by the liver where it gets converted into glycogen by a process called glycogenesis (2). When blood glucose levels decrease, the stored glycogen gets depolymerized and glucose gets exported back into the circulation (glycogenolysis) (3). Furthermore, when hepatic glycogen pools are depleted, the liver can synthesize glucose from amino acids or fatty acids by a process called gluconeogenesis (4). Free fatty acids are either taken up by the liver from the circulation or synthesized, under conditions when carbohydrates are abundant, from acetyl-CoA derived from glucose. Subsequently, free fatty acids undergo β-oxidation in mitochondria. The formed acetyl-CoA may either enter the citrate cycle to provide energy or it is used to form ketone bodies which are rapidly taken up by extra-hepatic tissues and get readily metabolized. Fatty acids can also be esterified to form triglycerides and cholesteryl esters which are stored in lipid droplets or they are packaged into lipoproteins and released into circulation. However, it has been shown that liver disease is associated with disturbances of metabolic homeostasis (5,6).

1.2. Cirrhosis

Cirrhosis is a major cause of death worldwide accounting for 1.03 million deaths per year and rates are increasing (7,8). Patients with cirrhosis are susceptible to bacterial infections that frequently result in acute-on-chronic liver failure (9-11). Cirrhosis is thought to be the most common immunodeficiency syndrome worldwide with an occurrence of bacterial infections in 30 to 50% of hospitalized patients (12,13). The overall median mortality rate of infected cirrhotic patients is

38% (14). In cirrhosis, sepsis is frequently accompanied by an imbalanced cytokine response leading to excessive inflammation. Bacterial infections develop as a consequence of immune dysfunction that occurs progressively during the course of cirrhosis. (15,16). During severe bacterial infections, disruption of the integrity of the endothelial barrier markedly increases permeability to fluids, solutes and inflammatory cells. The activation status of leukocytes has been shown to be associated with increased morbidity and mortality (17-19). Of particular interest, many of these studies demonstrated activation rather than a depression of peripheral mononuclear cells in cirrhosis (20-23). Given that the liver plays an essential role in several stages of lipid synthesis, transportation and metabolism, hypocholesterolemia is frequently found in patients with decompensated cirrhosis (24). Interestingly, low circulating cholesterol levels show a strong correlation with survival (25).

1.3. Lipoproteins

Lipoproteins are complex macromolecules composed of lipids and proteins. The core comprises of cholesteryl esters, triglycerides and fat soluble vitamins enveloped by a layer consisting of free cholesterol, proteins and amphipathic phospholipids. According to their density, lipoproteins are classified into 4 classes with several subclasses which vary in their lipid and protein content (Table 1). Chylomicrons (CMs) are very large, triglyceride rich particles which carry dietary lipids. Very low density lipoproteins (VLDLs) are smaller than CMs. They carry mostly endogenous triglycerides and to a lesser extent cholesterol. Low-density lipoproteins (LDLs) are cholesterol rich particles which contain only small amounts of triglycerides. High-density lipoproteins (HDLs) are the smallest particles. They are rich in phospholipids and proteins, and mediate the transport of excess cholesterol from the periphery to the liver.

Table 1. Lipoprotein classification

	CM	VLDL	IDL	LDL	HDL
MW (Da)	400 x 10^6	10-80 x 10^6	5-10 x 10^6	2.3 x 10^6	1.7-4.1 x 10^5
Size (nm)	75-1200	30-80	25-35	18-25	5-12
Apoproteins	B-48, A-I, A-II,A-IV, C-I, C-II, C-III, E	B-100, C-I, C-II, C-III, E	B-100, C-I, C-II, C-III, E	B-100	A-I, A-II, A-IV, C-I, C-II, C-III, E
Density (g/ml)	0.95	0.95-1.006	1.006-1.019	1.019-1.063	1.063-1.21
Lipids (%)					
TG	80-95	55-80	20-50	5-15	5-10
Chol	2-7	5-15	20-40	40-50	15-25
PL	3-9	10-20	15-25	20-25	20-30

MW, molecular weight; TG, triglyceride; Chol, cholesterol; PL, phospholipid; VLDL, very low-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein

1.3.1. Lipoprotein metabolism

Lipoprotein metabolism can be divided into an exogenous and an endogenous pathway as depicted in Figure 1. The exogenous pathway starts with the absorption of dietary fats, which consist mainly of triglycerides (90%-95%) but also of phospholipids, fat soluble vitamins and cholesterol. Dietary lipids are emulsified with bile acids and thereby formed micelles are processed enzymatically by pancreatic lipase, pancreatic phospholipase A2, cholesterol esterase and bile salts. Pancreatic lipase hydrolyzes triglycerides to generate free fatty acids and 2-monoglycerides. Free fatty acids can enter the enterocyte via passive diffusion or they are taken up by fatty acid transporters (26). Cholesterol uptake is mediated via Niemann-Pick C1-like 1 protein (27). In the enterocyte, free fatty acids and cholesterol are re-esterified by the enzyme acyl-CoA:cholesterol acyltransferase, are packaged together with phospholipids, triglycerides and apoB-48 into CMs and are finally released into circulation (28). For a proper lipoprotein assembly and secretion, the microsomal triglyceride transfer protein is required (29). ApoB-48 is only found on CMs. It is a truncated form of apoB-100 which lacks the C-terminal region which contains the domain recognized by the LDL receptor. In the

circulation, CMs acquire apoC-I, apoC-II, apoC-III, and apoE. ApoC-II is an activator of the endothelial bound lipoprotein lipase (LPL) which hydrolyzes triglycerides to free fatty acids which are used for energy production in muscle or are stored in lipid droplets in adipocytes (30). The triglyceride depleted, cholesterol-rich chylomicron remnants are taken up by the liver via binding of apoE to the LDL-receptor or to the lipoprotein receptor-related protein on hepatocytes (31). Endogenous lipids are transported to peripheral tissues by VLDL. In the liver, cholesterol, which is synthesized *de novo* by the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase or derives from diet, is packaged together with triglycerides and apoB-100 into VLDLs. Once in the bloodstream, they encounter apoC-I, apoC-II, apoC-III and apoE from HDL. In the circulation, the triglyceride-rich core of VLDL is hydrolyzed by LPL. During lipolysis, coupled to a loss of apoCs, VLDL is converted to intermediate-density lipoprotein which is enriched in apoE. Intermediate-density lipoprotein can either be internalized by hepatocytes via the interaction of LDL-receptor with apoE or can undergo further hydrolysis by LPL and hepatic lipase (HL) yielding LDL with a loss of apoE. LDL can interact with the LDL-receptor through the presence of the sole remaining protein apoB-100. LDL transports cholesterol from the liver to the peripheral tissues where it is endocytosed via the LDL-receptor. Cholesterol in non-hepatic tissue can be stored as an ester or be used for cell membrane synthesis or hormone production.

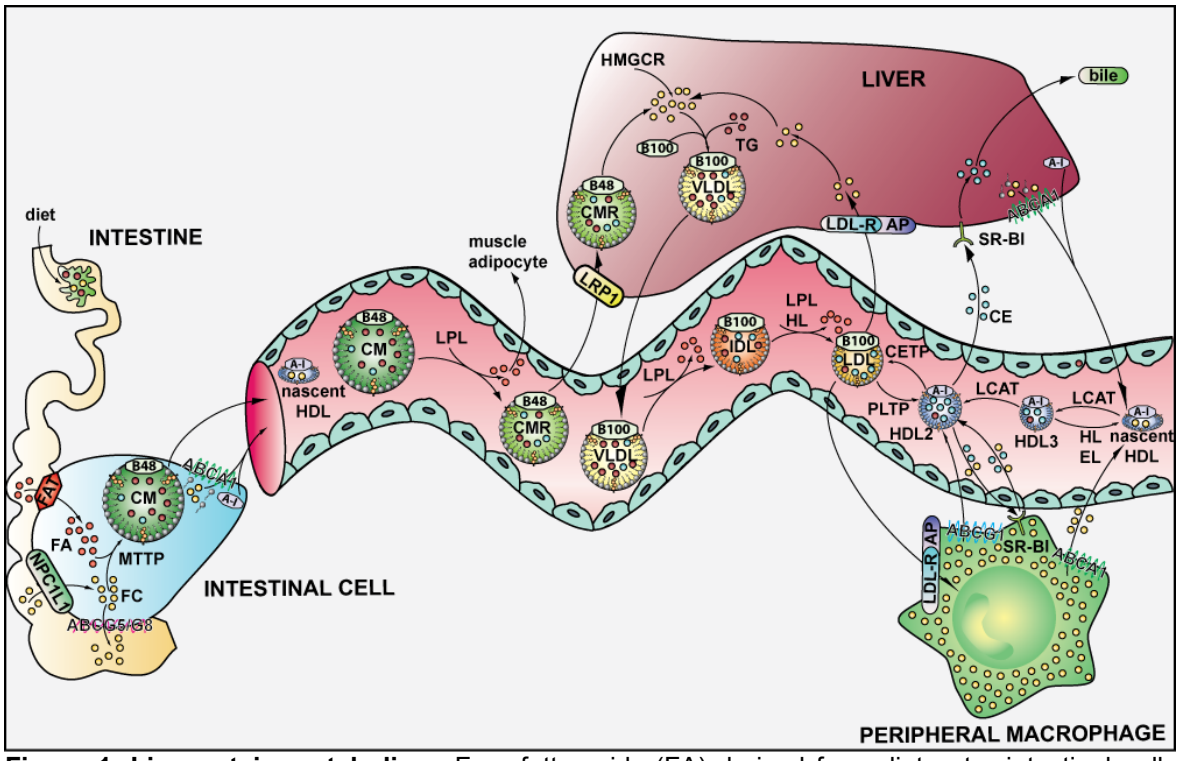


Figure 1. Lipoprotein metabolism. Free fatty acids (FA) derived from diet enter intestinal cells (enterocytes) via the fatty acid transporter (FAT) while cholesterol is transported into enterocytes via the Niemann-Pick C1-like 1 (NPC1L1) protein. In enterocytes, free cholesterol (FC) is packaged together with FA and apolipoprotein (apo)-B48 by the microsomal triglyceride transfer protein (MTTP) into chylomicrons (CMs) which are released into the circulation. In the bloodstream, the triglyceride (TG)-rich core of CMs is hydrolyzed by lipoprotein lipase (LPL). The released FAs are either used for energy production in the muscle or are stored as TGs in adipocytes. The TG-depleted, cholesterol rich CM remnants are taken up by the hepatic LDL-receptor like protein-1 (LRP1). In the liver, cholesterol, endogenously synthesized by the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) or derived from diet, is packaged with TG and apoB-100 into very low-density lipoprotein (VLDL). In circulation, VLDL is hydrolyzed by LPL leading to the formation of intermediate-density lipoprotein (IDL) which is further hydrolyzed by LPL and hepatic lipase (HL) yielding low-density lipoprotein (LDL). LDL is endocytosed by hepatocytes or peripheral cells via the LDL receptor (LDL-R), assisted by an adaptor protein (AP).

The initial step in the biosynthesis of HDL is the secretion of apoA-I from the liver or intestine where it is immediately lipidated to form nascent HDL. This is achieved by the binding of apoA-I to the ATP-binding cassette transporter (ABC) A1. In the periphery, nascent HDL obtains phospholipids and free cholesterol via ABCA1. The nascent HDL gets converted to its mature, spherical form by a series of enzymatically-driven modifications starting with the action of lecithin-cholesterol transferase (LCAT). LCAT mediates the conversion of free cholesterol to cholesteryl ester (CE) which is sequestered into the core of HDL, making HDL spherical. Through the continuous action of LCAT and, additionally, phospholipid transfer protein (PLTP), HDL becomes the mature HDL2 form. PLTP mediates the transfer of phospholipids and cholesterol from TG rich lipoproteins to HDL. The mature HDL particles are the main acceptor for ABCG1- and scavenger receptor-BI (SR-

BI)-mediated cholesterol export. Large HDL particles can be converted into smaller ones by the action of CE transfer protein (CETP), hepatic lipase (HL) and endothelial lipase (EL). CETP catalyzes the transfer of CEs in exchange with TGs between HDL and apoB-containing lipoproteins, which are subsequently taken up by the liver. The last step in the reverse cholesterol transport is the transport of CE back to the liver via SR-BI. Excess cholesterol is excreted via the bile.

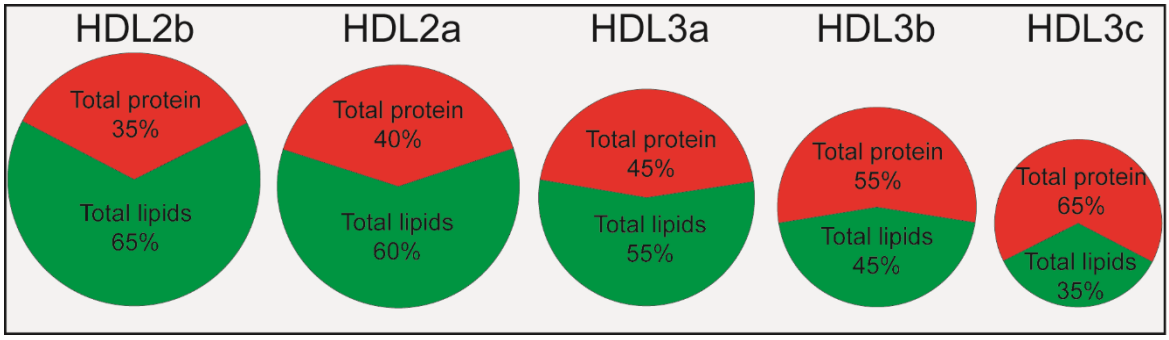
1.4. HDL

1.4.1. Structure, Composition and Heterogeneity of HDL

HDL consists of a group of particles which are highly heterogeneous in their physicochemical properties including size, electrophoretic mobility, lipid- and protein composition (32) (Figure 2). Traditionally, HDL is fractionated on the basis of density by ultracentrifugation into two major subfractions, HDL_2 (1.063-1.125 g/ml), which are large, cholesteryl ester-rich, and HDL_3 (1.125-1.21 g/ml) which are small, cholesteryl ester-poor. However, these subclasses can be further separated by a nondenaturing polyacrylamide gradient gel electrophoresis on the basis of particle size into five distinct subclasses: HDL_{2b} (10.6 nm), HDL_{2a} (9.2 nm), HDL_{3a} (8.4 nm), HDL_{3b} (8.0 nm) and HDL_{3c} (7.6 nm) (33). Additionally, HDL can be separated on the basis of electrophoretic mobility into α- and pre-β-migrating particles or can be divided on the basis of apolipoprotein content into particles containing only apoA-I (LpA-I) and both apoA-I and apoA-II (LpA-I/A-II) (34,35).

Depending on the isolation procedure, upwards of 85 proteins have been identified on HDL (36). ApoA-I is the main apolipoprotein on HDL particles accounting for about 70% of the total protein content, whereas ApoA-II is the second most abundant apolipoprotein representing ~20%. In addition, the presence of apoE, apoA-IV, apoA-V, apoJ, apoC-I, apoC-II, apoC-III, apoM have been confirmed as well as proteins involved in the complement system, immune response, and lipid metabolism as well as acute phase response proteins, protease inhibitors, and even metal-binding proteins (36,37). Due to this complexity, it is most likely that specific particles fulfill distinct functions. Therefore, routine measurement of HDL cholesterol has to be treated with caution since this measurement primarily reflects

levels of the large, cholesterol-rich HDL particles while no information about the small, cholesterol-poor HDL particles is provided.



	HDL2b	HDL2a	HDL3a	HDL3b	HDL3c
Diameter (nm)	10.4	10.3	9.9	8.0	7.3
Density (g/ml)	1.099	1.107	1.123	1.155	1.186
MW (Da)	4.1×10^5	4.0×10^5	3.6×10^5	2.0×10^5	1.6×10^5
			mol/mol HDL		
Apolipoproteins					
ApoA-I	4-5	4	3-4	3	2-3
Others	≤ 2	≤ 2	≤ 2	1	≤ 1
Surface lipids					
PL	130	140	120	45	25
FC	70	40	25	15	10
Core lipids					
CE	180	160	140	70	40
TG	30	20	15	10	5

Figure 2. HDL subclasses. MW, molecular weight; PL, Phospholipid; FC, free cholesterol; CE, cholesteryl ester; TG, triglyceride

1.4.2. HDL-metabolism and reverse cholesterol transport

A schematic overview about HDL metabolism is depicted in Figure 1. The initial step in HDL biosynthesis is the lipidation of apoA-I, the main apolipoprotein on HDL which is mainly produced in the liver and in the intestine, to produce lipid-poor, discoidal, nascent HDL. This is achieved by the binding of apoA-I to the

adenosine triphosphate (ATP) binding cassette transporter (ABC) A1, an integral membrane transporter protein that mediates the export of phospholipids and cholesterol to lipid-poor, nascent HDL (38). The crucial role for ABCA1 in HDL metabolism was highlighted by the finding of genetic defects in the ABCA1 gene as it occurs in Tangier disease leading to relatively low levels of HDL cholesterol, accumulation of cholesterol in peripheral tissues, and premature development of atherosclerosis (39,40). Additional support for the pivotal role of ABCA1 in cholesterol efflux to lipid poor HDL was given by the observation that apoA-I mediated efflux was severely impaired by pharmacological inhibition of ABCA1, but significantly increased by overexpression of ABCA1 (41). Nascent HDL particles are heterogeneous in respect to size and lipid content. They comprise 2-3 apoA-I per particle. However, some particles contain only one apoA-I. This species is termed pre-β HDL. The nascent HDL eventually gets converted to its mature, spherical form by a series of enzymatically-driven modifications starting with the action of lecithin-cholesterol transferase (LCAT). LCAT transfers the 2-acyl group of phosphatidylcholine (lecithin) or phosphatidylethanolamine to the free hydroxyl residue of cholesterol thereby generating cholesteryl ester, which is then sequestered into to the core of HDL, eventually making the particle spherical (42). The spherical form is the major form of HDL in circulation and is responsible for cholesterol transport to the liver. A crucial role for LCAT in HDL maturation is supported by the finding that in LCAT-deficient subjects the apoA-I and apoA-II catabolism is accelerated and that in those subjects HDL circulates predominantly as pre-β particle (43). Through the continuous action of LCAT and, additionally, phospholipid transfer protein (PLTP) HDL becomes the mature HDL_2 form. PLTP is ubiquitously expressed in human tissues and mediates the transfer of phospholipids and cholesterol from triglyceride rich lipoproteins to HDL. PLTP also plays an important part in the remodeling of HDL into larger and smaller particles by initiating particle fusion, accompanied by a dissociation of apoA-I (44). A critical role for PLTP in HDL homeostasis was shown in PLTP knockout mice in which markedly reduced HDL levels could be observed (45). The mature HDL particles are the main acceptor for ABCG1- and scavenger receptor-BI (SR-BI)-mediated cholesterol export. Despite SR-BI and ABCA1- mediated efflux, ABCG1 efflux is not dependent upon interaction with acceptor proteins. Instead, it has been proposed that ABCG1 enriches the plasma membrane with cholesterol, thereby

increasing the rate constant for passive diffusional cholesterol efflux to acceptor lipoproteins along a cholesterol gradient (46). Targeted disruption of ABCG1 in mice resulted in massive lipid accumulation in various tissues, whereas overexpression of ABCG1 protected against lipid deposition (47). SR-BI mediates the bidirectional flux of cholesterol between cells and HDL. In addition to ABCG1, SR-BI also requires phospholipid-rich cholesterol acceptors. It has been suggested that SR-BI mediates cholesterol efflux in a process independently of HDL tethering to the cell surface. SR-BI rather promotes the generation of a hydrophobic environment in cell membranes promoting bidirectional cholesterol movement (48). However, other studies with mutant forms of apoA-I and SR-BI suggest that lipoprotein binding is essential for a proper cholesterol transport (49,50). Another mechanism by which excess cholesterol can be removed from lipid-laden macrophages onto HDL is aqueous diffusion. Diffused free cholesterol can collide in the extracellular aqueous phase with HDL leading to rapid uptake into the lipoprotein acceptor. Interestingly, this process is independent of the particle size of HDL, meaning that all subclasses are equally effective cholesterol acceptors (51). Large HDL particles can be converted into smaller ones by the action of cholesteryl ester transfer protein (CETP), HL and endothelial lipase (EL). CETP catalyzes the transfer of cholesteryl esters in exchange with triglycerides between HDL and apoB-containing lipoproteins which are subsequently taken up by the liver. This pathway is referred to be the indirect cholesterol transport and accounts for the majority of cholesterol transported to the liver where it is excreted as bile acids. In dyslipidemia, CETP levels are elevated and associated with higher cardiovascular risk (52). Subjects with a mutation in the CETP gene were shown to have significantly elevated HDL-cholesterol and apoA-I levels whereas LDL-cholesterol and apoB-levels were reduced (53). HL is predominantly synthesized in the liver and catalyzes the hydrolysis of triglycerides and phospholipids on HDL and LDL, generating small HDL and small LDL. HDL particles in HL-deficient subjects are enriched in phospholipids and triglycerides (54). Through the concerted action of CETP and HL; the large HDL particle is getting smaller in size, pre-β HDL is formed and apoA-I dissociates which can be re-lipidated by ABCA1 in a next lipidation cycle (55). Besides LPL and HL, EL also belongs to the LPL family but, in contrast to the two others, EL mainly acts as a phospholipase and hydrolyzes phospholipids on HDL at the *sn*1 position (56). EL

is primarily synthesized by endothelial cells. It possesses a heparin- and lipid-binding domain. Thus EL has additionally to its catalytic phospholipase activity a noncatalytic bridging activity. After secretion, it binds directly to proteoglycans on the endothelium and to circulating lipoproteins, thereby; EL acts as a bridge and promotes lipoprotein incorporation (57). In humans, EL concentration is negatively correlated with HDL-cholesterol levels and positively associated with features of the metabolic syndrome and atherosclerosis (58).

To sum up, efflux of cholesterol is essential for maintaining homeostasis. An overload of excess cholesterol in the periphery can lead to foam cell formation and subsequently to the development of atherosclerotic plaques. Therefore, the ability of HDL to remove cholesterol from lipid laden macrophages via different pathways is essential in preventing or even regressing atherosclerosis. Recent data have clearly shown that the cholesterol efflux potential of HDL is a strong inverse predictor for coronary artery disease independent of HDL-cholesterol levels (59).

1.4.3. Other important functions of HDL

HDLs ability to mobilize cholesterol from cells is considered to be a fundamental function of HDL. However, recent insights have provided clear evidence of additional HDL-mediated functions which are thought to be of particular importance (Figure 3).

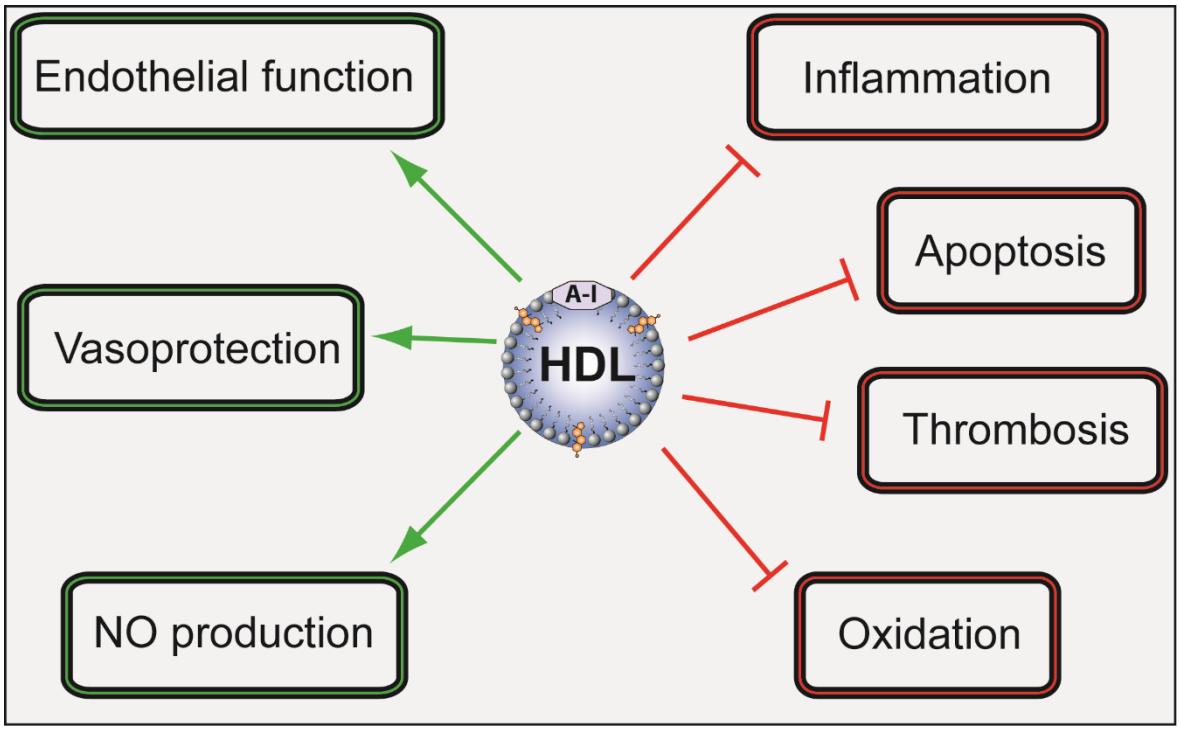


Figure 3. Non-reverse cholesterol transport functions of HDL

1.4.3.1. Anti-oxidant activity

A growing body of evidence suggests that HDL possesses potent anti-oxidant activity by counteracting the proatherogenic oxidation of LDL which is considered as an initial step in the development of atherosclerosis. This is usually attributed to HDL-associated proteins like apoA-I, paraoxonase (PON) and platelet activating factor acetylhydrolase, also known as lipoprotein associated phospholipase A2. PON catalyzes the breakdown of oxidized phospholipids in LDL. An important role for PON in atheroprotection was supported by several findings in animal studies. Transgenic mice lacking PON were significantly more susceptible to atherosclerosis than control mice (60). Additionally, PON activity was significantly reduced in apoE -/- and LDL-receptor -/- mice which are prone to atherosclerosis (61). Furthermore there is strong evidence for a mechanistic link between PON activity with oxidative stress and cardiovascular risk in humans (62). Platelet activating factor acetylhydrolase promotes the hydrolysis of platelet activating factor, a potent pro-inflammatory lipid mediator. Overexpression of human apoA-I in apoE -/- mice increased platelet activating factor acetylhydrolase activity with a simultaneous reduction of oxidative stress (63). In addition, HDL also has intrinsic anti-oxidant properties which are independent of PON and platelet activating factor acetylhydrolase. ApoA-I possesses several methionine sulfoxides. Thereby it can

directly act as an anti-oxidant and reduce the formation of hydroperoxides of cholesteryl esters and phosphatidylcholine (64).

1.4.3.2. Anti-inflammatory activity

Activation of the vascular endothelium and a subsequent expression of adhesion molecules (such as E-selectin, vascular cell adhesion molecule-1, intercellular adhesion molecule-1) and the release of pro-inflammatory mediators like monocyte chemotactic protein-1 (MCP-1) is a crucial event in numerous chronic inflammatory disorders. It was shown by several groups that both HDL and reconstituted HDL (rHDL) inhibit the cytokine induced expression of E-selectin, vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 *in vitro* (65-67). Interestingly, the inhibitory effect was persistent even when HDL was removed before stimulation with tumor necrosis factor-α (TNF-α) (68). These inhibitory effects of HDL on endothelial adhesion molecule expression have also been observed *in vivo* in animal models (69,70). In addition, previous studies reported that lipopolysaccharide (LPS)-induced overproduction of pro-inflammatory cytokines by monocytes can be abolished by incubation of whole blood with rHDL (71,72). The production of MCP-1, a key regulator of monocyte recruitment to sites of vascular inflammation, can be inhibited by HDL-associated lysosphingolipids (73).

1.4.3.3. Endothelium regenerative activity

The endothelium, an interface between the vascular wall and the blood flow, plays a pivotal role for vascular homeostasis. Alterations in endothelium integrity and functions, including a decreased nitric oxide (NO) bioavailability and increased affinity for leukocytes, contribute to the development of atherosclerosis. HDL was shown to promote activation of the endothelial nitric oxide synthase (eNOS) *in vitro* in human endothelial cells (74), *in vivo* using animal models (75), and also in humans after intravenous rHDL infusion (76). Notably, HDL-mediated stimulation of eNOS is impaired in patients with chronic heart failure. However, exercise training can improve the compromised HDL-mediated vascular effects (77). Mechanistically, HDL regulates eNOS activation through the endothelial SR-BI receptor with a subsequent activation of Src Tyrosine kinase, phosphatidylinositol-

3-kinase (PI-3K) kinase, Akt kinase and ERK1/2 MAP kinase leading to the phosphorylation of eNOS at Ser^{1177} (78). In addition, the HDL-associated sphingosine 1 phosphate (S1P) is importantly involved in the activation of eNOS. After binding to $S1P_1$ and $S1P_3$ receptors, intracellular Ca^{2+} is released leading to an activation of the PI-3K/Akt pathway (79).

1.4.3.4. Anti-apoptotic activity

HDL promotes endothelial repair mechanisms by enhancing the growth of endothelial progenitor cells at sites of a damaged endothelium (80) and reduces endothelial cell apoptosis which can be induced e.g. by oxidized LDL or TNF-α (81,82). Moreover, HDL has been shown to stimulate cell migration and anti-apoptotic effects also through the binding to SR-BI but independently of eNOS activation (83). In addition, HDL protects macrophages from apoptosis by promoting efflux of 7-ketocholesterol and oxysterols via ABCG1 (84).

1.4.3.5. Anti-thrombotic activity

HDL has multiple anti-thrombotic actions. It increases blood flow through the enhancement of prostacyclin synthesis and activation of eNOS and the subsequent increased bioavailability of NO. Both, prostacyclin and NO, induce vaso-relaxation and inhibit platelet activation (85). It has been shown that, when infused into isolated rat hearts, HDL increases prostacyclin production (86). In addition, HDL reduces platelet activity by suppressing the biosynthesis of thromboxane A2. Together with the increased synthesis of prostacyclin this leads to decreased platelet aggregation (85).

1.4.1. HDL as a drug target

HDL was first identified in the late 1940s (87). However, it took more than 20 years to identify an inverse relationship between HDL-cholesterol levels and cardiovascular disease (88). Over the last decades, the proposition that HDL is cardioprotective was further strengthened by several observations in human population studies and animal studies. Plasma concentrations of HDL-cholesterol and of apoA-I, the main apolipoprotein of HDL, were shown to be independent, inverse risk factors for cardiovascular events in humans (89-91). Transgenic

overexpression of apoA-I and increased HDL levels in mice were protective against the development of fatty streak lesions (92). Additionally, intravenous infusion of HDL led to atherosclerotic lesion regression in rabbits (93). As a proof-of-principle, infusion of rHDL, consisting of apoA-I and phosphatidylcholine, was protective against coronary plaque burden and significantly improved the coronary score as assessed by coronary angiography (94,95). However, pharmacological interventions that raise HDL-cholesterol levels could not yet be translated into a reduction of morbidity and mortality associated with cardiovascular diseases. The CETP inhibitors torcetrapib, dalcetrapib and evacetrapib successfully increased HDL-cholesterol and apoA-I levels. However, neither dalcetrapib nor evacetrapib or torcetrapib reduced cardiovascular events. Torcetrapib even caused harm, possibly because of off-target effects, likely by an increased blood pressure (96,97). The most widely treatment strategy to raise HDL-cholesterol is the use of niacin. Niacin significantly raises HDL-cholesterol and apoA-I levels by inhibition of HDL catabolism. However, two large randomized controlled trials of niacin have shown no beneficial effects on cardiovascular events and demonstrated that niacin may have severe adverse effects (98). These studies could not strengthen the theory that simply raising HDL-cholesterol levels would be beneficial. However, one has to keep in mind that those pharmacological treatment strategies delayed HDL catabolism to raise HDL-cholesterol levels. Thereby large, cholesterol-rich HDL_2 particles are generated which do not, in contrast to small, dense HDL_3 particles, interact efficiently with ABCA1 (99). ABCA1 is the major mediator of cholesterol efflux to small HDL_3 particles. It has been shown recently that the cholesterol efflux potential of HDL is inversely associated with cardiovascular events independent of established cardiovascular risk factors (59). Patients with apoA-I_{Milano}, a mutant form of apoA-I (Arg173 → Cys mutation) have very low levels of HDL (100), consisting primarily of HDL_3 particles with a nearly total absence of HDL_2 particles (101). The findings that those patients have no increase in atherosclerosis and infusion of reconstituted apoA-I_{Milano} in patients with acute coronary syndrome even leads to a regression of atherosclerosis (102), supports the concept of a divergence between HDL quantity and quality. HDL is a very heterogeneous particle. Therefore it is essential to determine the functionality of individual HDL subpopulations and to focus on interventional studies aiming on elevating the functional activity of HDL.

1.4.2. HDL dysfunction

There is compelling evidence from recent studies that inflammatory diseases such as chronic kidney disease, psoriasis, cardiovascular disease, rheumatoid arthritis and also aging, lead to alterations in HDL composition and function. Thereby, HDL can be rendered dysfunctional or even pro-inflammatory (103-106). It was shown that accumulation of acute phase protein serum amyloid A (SAA) on HDL decreased its protective functions (107). In addition, enrichment in lysophosphatidylcholine and phosphatidic acid also render HDL dysfunctional (108), and oxidized lipids in HDL can promote monocyte adhesion to the endothelium (109). These compositional alterations are linked to changes in HDL functions including the cholesterol efflux potential, the anti-oxidative and anti-inflammatory capacity, and PON activity (104,107,109-111).

1.4.3. HDL in cirrhosis

The liver plays a pivotal role in lipid metabolism and cholesterol homeostasis. Therefore, it is not surprising that abnormalities in the lipid and lipoprotein profile are often observed in patients with cirrhosis. Studies have shown that cirrhosis is accompanied by a reduction in serum levels of HDL, LDL, and total cholesterol (112,113) most likely because of an impaired biosynthesis. In line with this notion, HDL, LDL and total cholesterol levels decrease progressively with the severity of liver disease (114). It has been observed that HDL cholesterol levels were decreased in alcohol-induced liver disease but returned to normal values with abstinence (115). In addition to reduced HDL-cholesterol levels, it was also reported that apoA-I levels decreased progressively with advancing stages of liver disease (116,117). Of particular interest, HDL-cholesterol was shown to be associated with 6- and 12-month mortality (118).

Up to now, all studies about lipoproteins in cirrhosis only determined changes in HDL-cholesterol levels in patients. On the basis of the discovery that HDL might be modified in different diseases leading to HDL dysfunction, it becomes increasingly apparent that the steady-state assessment of circulating HDL concentrations may only incompletely reflect *in vivo* functionality. Therefore, direct

measures of HDL function are rather needed than relying on surrogate markers such as HDL cholesterol levels.

1.5. Aim of the study

This study aimed to elucidate possible compositional and functional changes of HDL in cirrhosis.

Since lipoproteins are essential components controlling cellular functions and homeostasis, we hypothesized that an altered lipid profile in cirrhosis patients is linked to disturbed anti-inflammatory and endothelial regenerative activities of HDL. In the present study, we assessed PON activity of serum HDL from patients with compensated and decompensated cirrhosis and its ability to suppress monocyte activation, to promote cholesterol efflux, to stimulate the activation of eNOS and to induce endothelial barrier function. Furthermore, we were interested to identify possible alterations in the protein and lipid composition of HDL of patients with cirrhosis compared to healthy subjects.

2. Material and Methods

2.1. Materials

Table 2. List of materials

Name	Pr. Nr.	Company
[^{3}H]-cholesterol	ART0255	Hartman Analytic
2,2'Azobis(2-methylpropionamidine)dihydrochloride (AAPH)	44091-4	Sigma
8-(4-chlorophenylthio)-adenosine 3',5'-cyclic monophosphate sodium salt	C3912	Sigma
Acetic acid	67551	Roth
Acrylamide	7906.2	Roth
Apolipoprotein AI	courtesy of Dr. Christian Wadsack	
Apolipoprotein AI Human ELISA Kit	ab108804	Abcam
Calcium chloride	208290	Merck
Chelex-100	C7901	Sigma
Chloroform	2445	Merck
Cholate	C6445	Sigma
Cholesterol	C3045	Sigma
Cholesterol FS Kit	11300/1350	DiaSys
Cholesteryl-ester transfer kit	KA0790	Abnova
Dihydrorhodamine	D1054	Sigma
Dimethyl sulfoxide (DMSO)	109678	Merck
Ethylenediaminetetraacetic acid (EDTA)	8043.2	Roth
Gelatine	4078	Merck
Glycine	3908.2	Roth
HEPES, iron-free	H4034	Sigma
Human Basic Kit FlowCytomix	BMS8420FF	eBioscience
IGEPAL CA-630	I3021	Sigma
Isopropanol	I9516	Sigma
L-cysteine	C7352	Sigma
Lecithin-cholesterol assay kit	428900	Merck
Lipopolysaccharide (LPS)	L3012	Sigma
Methanol	T169.2	Roth

N,N'-Methylene bisacrylamide	29195.02	Serva
NativeMARK™ Unstained Protein Standard	LC0725	Life Technologies
PD Spintrap G-25	28-91-80-04	GE Healthcare
PD-10 columns	17-0851-01	GE Healthcare
Phenylacetate	108723	Sigma
Phosphatidylcholine	153189	Avanti Polar Lipids
Phosphatase-Inhibitor-Mix II	39055.01	Serva
Phospholipid assay kit	KA0791	Abnova
Phospholipid FS kit	15741	DiaSys
Pierce™ ECL Western Blotting Substrate	32106	Thermo Fisher
Polyethylenglycol	P1458	Sigma
Potassium bromide	60093	Sigma
Potassium phosphate	P5655	Sigma
Protease-Inhibitor-Mix M	39102.01	Serva
Sandoz 58-035	S9318	Sigma
*sn*1-lipase activity kit	700640	Cayman
Sodium chloride (NaCl)	3957.1	Roth
Sodium dodecyl sulfate (SDS)	5136.1	Roth
Sodium-Deoxycholate	3484.2	Roth
Sudan black	199664	Sigma
Tetramethylethylenediamine (TEMED)	2367.1	Roth
Triglyceride FS Kit	157609910021	DiaSys
TRIS	5429.2	Roth
Triton X-100	6683.1	Roth

Table 3. List of antibodies

Name	Dilution	Pr. Nr.	Company
Anti-apoA-I, mouse monoclonal	1:1,000	NB100-65491	Novus Biol.
Anti-eNOS ($pSer^{1177}$), mouse monoclonal	1:2,000	612392	BD Bioscience
Anti-eNOS ($pThr^{495}$), mouse monoclonal	1:2,000	612706	BD Bioscience
Anti-mouse IgG, goat polyclonal	1:1,000	31430	Thermo Fisher
Anti-mouse IgG-Biotin, goat polyclonal	1:5,000	B7264	Sigma
Anti-rabbit IgG-HRP, goat polyclonal	1:1,000	7074s	Cell Signaling
NOS3 (C-20), rabbit polyclonal	1:500	sc-654	Santa Cruz
Streptavidin-HRP	1:2,000	21124	Thermo Fisher

Table 4. List of cell culture materials

Name	Product Number	Company
Bovine serum albumin (BSA), fatty acid free	A8806	Sigma
Dulbecco's Modified Eagle's Medium (DMEM)	41965-039	Gibco
Phosphate buffered saline (PBS) with Ca & Mg	P04-35500	PAN
Endothelial growth basal medium (EBM)-2	CC-3156	Lonza
Endothelial cell growth medium (EGM)-2 MV Single Quots	CC-4147	Lonza
Fetal bovine serum (FBS) Gold	SV30180.03	GE Healthcare
Penicillin/Streptomycin 100x	P06-07100	PAN
Roswell Park Memorial Institute medium (RPMI) 1640	P04-16500	PAN

2.2. Methods

Since parts of this work have previously been published as an original article in “BBA - Molecular and Cell Biology of Lipids” the Method chapter has been partially adopted from there (119).

2.2.1. Cell culture

RAW264.7 macrophages: Male, murine RAW264.7 macrophages were cultivated in DMEM with stable L-glutamine, 4.5 g/L D-glucose and supplemented with 10% FBS and 1% PS (penicillin (100 units/ml) /streptomycin (100 µg/ml)). Cells were passaged when 80% to 90% confluence was reached.

U937 monocytes: Human monocytic U937 cells were maintained in RPMI 1640 with stable glutamine and supplemented with 10% FBS and 1% PS (100 units/ml/100 µg/ml).

HCAEC: Human coronary artery endothelial cells were cultivated in EBM-2 supplemented with 5% FBS, 0.04% hydrocortisone, 0.4% human fibroblast growth factor-beta (hFGF-β), 0.1% vascular endothelial growth factor (VEGF), 0.1% R3-insulin-like growth factor-1 (R3-IGF-1), 0.1% ascorbic acid, 0.1% human epidermal growth factor (hEGF) and 1% GA-1000 (gentamicin (30 mg/ml) / amphotericin (15 µg/ml). Cells were passaged when 80% to 90% confluence was reached and used within 4 passages for experiments.

HAEC: Human aortic endothelial cells were maintained in the same culture medium as described for HCAECs.

2.2.2. Subjects

The study population of this explorative cross sectional study comprised 59 consecutive patients with clinical and radiological evidence of cirrhosis, and/or biopsy proven cirrhosis. Patients with a Child-Pugh score > 11, abstinence from alcohol for < 2 weeks, clinical evidence of active infection, antibiotic treatment within 7 days prior to enrolment (except for primary or secondary prophylaxis of spontaneous bacterial peritonitis), gastrointestinal hemorrhage within the previous 2 weeks, use of immune modulating agents within one month (steroids etc.), renal failure (such as hepatorenal syndrome), creatinine >1.5x ULN, hepatic encephalopathy II to IV, pancreatitis, other organ failure, hepatic or extra-hepatic malignancy, and pregnancy were excluded. In addition, 21 age- and sex-matched consecutive cirrhotic patients who were admitted with acute decompensation of their liver disease were included. There were no restrictions regarding etiology of cirrhosis or reason for decompensation. Furthermore, 20 sex-matched healthy

controls were included after they passed following exclusion criteria: any history of cardiovascular disease, pregnancy, obesity, dyslipidemia, liver disease, renal disease, diabetes or clinical signs of inflammation. Control subjects were free of lipid-lowering medication and anti-inflammatory drugs. Blood was sampled from patients and healthy volunteers in serum tubes (Greiner, Kremsmünster, Austria) after obtaining written informed consent. The study protocol was approved by the Ethics Committee of the Medical University of Graz and informed consent was obtained in accordance with the Declaration of Helsinki (No. 23-056 ex. 10/11, No. 23-285 ex 10/11 and No. 21-523 ex. 09/10). This subject description has also been published in an original article (119).

2.2.3. Determination of serum lipid composition

Levels of total cholesterol and phospholipids were measured enzymatically at the Clinical Institute of Medical and Chemical Laboratory Diagnostics at the Medical University of Graz. Low-density lipoprotein (LDL) cholesterol was calculated according to the Friedewald equation using HDL cholesterol values measured in the supernatant of the phosphotungstic acid/$MgCl_2$ precipitation. This methodological description has also been published in an original article (119).

2.2.4. Isolation of HDL and lipoprotein deficient sera

Serum density was adjusted with potassium bromide (KBr) to 1.24 g/ml, and a two-step density gradient was generated in centrifuge tubes (16 × 76 mm, Beckman) by layering the density-adjusted serum underneath a KBr density solution (1.063 g/ml) as described (103). Tubes were sealed and centrifuged at 60,000 rpm for 5 h in a 90Ti fixed angle rotor (Beckman Instruments, Krefeld, Germany). After centrifugation, the HDL-containing band and the lipoprotein deficient sera (bottom fraction) were collected, and desalted via PD10 columns (GE Healthcare, Vienna, Austria). This methodological description has also been published in an original article (119).

2.2.5. Preparation of serum HDL

The apolipoprotein B containing lipoproteins LDL and VLDL were depleted from serum by addition of 40 µL polyethylenglycol (20% in 200 mmol/L glycine buffer) to 100 µL serum (120). Serum was incubated at room temperature for 20 minutes and the supernatant recovered after centrifugation (10,000 rpm, 20 minutes, 4°C). The recovered apoB-depleted serum is termed “serum HDL”. This methodological description has also been published in an original article (119).

2.2.6. Preparation of reconstituted HDL

Discoidal reconstituted HDL was prepared using the cholate dialysis method as described (121). L-α-phosphatidylcholine and free cholesterol were dissolved in chloroform to 25 mg/ml and 2.5 mg/ml respectively, while apoA-I was dissolved in 0.2 mol/L potassium phosphate buffer at a pH of 7.4 to 3 mg/ml. From these stock solutions, 216 µl (5.4 mg) PC was mixed with 56 µl (0.14 mg) FC and chloroform was evaporated under a stream of argon. The dried PC/FC was resuspended during vortexing by drop wise addition of sodium-cholate (3% solution in 0.2 mol/L potassium phosphate buffer at a pH of 7.4) until a clear solution was achieved. Under steadily vortexing 667 µl (2 mg) apoA-I was added drop wise and vortexed twice for 30 seconds after addition and filtrated through PD-10 columns.

2.2.7. HDL particle size analysis

Serum HDL (2 µL) was separated by native gradient gel electrophoresis (4–16% NativePage; Life Technologies, Vienna, Austria). Gels were run for 120 min at constant voltage of 150 V, in NativePage running buffer (Life Technologies, Vienna, Austria). Gels were subsequently either fixed (with 25% isopropanol/10% acetic acid for 10 min) and neutral lipids stained with Sudan black, or proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane (for 45 min at 100 V). Membranes were probed using a primary monoclonal apoA-I antibody overnight at 4°C and secondary goat anti-mouse IgG for 120 min at room temperature. Size distribution of HDL was analyzed using ImageJ software. Intensity blots of individual samples were obtained and the peak areas of HDL_2, HDL_3 and small HDL_3 particles identified using standard proteins. This methodological description has also been published in an original article (119).

2.2.8. LC-MS/MS Analysis

Proteomic profiling of HDL was performed in collaboration with Dr. Ruth Birner-Grünberger at the Core Facility Mass Spectrometry at the Medical University of Graz. HDL was digested with trypsin, and the resulting peptides were separated after online desalting by nano-HPLC (Dionex Ultimate 3000, Thermo Scientific, Waltham, MA) using a flow rate of 300 nl/min and a 2 hour gradient. The samples were ionized in the nanospray source equipped with nanospray tips and analyzed in an Orbitrap velos pro mass spectrometer (Thermo Scientific, Waltham, MA) in positive ion mode, applying alternative full scan MS (m/z 380-2000) at 60,000 resolution and MS/MS by collision induced dissociation of the 20 most intense peaks in the ion trap with dynamic exclusion enabled. Data were analyzed by searching the human SwissProt public database downloaded on Jul. 13, 2012, with Proteome Discoverer 1.4 (Thermo Scientific, Waltham, MA) and Mascot 2.3 (Matrixscience, London, UK). Detailed search criteria were as follows: trypsin; maximum missed cleavage sites = 2; fixed modification = carbamidomethylation at cysteine; variable modification = oxidized methionine; precursor mass tolerance = ±0.05 Da; and product mass tolerance = ±0.7 Da. Protein hits were subjected to automatic validation by Mascot using a decoy search and an FDR of 1%. Changes in HDL proteome (by comparison of relative protein abundances of the same protein between groups) were evaluated by label free quantitation from areas under the curve of precursor ions (i.e. mean areas of extracted ion chromatograms of the individual peptides matched to a protein) normalized to the total area under the curve of all proteins in each sample. This methodological description has also been published in an original article (119).

2.2.9. Serum enzyme activities

Commercial available kits were used to assess PLTP, CETP, LCAT, and *sn*1-lipolytic activity in sera according to the manufactures instructions.

2.2.9.1. CETP and PLTP

The kits for both activity assays use a self-quenched fluorescent neutral lipid or phospholipid that can be measured when transferred to an acceptor molecule. The transfer results in an increase in fluorescence.

The experimental setting was as follows: 2 µl of serum were transferred to a 384-well plate. Then 70 µl of a master mix consisting of 5% of the donor molecule, 5% of the acceptor molecule, 10% of a supplied buffer and 80% distilled water were added to each well. The fluorescence intensity was measured at an excitation wavelength of 465 nm and an emission wavelength of 535 nm. Upon transfer, the fluorometric intensity (535 nm) is directly proportional to the amount of lipids transferred. Readings were taken every 2.5 minute for 60 minutes. To calculate the enzyme activity, we used the slope of the kinetic chart and expressed it in fluorescence per minute. A standard curve of the donor molecule (0-100 pmol) was used to convert the enzymatic activities expressed in fluorescence per minute into pmol donor molecule transferred per minute.

2.2.9.2. *sn*1 lipases

The used kit was fluorescence based. Lipases (LPL, EL, and HL) in serum hydrolyze arachidonyl-1-thioglycerol to arachidonic acid and thioglycerol which reacts with a thiol fluorometric detector yielding a fluorescent product at 510 nm.

The experimental setting was as follows: 5 µl of serum (diluted 1:75) were transferred to a 384-well plate. Then 65 µl of a master mix consisting of 5% of thiol detector, 5% of lipase substrate and 90% assay buffer were added to each well. The fluorescence intensity was measured at an excitation wavelength of 385 nm and an emission wavelength of 510 nm. Readings were taken every minute for 15 minutes. To calculate the enzyme activity, we used the slope of the kinetic chart and expressed it in fluorescence per minute. A standard curve of thiolglycerol (0-80 µM) was used to convert the enzymatic activities expressed in fluorescence per minute into nmol arachidonyl-1-thioglycerol hydrolyzed per minute.

2.2.9.3. LCAT

The LCAT activity assay kit is fluorescence based. A LCAT substrate fluoresces at 470 nm. Upon hydrolysis by LCAT, a monomer is released from the LCAT substrate that fluoresces at 390 nm. LCAT activity is assessed as a change in 470/390 emission intensity.

The experimental setting was as follows: 1 µl of serum was transferred to a tube. Then 40 µl of buffer containing 150mM NaCl, 10mM TRIS/HCl, 1mM EDTA, 4mM 2-mercaptoethanol and 0.2 µl of LCAT substrate at a pH of 7.4 were added to each tube. After incubation for 5 hours at 37°C, 120 µl READ reagent were added to the mixture. After vortexing, 80 µl was transferred to a 384-well plate. The fluorescence intensity was measured at an excitation wavelength of 340nm and an emission wavelength of 470 nm (representing the non-hydrolyzed substrate) and 390 nm (representing the hydrolyzed substrate).

2.2.10. HDL cholesterol efflux capability

Cholesterol efflux capacity was assessed using an established assay (59,122). RAW264.7 macrophages, maintained in DMEM with 10% fetal bovine serum ($DMEM^+$) were plated on 48-well plates (300,000 cells/well). After 24 hours cells were labeled for another 24 hours in $DMEM^+$ containing 1 µCi/mL [^{3}H]-cholesterol. To upregulate ABCA1, cells were concurrently stimulated with 0.3 mmol/L cAMP. After labeling, cells were washed twice with serum free DMEM and subsequently equilibrated in serum free DMEM containing 0.2% BSA for 2 hours. After additional two washing steps, [^{3}H]-cholesterol efflux was determined by incubating cells for 3 hours with serum free DMEM containing 2.8 % serum HDL. Radioactivity (cpm) in the supernatant was quantified by liquid scintillation counting. To determine the total radioactivity-label, cells were lysed with 0.1% SDS in 0.3N NaOH. Cholesterol efflux was expressed as the radioactivity in the medium relative to total radioactivity in medium and cells. In some experiments RAW264.7 macrophages were grown in the presence and absence of cAMP to assess cAMP specific efflux (ABCA1 dependent efflux) of 2.8 % serum HDL or 50 µg/ml HDL-protein. All steps were performed in the presence of 1% PS and 2 µg/mL of the acyl coenzyme A cholesterol acyltransferase inhibitor Sandoz 58-035. This methodological description has also been published in an original article (119).

2.2.11. Paraoxonase activity

Human serum PON is known to have arylesterase activity (123). Arylesterase activity was determined with a photometric assay using phenylacetate as substrate. 5 µg HDL or 2.5 µl of serum HDL (1:10 diluted) were placed on a 96-

well plate. Added to the wells were 200 µl of a reaction mixture containing 100mM TRIS/HCl, 2mM $CaCl_2$ and 1mM phenylacetate at pH of 8. The rate of hydrolysis was determined by monitoring the absorbance at 270 nm. Readings were taken every 15 sec for 5 min. ΔAb_{270nm} / min was calculated from the slope of the kinetic chart. The enzymatic activity was calculated from the molar extinction coefficient of 1310 $L*mol^{-1}*cm^{-1}$ with the Lambert-Beer Law and a path length correction of 0.5 cm.

2.2.12. Anti-oxidative capacity of HDL

The anti-oxidative capacity of serum HDL was determined by assessing HDL's ability to inhibit oxidation of dihydrorhodamine (DHR) (124). 10 µl of serum HDL (1:10 diluted) were placed in a 384-well plate and 90 µl assay buffer containing 20mM HEPES, 150mM NaCl, 10 g/L Chelex-100, 1mM AAPH and 7.5µM DHR at a pH of 7.4 were added to each well. The fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Readings were taken every 5 minutes for 60 minutes. The increase in fluorescence per minute due to oxidation of DHR was determined for samples containing only DHR and for samples containing DHR and individual serum HDL probes from study subjects.

2.2.13. Determination of nuclear factor κB (NF-κB) expression and cytokine releases

U937 monocytic cells containing a 5x NF-κB green fluorescence protein reporter cassette (125) were cultivated in RPMI 1640 containing 7.5% fetal bovine serum in FACS tubes (25,000 cells/tube). The cells were pretreated for 90 minutes with rHDL (1-50 µg/mL), 10% lipoprotein deficient serum or 7% serum HDL. Subsequently, the cells were stimulated for 24 hours by adding serum-free RPMI 1640 containing 100 ng/ml LPS (final concentration = 50 ng/mL), collected by centrifugation at 400 g for 7 minutes and fixed with a solution consisting of 75% BD FACSFlow™, 22.5% aqua dest. and 2.5% BD CellFIX™ (BD Biosciences, Franklin Lakes, NJ, USA). All steps were performed in the presence of 1% PS. The expression of NF-κB was assessed by flow cytometry, and 8 different cytokines in the supernatant were quantified using a multiplex bead-based

immunoassay (FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA). Per sample, 12.5 µl of an Bead Mixture were prepared containing 1/20 of beads for interleukin (IL)-12, IL-10, IL-8, IL-6, IL-1β, TNF-α and MCP-1. Additionally, per sample 25 µl of a Biotin-Conjugate Mixture were prepared containing 1/20 of each biotin-conjugate. 12.5 µl of a standard dilution series and of sample were transferred to FACS tubes. Then 12.5 µl of the Bead Mixture and 25 µl of the Biotin-Conjugate Mixture were added to each tube. After mixing the content well the tubes were protected from light and incubated for 2 hours at room temperature followed by two washing steps. For this purpose, 500 µl assay buffer were added to each tube and centrifuged at 200 g for 5 minutes. After discarding all of the supernatant except of 50 µl, 300 µl assay buffer were added and centrifuged as mentioned above. All of the supernatant was discarded except of 50 µl and 25 µl of streptavidin-phycoerythrin were added to the tubes. After mixing, the tubes were incubated in the dark for one hour at room temperature, followed by two washing steps as described above. 250 µl of assay buffer were added to each sample and analyzed by flow cytometry. This methodological description has also been published in an original article (119).

2.2.14. Assessment of cytokine concentration in serum

The concentrations of IL-12, IL-8, IL-6, IL-1β, TNF-α, and MCP-1 in serum were assessed as described above.

2.2.15. Endothelial barrier measurements

Impedance measurements at 4,000 Hz were performed using the Electric Cell-substrate Impedance Sensing system (ECIS) (Applied Biophysics, Troy, NY). To maintain electrode capacitance, each well of an 96W1E+ polycarbonate array containing gold microelectrodes (Applied Biophysics, Troy, NY) were incubated with 10mM cysteine for 10 minutes at 37°C, followed by washing twice with distilled water. After coating the cells with 1% gelatin for 30 minutes at 37°C, HCAECs, maintained in EBM-2 containing EGM-2 MV Single Quots, were seeded (50,000 cells/well) and grown to confluence. Cells were starved for 2 hours in EBM-2 without supplements, subsequently electrically wounded (4 seconds at 2,400 µA and 48 kHz) resulting in severe electroporation and killing of cells

situated on the electrodes. Immediately after wounding, cells were incubated with HDL (50 µg/mL), 3% serum HDL or 3% lipoprotein deficient serum to assess repopulation of the wound and barrier promoting capability. Impedance was continuously monitored. This methodological description has also been published in an original article (119).

2.2.16. Determination of endothelial nitric oxide synthase (eNOS) phosphorylation by SDS PAGE and Western blot

HAECs, maintained in EGM-2 medium, were plated on 12-well plates (17,000 cells/well) until 80% - 90% confluence. Cells were incubated with 10% serum HDL for 0, 5, 10, 15, 30, and 60 minutes. Thereafter, cells were gently washed twice with DPBS followed by the addition of ice-cold lysis buffer consisting of 50mM TRIS/HCl. 150mM NaCl, 1mM EDTA, 1% NP-40, 0.25% Na-deoxycholate and containing protease-inhibitor mix M and phosphatase-inhibitor mix II and shock-freezing in liquid nitrogen. SDS lyse buffer was added to harvested cells and heated for 10 minutes at 99°C. Half of the cell lysate was separated on a denaturing polyacrylamide gel and transferred to a PVDF membrane (for 2 hours at 400 mA). Membranes were either probed using a primary monoclonal anti-eNOS ($pSer^{1177}$) antibody or a primary monoclonal anti-eNOS ($pThr^{495}$) antibody overnight at 4°C, a secondary goat anti-mouse IgG-Biotin for 60 minutes at room temperature and with streptavidin-HRP for 60 minutes at room temperature. After detection of specific proteins, the membranes were probed using a polyclonal anti-eNOS antibody overnight at 4°C and a secondary goat anti-rabbit IgG-HRP for 60 min at room temperature. For the evaluation of serum HDL induced phosphorylation at the respective protein, the maximal stimulation was used. All samples were analyzed in duplicates.

2.2.17. Statistical analysis

Comparison of normal distributed groups was performed with One-Way ANOVA and Bonferroni post-hoc test and non-parametric groups were compared with Kruskal-Wallis test. Correlations were determined using the Pearson product-moment estimates. Group differences were considered statistically significant for $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. Statistical analyses were performed using

GraphPad Prism (Version 4.0, GraphPad Software) or SPSS Statistics Version 20. This methodological description has also been published in an original article (119).

3. Results

Since parts of this work have previously been published as an original article in "BBA - Molecular and Cell Biology of Lipids" the Result chapter has been partially adopted from there (119).

3.1. Clinical characteristics of study subjects

Clinical characteristics of the study subjects are given in Table 5. Patients with decompensated cirrhosis had significantly increased levels of C-reactive protein while levels of total cholesterol, HDL-cholesterol and apoA-I were significantly decreased compared to healthy controls. Patients with compensated cirrhosis showed significantly reduced levels of HDL-cholesterol and apoA-I. When compared to compensated cirrhotics, cirrhotic patients with decompensation had significantly reduced levels of total cholesterol, LDL-cholesterol, HDL-cholesterol, apoA-I and albumin while bilirubin was increased. These findings have been published in a similar fashion in an original article (119).

Table 5. Clinical characteristics of study subjects

		cirrhosis	
	control	**compensated**	**decompensated**
n	20	59	21
Age (y)	67 (66-69)	58 (53-64)	58 (50-66)
Male/female	15/5	42/17	16/5
Total cholesterol (mg/dL)	169 (152-188)	173 (134-209)	86 (64-123)***††
Triglycerides (mg/dL)	103 (70-145)	81 (60-105)	77 (62-123)
LDL-cholesterol (mg/dL)	78 (60-102)	107 (72-133)	63 (42-74)††
HDL-cholesterol (mg/dL)	65 (55-80)	44 (25-61)*	10 (6-19)***††
Apolipoprotein A-I (mg/dL)	79 (57-113)	37 (27-52)***	17 (15-24)***†
CRP (mg/L)	2 (1-3)	3 (1-5)	25 (12-46)***††
Child-Pugh Score	-	5 (5-6)	10 (8-12)
MELD Score	-	10 (8-14)	17 (15-19)
Etiology of liver disease (%)			
Alcohol	-	49.1	95.2
Viral infection	-	21.1	4.8
Other	-	29.8	-
Albumin$ (g/dL)	-	4.0 ± 0.6	2.9 ± 0.7†
Bilirubin$ (mg/dL)	-	1.7 ± 1.6	5.3 ± 4.7†
Creatinine$ (mg/dL)	0.9 ± 0.3	0.9 ± 0.2	1.0 ± 0.6

Values are given as medians with the interquartile range. $Values are given as mean ± SD. CRP, C-reactive protein; MELD, model of end stage liver disease. *$P < 0.05$, ***$P < 0.001$ vs. control. †$P < 0.01$, ††$P < 0.001$ vs. compensated. Adopted from (119).

3.2. Cirrhosis alters serum enzyme activities involved in HDL metabolism

HDL particles are emerging as relevant players in innate and adaptive immunity (126). Given that HDL-levels are markedly altered in cirrhotic patients, we first assessed activities of key regulators in HDL maturation and metabolism, such as PLTP, LCAT and CETP. We further sought to analyze serum lipolytic activities releasing acyl chains in *sn1* position, since activities of lipases are altered under inflammatory conditions and can profoundly impact HDL composition and function by mediating the hydrolysis of triglycerides and phospholipids within circulating lipoproteins. In sera of patients with cirrhosis, we observed a significant decrease in the activities of CETP (Figure 4A), *sn*1-lipolytic activity (Figure 4C) and LCAT (Figure 4D). This reduction was even more profound in patients with acute decompensation. Additionally, in sera of decompensated patients, PLTP activity was significantly reduced while there was a trend towards a decreased activity in sera of patients with compensated cirrhosis (Figure 4B). These findings have been published in a similar fashion in an original article (119).

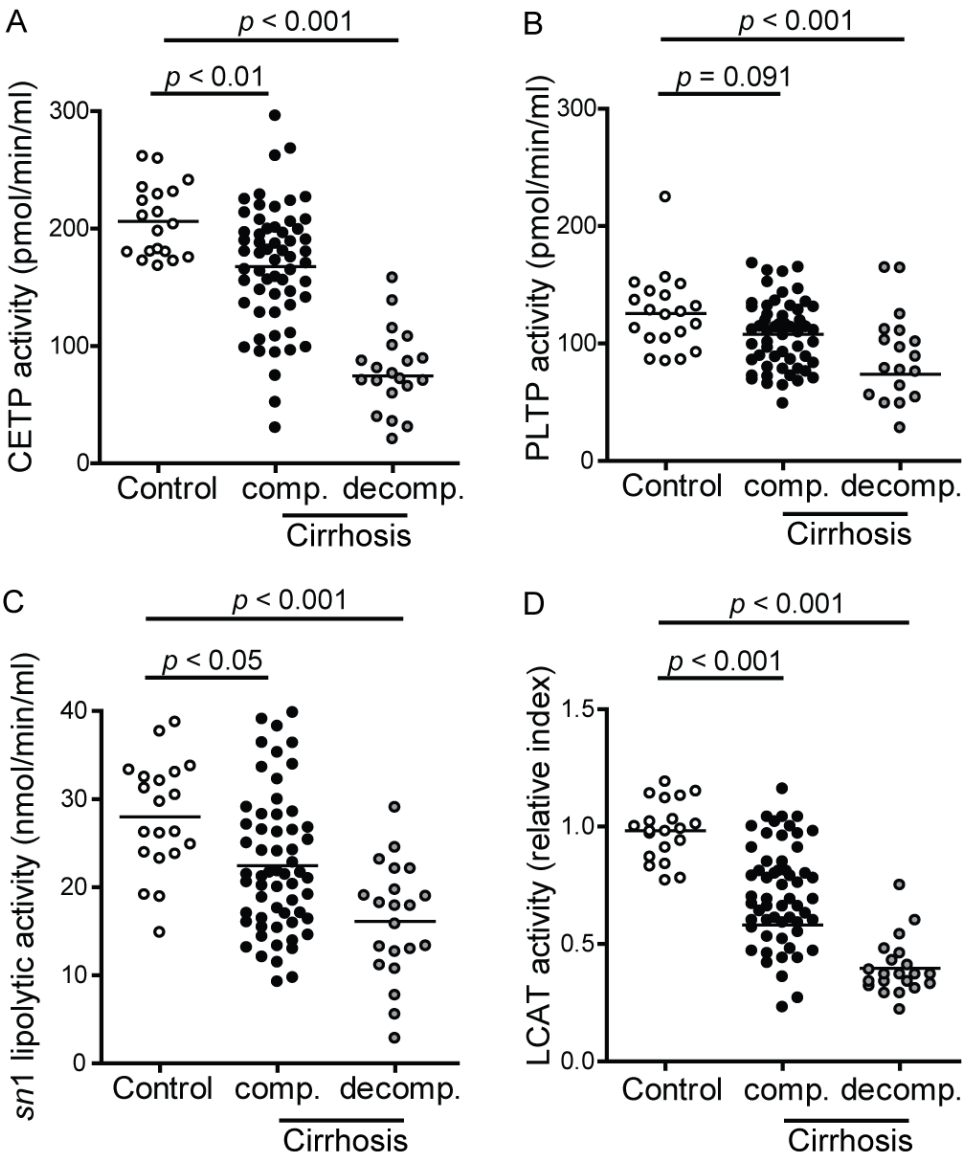


Figure 4. Cirrhosis alters serum enzyme activities involved in HDL metabolism. Sera of healthy subjects (control, n = 20), patients with compensated (comp., n = 59) and acutely decompensated (decomp., n = 21) cirrhosis were examined for activities of (A) cholesteryl-ester transfer protein (CETP), (B) phospholipid transfer protein (PLTP), (C) *sn*1-lipolytic activity and (D) lecithin-cholesterol acyltransferase (LCAT). Adopted from (119).

3.3. Cirrhosis affects HDL subclass distribution

HDL consists of a group of particles which are highly heterogeneous in size and function. Given the tremendous changes in activities of serum enzymes involved in HDL metabolism, we next sought to investigate possible alterations in HDL particle distribution. By examining serum HDL (serum depleted of LDL and VLDL) with native gel, we observed a clear shift from the small, dense HDL_3 particle towards the large, cholesterol-rich HDL_2 subclass in patients with cirrhosis causing in an

increased HDL_2/HDL_3 ratio (Figure 5A, B, C). In addition, western blot analysis revealed a significant decrease of small HDL_3 content in cirrhotic patients (Figure 5A, B, D). These findings have been published in a similar fashion in an original article (119).

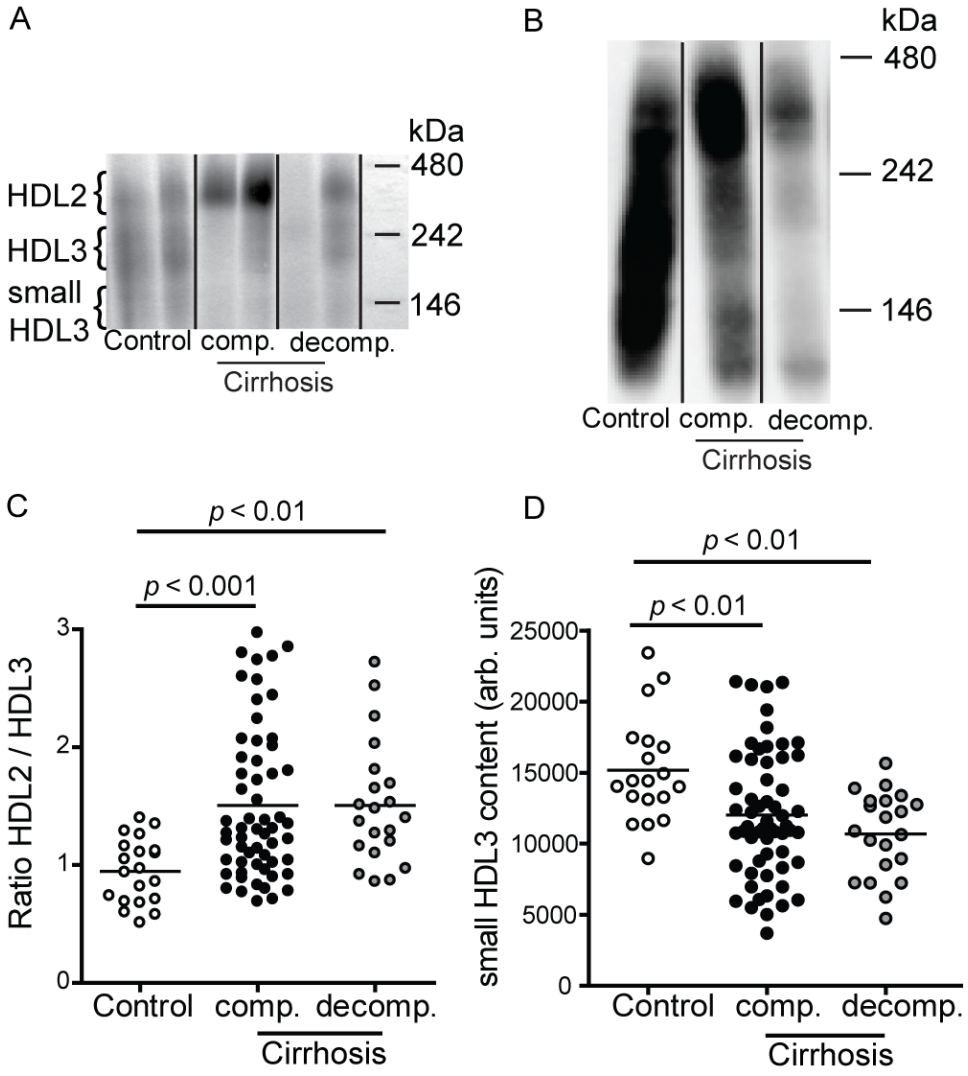


Figure 5. HDL particle distribution in cirrhosis is altered. (A) Serum HDL of healthy subjects (control, n = 20), patients with compensated (comp., n = 59) and acutely decompensated (decomp., n = 21) cirrhosis was separated by native gradient gel electrophoresis and lipids were stained with Sudan Black. (B) Serum HDL was separated by native gradient gel electrophoresis and blotted onto a PVDF membrane. ApoA-I was detected using a monoclonal apoA-I antibody. (C,D) Intensity blots of individual samples were obtained and HDL_2/HDL_3 ratios (C) and small HDL_3 content (D) were calculated. Adopted from (119).

3.4. Serum HDL of cirrhotic patients depicts impaired anti-inflammatory capacity

Patients with cirrhosis are susceptible to bacterial infections which are a common precipitating event in acute-on-chronic liver failure (9-11). Several lines of evidence suggest that HDL acts as an endogenous inhibitor of inflammatory responses (127). It was shown that HDL induced cholesterol efflux depletes membrane cholesterol from immune cells, thereby dampening inflammatory signaling via Toll-like receptors, resulting in the inhibition of cytokine production (128). To demonstrate that HDL is able to directly diminish activation of the transcription factor NF-κB, a key player regulating the immune response in infection, we pretreated U937 monocytes with increasing concentrations of HDL followed by stimulation with LPS. Because plasma HDL is a heterogeneous group of lipoproteins with varying lipid composition and functionality (129), reconstituted HDL was prepared using human apoA-I as the sole protein and phosphatidylcholine as the sole phospholipid. As shown in Figure 6A, rHDL effectively and dose-dependently inhibited LPS induced NF-κB activation. Notably, when all lipoproteins were removed from serum by ultracentrifugation, the remaining lipoprotein deficient serum showed no inhibitory activity (Figure 6B). These data clearly suggest that HDL is required to suppress LPS-induced activation of NF-κB in monocytes. To study anti-inflammatory properties of HDL from patients, we stimulated U937 monocytes with the Toll-like receptor 4 agonist LPS in the presence or absence of serum HDL (of patients and of healthy controls) and measured the activation of NF-κB. Strikingly, in the majority of cases, serum HDL of cirrhotic patients showed a markedly reduced ability to suppress LPS-induced activation of NF-κB when compared to controls (Figure 6C). These findings have been published in a similar fashion in an original article (119).

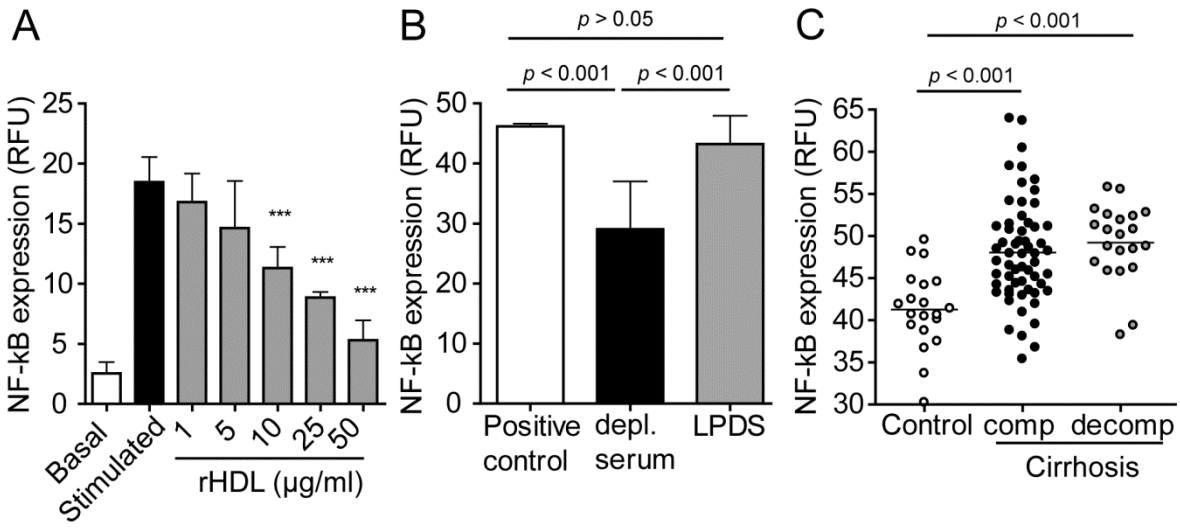


Figure 6. Cirrhosis is associated with a reduced anti-inflammatory capacity of serum HDL. Serum HDL of healthy subjects (control, n = 20), patients with compensated cirrhosis (comp., n = 59), and of cirrhotic patients with acute decompensation (decomp., n = 21) were analyzed for their ability to inhibit lipopolysaccharide (LPS)-induced nuclear factor-κB (NF-κB) activation in monocytes. U937 monocytes containing a reporter cassette for NF-κB, were pretreated with (A) increasing concentrations (1-50 µg/mL) of reconstituted HDL (rHDL), (B) 10% lipoprotein deficient sera (LPDS) or (B,C) 7% serum HDL. After 90 minutes, cells were stimulated with LPS (50 ng/mL) for 24 hours, followed by assessment of GFP expression by flow cytometry. RFU, relative fluorescence units. ***$P < 0.001$ vs. stimulated. Adopted from (119).

3.5. Impaired ability of serum HDL of cirrhotic patients to suppress LPS induced production of cytokines by monocytes

NF-κB is a central pro-inflammatory mediator activating various genes including IL-6, TNF-α, MCP-1, IL-1β, IL-8 and IL-12 (130-135). In line with the reduced ability of serum HDL of cirrhotic patients to inhibit NF-κB activity (Figure 6C), we observed an impaired ability of serum HDL of patients with cirrhosis to inhibit the production of the inflammatory cytokines IL-6 (Figure 7A), TNF-α (Figure 7B) and MCP-1 (Figure 7C). No significant differences were observed for IL-1β (Figure 7D), IL-8 (Figure 7E) and IL-12 (Figure 7F). These findings have been published in a similar fashion in an original article (119).

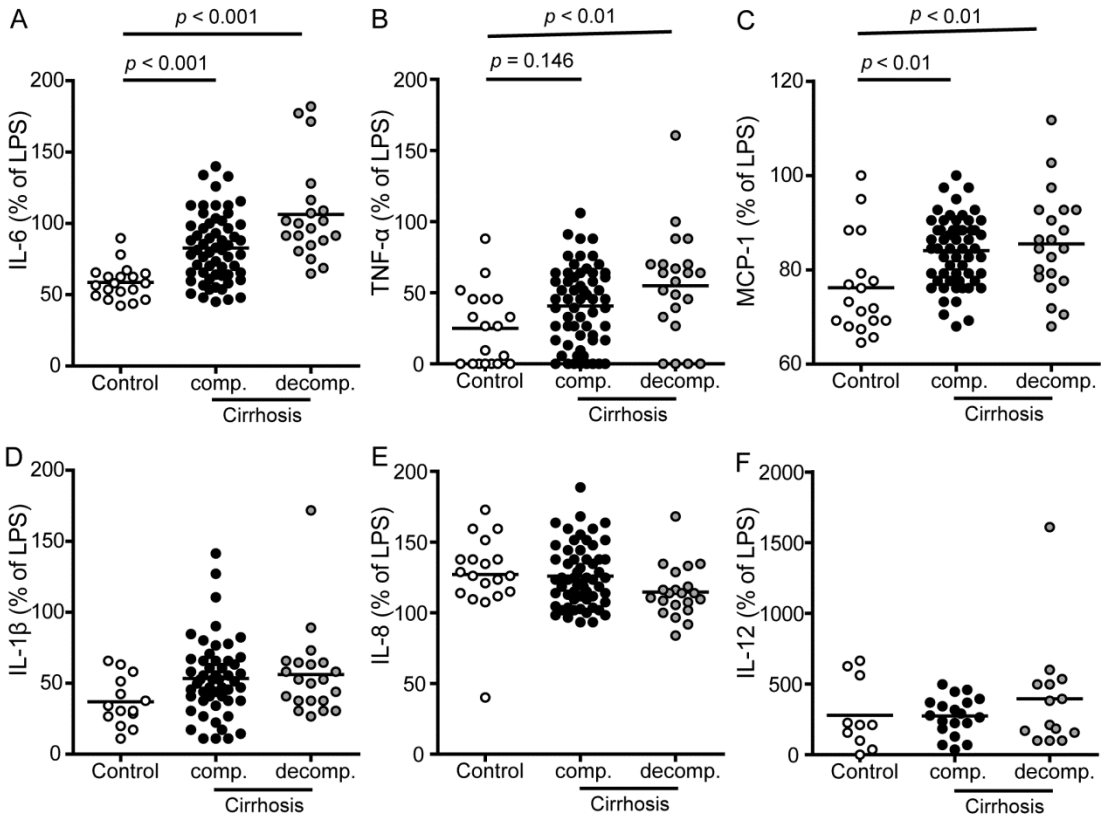


Figure 7. Cirrhosis is associated with a reduced ability of serum HDL to suppress the production of pro-inflammatory cytokines. (A-E) The supernatants of LPS stimulated monocytes which were pretreated with 7% serum HDL of healthy subjects (control, n = 20), of patients with compensated (comp., n = 59) cirrhosis and of cirrhotic patients with acute decompensation (decomp., n = 21) were analyzed for (A) interleukin (IL)-6, (B) tumor necrosis factor-α (TNF-α), (C) monocyte chemoattractant protein-1 (MCP-1), (D) IL-1β, (E) IL-8 and IL-12 concentrations using flow cytometry. All values shown represent means of two independent experiments measured in duplicates. Adopted from (119).

3.6. Levels of pro-inflammatory cytokines are increased in serum of patients with cirrhosis

In line with the *in vitro* finding that serum HDL of patients with cirrhosis failed to efficiently suppress production of inflammatory cytokines, we observed increased levels of the pro-inflammatory cytokines IL-6 (Figure 8A), IL-1β (Figure 8D), and IL-8 (Figure 8E) in serum of patients with cirrhosis. Interestingly, the levels of elevated, secreted pro-inflammatory cytokines correlated inversely with HDL-cholesterol levels as shown in Table 6. The levels of TNF-α (Figure 8B), MCP-1 (Figure 8C) and IL-12 (Figure 8F) were unaltered between the groups.

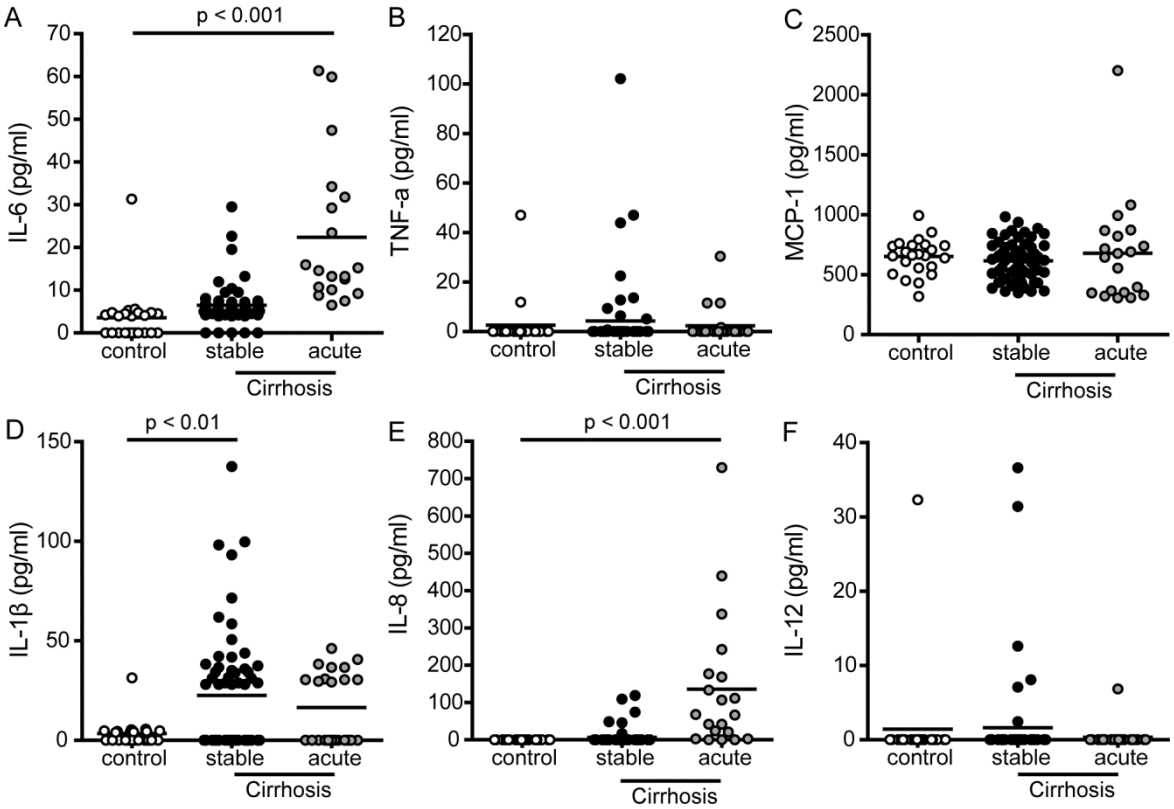


Figure 8. The concentrations of several pro-inflammatory cytokines are increased in patients with cirrhosis. (A-F) Sera of healthy subjects (control, n = 20), of patients with compensated (comp., n = 59) cirrhosis and of cirrhotic patients with acute decompensation (decomp., n = 21) were analyzed for (A) interleukin (IL)-6, (B) tumor necrosis factor-α (TNF-α), (C) monocyte chemoattractant protein-1 (MCP-1), (D) IL-1β, (E) IL-8, and (F) IL-12 concentrations using flow cytometry. All values shown represent means of two independent experiments measured in duplicates. RFU, relative fluorescence units.

Table 6. Correlation of HDL-cholesterol levels with anti-inflammatory capacity and serum levels of pro-inflammatory cytokines.

	HDL-cholesterol (mg/dl)	
	r	***p***
NF-κB expression (RFU)	-0.434	< 0.001
IL-6 (pg/ml)	-0.398	< 0.001
TNF-α (pg/ml)	-0.092	0.367
MCP-1 (pg/ml)	-0.045	0.661
IL-1β (pg/ml)	-0.091	0.371
IL-8 (pg/ml)	-0.365	< 0.001
IL12 (pg/ml)	-0.079	0.436

3.7. Cholesterol efflux potential is significantly reduced in patients with cirrhosis and predicts one year mortality

HDLs ability to mobilize cholesterol from lipid laden macrophages in the periphery is considered to be a fundamental protective function of HDL. In contrast to HDL-cholesterol level which associates with multiple traditional risk factors and metabolic variables, cholesterol efflux capacity depicts minimal association with these factors (136). There is strong evidence that cholesterol efflux by the ABCA1 pathway is a strong predictor for coronary artery disease even after adjustment for HDL-cholesterol levels (59). By analyzing the serum HDL efflux capacity of healthy subjects and patients with cirrhosis, we observed a significantly decreased ABCA1 mediated efflux in patients with cirrhosis (Figure 9A, C). Serum HDL of healthy subjects showed the highest ABCA1 mediated efflux (44% of total efflux) compared to patients with compensated and decompensated cirrhosis (26% and 23% of total efflux) (Figure 9A). Of particular interest, cholesterol efflux of isolated HDL of patients with cirrhosis was unaltered and the ABCA1 mediated efflux was generally low (Figure 9B). This can be explained by the shedding of small pre-β like particles during the ultracentrifugation procedure. Pre-β like particles are mainly responsible for ABCA1 mediated efflux. These findings have been published in a similar fashion in an original article (119).

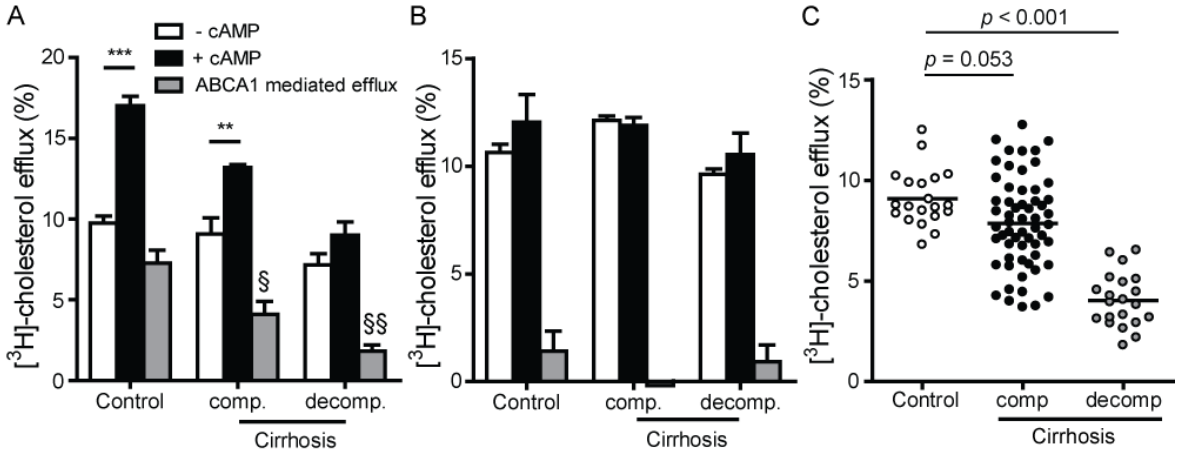


Figure 9. Cholesterol efflux potential is reduced in patients with cirrhosis. Pooled fractions of sera of healthy subjects (control, n = 10), patients with compensated (comp., n = 10) and acutely decompensated (decomp., n = 10) cirrhosis were used to isolate HDL and to generate serum HDL. Serum HDL (A) or HDL (B) were examined for their ability to promote [^{3}H]-cholesterol efflux from

macrophages. [^{3}H]-cholesterol-labeled RAW264.7 macrophages were incubated in the absence and presence of L 8-(4-chlorophenylthio)-cyclic AMP (cAMP) to stimulate ATP-binding cassette transporter A1 (ABCA1) expression. Cells were either exposed to 2.8% serum HDL (A) or 50 µg/mL HDL-protein (B) for 3 hours. ABCA1 mediated efflux was calculated by subtracting effluxed [^{3}H]-cholesterol of macrophages not exposed to cAMP from effluxed [^{3}H]-cholesterol of cAMP stimulated cells. Cholesterol efflux is expressed as radioactivity in the supernatant relative to total radioactivity (in supernatant and cells). The values represent means of two independent experiments measured in duplicates. $^{\S}P < 0.05$, $^{\S\S}P < 0.01$ vs. control. Serum HDL of healthy subjects (control, n = 20), patients with compensated (comp., n = 59) and acutely decompensated (decomp., n = 21) cirrhosis was examined for its ability to (C) promote [^{3}H]-cholesterol efflux from macrophages. [^{3}H]-cholesterol-labeled and cAMP stimulated RAW264.7 macrophages were exposed to 2.8% serum HDL for 3 hours. Cholesterol efflux is expressed as radioactivity in the supernatant relative to total radioactivity (in supernatant and cells). The values represent means of two independent experiments measured in duplicates. Adopted from (119).

3.8. Barrier promoting activity of serum HDL of patients with decompensated cirrhosis is significantly impaired

The disruption of endothelial monolayer integrity is an important contributing factor in multiple inflammatory disorders and considerably increases permeability to fluids, solutes and inflammatory cells during inflammation. Based on the observation that HDL can promote endothelial regeneration (83), we assessed whether endothelial regenerative and barrier promoting activities of serum HDL are altered in cirrhotic subjects using the electric cell-substrate impedance sensing system. Interestingly, addition of serum HDL of cirrhotic patients with acute decompensation to HCAECs showed a severe reduction in the re-endothelialization capacity of electrically wounded cells (Figure 10A) and tended to decrease in cells treated with serum HDL of compensated cirrhotic patients (Figure 10D). Importantly, addition of HDL successfully promoted barrier function (Figure 10B) while lipoprotein deficient serum was less effective (Figure 10C), clearly suggesting that very low levels of HDL observed in patients with decompensated cirrhosis are linked to suppressed endothelial barrier promoting activity. These findings have been published in a similar fashion in an original article (119).

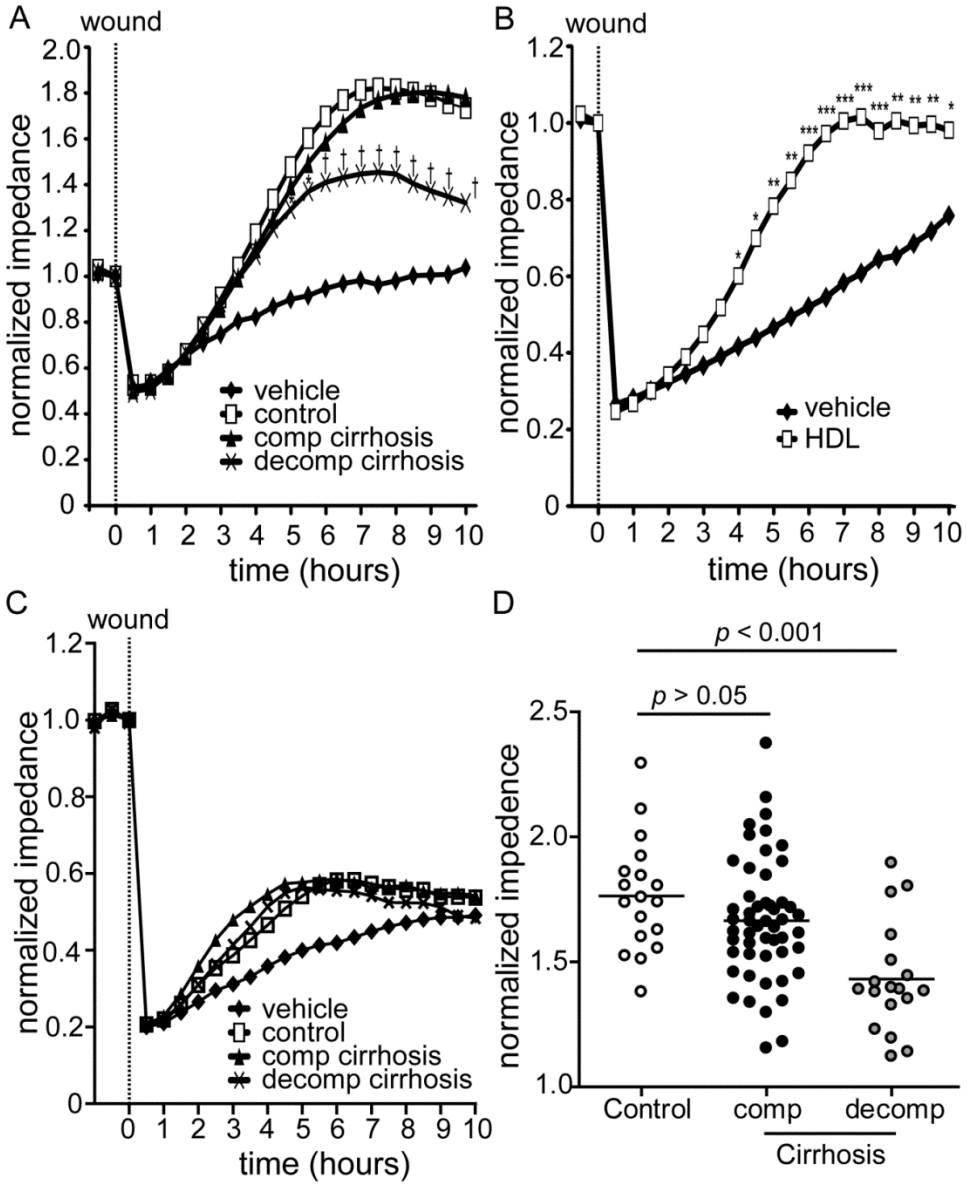


Figure 10. Endothelial regenerative activity is impaired in patients with decompensated cirrhosis. Human coronary artery endothelial cells were serum-starved for 2 hours and a stable baseline (8-10 kΩ) for normalization was recorded. Cells were electrically wounded using the electric cell-substrate impedance sensing system. Immediately after wounding, cells were treated with (A) HDL (50 µg/mL), (B) 3% serum HDL of healthy subjects (control, n = 18), patients with compensated (comp., n = 52) and acutely decompensated (decomp., n = 18) cirrhosis or (C) 3% of lipoprotein deficient sera (pooled fractions) of healthy subjects (n = 5), compensated (n = 5) and decompensated (n = 5) cirrhotic subjects. Re-endothelialization was monitored over time. (D) Scatter blot of serum HDL induced re-endothelialization at 6 ½ hours. Values shown represent means of two individual experiments measured in duplicates. *$P < 0.05$ vs. control, $^{\dagger}P < 0.001$ vs. control. Adopted from (119).

3.9. The ability to induce activation of the endothelial nitric oxide synthase is impaired in patients with cirrhosis

HDL exerts several important activities to prevent endothelial dysfunction. In particular, several studies have shown that HDL stimulates the activation of eNOS, thereby increasing NO bioavailability (137). HDL bound to endothelial SR-BI promotes downstream activation of eNOS by phosphorylation of the enzyme at Ser^{1177}. Interestingly, it was shown in patients with chronic heart failure that an altered HDL particle triggers the activation of PKC-βII and subsequently p70S6 kinase, thereby inhibiting protein kinase B-dependent regulation of eNOS. Additionally, activation of this pathway leads mainly to phosphorylation of eNOS at the inhibitory site Thr^{495} (77). Since we observed significant alterations in HDL metabolism and in HDL particle distribution, we next sought to determine the ability of HDL from patients with cirrhosis to induce phosphorylation of eNOS at various sites.

By incubating cells with serum HDL of healthy controls, eNOS phosphorylation at Ser^{1177} was increased 2.1 ± 0.2 fold compared with untreated cells. Interestingly, the stimulation of eNOS phosphorylation at this site was significantly lower when cells were incubated with serum HDL of patients with compensated cirrhosis (1.5 ± 0.1-fold versus untreated cells) (Figure 11A). There was no change in phosphorylation at Thr^{495} (1.5 ± 0.1 fold versus unstimulated cells for healthy subjects; 1.4 ± 0.1 fold versus unstimulated cells for patients with compensated cirrhosis) (Figure 11B).

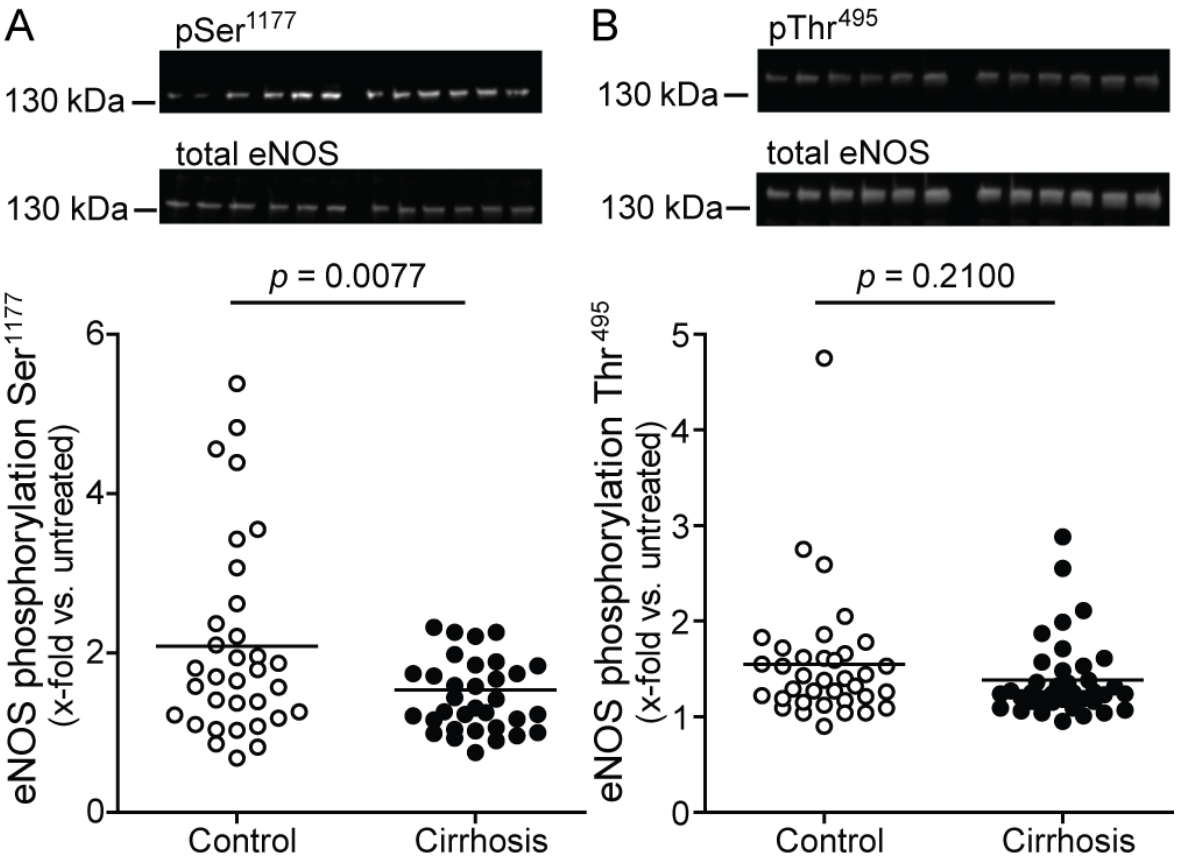


Figure 11. HDL-mediated endothelial nitric oxide synthase (eNOS) phosphorylation. Human aortic endothelial cells were incubated with 10% serum HDL of (A) healthy subjects (control, n = 33 (A), n = 36 (B)) and patients with compensated cirrhosis (cirrhosis, n = 33 (A), n = 38 (B)) and phosphorylation of eNOS at position Ser^{1177} (A) and Thr^{495} was evaluated by Western blot analysis. To compensate for protein loading differences, phosphorylation at specific sites was normalized to non-phosphorylated eNOS. For quantitative analysis, the x-fold increase in eNOS phosphorylation of cells treated with serum HDL vs untreated cells was determined. Representative examples of the Western blots are depicted on top of the figure.

3.10. Anti-oxidative activity of HDL is unaltered in cirrhosis

HDL has been reported to exhibit unique anti-oxidative activity (138). HDL has the ability to inhibit oxidative modifications of LDL thereby reducing the atherogenicity of these lipoproteins. Therefore, we assessed the anti-oxidative ability of serum HDL by measuring the inhibition of free radical-induced oxidation of dihydrorhodamine, a dye which highly increases fluorescence upon oxidation (124) (Figure 12). We observed no changes in the anti-oxidative capacity of serum HDL between healthy subjects and patients with cirrhosis.

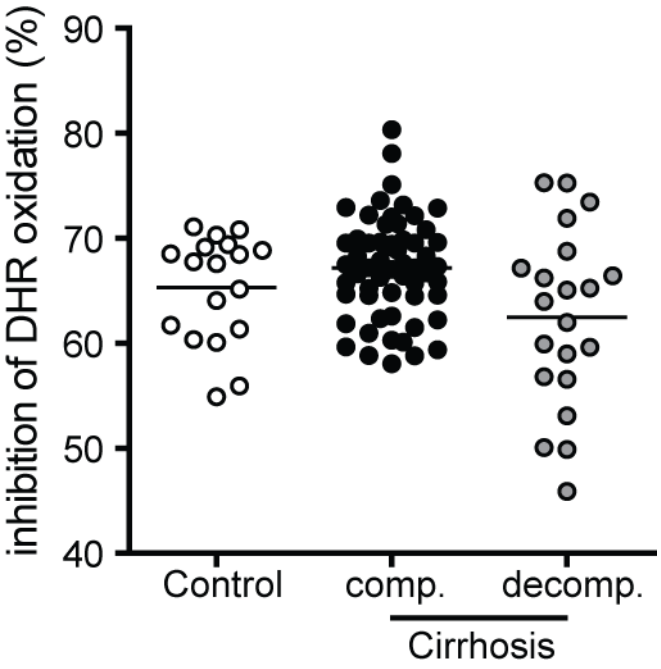


Figure 12. Anti-oxidative capacity is unaltered in cirrhosis. Serum HDL of healthy subjects (control, n = 20), patients with compensated (comp., n = 59) and acutely decompensated (decomp., n = 21) cirrhosis was analyzed for its anti-oxidative potency. Inhibitory activity of serum HDL on oxidation was measured by incubating it with dihydrorhodamine (DHR). Values shown represent means of two independent experiments measured in duplicates.

3.11. HDL of patients with cirrhosis carries a distinct protein cargo

Over the past years, our understanding in the proteomic diversity of HDL has been markedly increased and upwards of 85 proteins associated with HDL have been identified (36). Besides proteins involved in lipid metabolism, HDL also carries proteins which are members of the complement system, protease inhibitors, and proteins involved in the acute-phase response (37). Recent studies have clearly shown that upon inflammation, huge alterations in HDL composition and function can be observed (111,129). Therefore, we assessed possible alterations in the HDL proteome of patients with cirrhosis. HDL was isolated using a two-step density gradient ultracentrifugation method. Isolated HDL was digested, and the resulting peptides were analyzed by tandem mass spectrometry. A list of all identified proteins is shown in Table 7. The major proteins which were significantly altered are depicted in Figure 13. As expected, the most abundant proteins on HDL of healthy subjects, patients with compensated and decompensated cirrhosis

were apoA-I and apoA-II accounting for 64.0% ± 7.9%, 64.3% ± 5.7%, 49.7% ± 7.9% and 9.2% ± 1.5%, 8.7% ± 2.4%, 7.1% ± 1.3%, respectively. We identified apoA-I (Figure 13A), apoA-II (Figure 13B), apoC-I (Figure 13C), apoE (Figure 13F) and PON1 (Figure 13I) to be significantly decreased on HDL of patients with decompensated cirrhosis compared to healthy controls while SAA1 (Figure 13G) and SAA2 (Figure 13H) were significantly increased. A significant decrease of apoC-II was observed on HDL of patients with compensated cirrhosis (Figure 13D). ApoC-III was significantly decreased on HDL of both, patients with compensated and decompensated cirrhosis, respectively (Figure 13E). These findings have been published in a similar fashion in an original article (119).

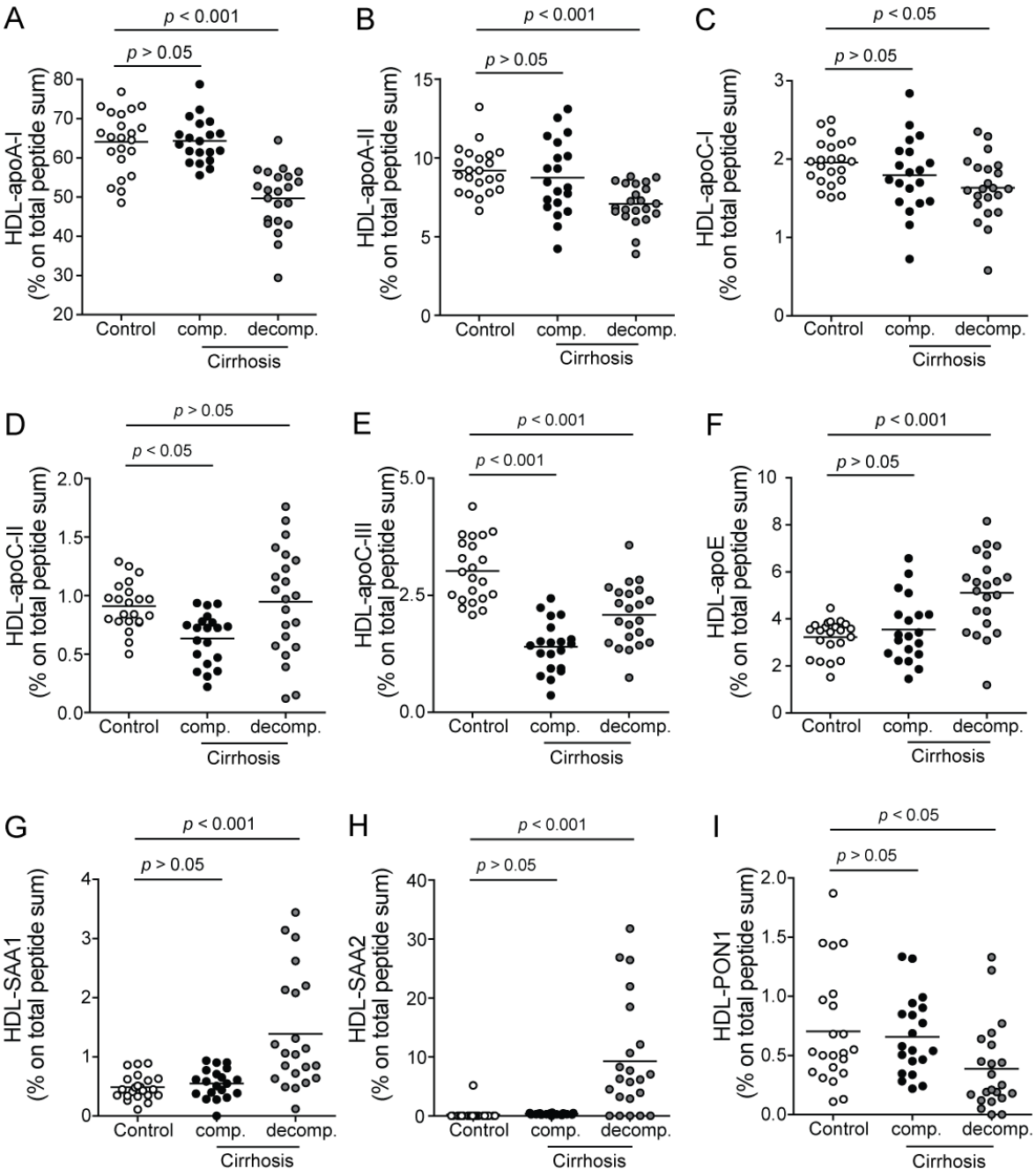


Figure 13. Identification of proteins in HDL isolated from healthy subjects and patients with cirrhosis. (A-I) HDL was isolated from healthy subjects (control, n = 22), patients with compensated (comp., n = 20) and acutely decompensated (decomp., n = 22) cirrhosis. The HDL proteome was analyzed on a LC-MS/MS system. Data were analyzed by searching the human SwissProt public database with Proteome Discoverer 1.4 (Thermo Scientific) and Mascot 2.3 (MatrixScience) and label free quantitation of precursor ion chromatograms. Values shown represent percentage of total peptide sum. Adopted from (119).

Table 7. Identification of HDL-associated proteins

	% HDL-derived peptides		
		Cirrhosis	
Protein	**Control**	**compensated**	**decompensated**
ApoA-I	64.0	64.3	49.7
Albumin	8.1	7.1	11.1
ApoA-II	9.2	8.7	7.1
ApoE	3.2	3.5	5.1
ApoD	1.4	1.5	1.2
ApoC-I	2.0	1.8	1.6
ApoC-III	3.0	1.4	2.1
SAA4	1.4	1.4	1.4
ApoM	0.9	0.9	0.7
ApoC-II	0.9	0.6	0.9
SAA1	0.5	0.6	1.4
Paraoxonase 1	0.7	0.7	0.4
ApoL-I	0.4	0.5	0.3
ApoF	0.2	0.2	0.2
ApoC-IV	0.4	0.2	0.4
Paraoxonase 3	0.2	0.1	0.1
α-1-antitrypsin	0.3	0.3	0.6
ApoA-IV	0.4	0.7	0.9
Apo(a)	0.2	0.1	0.0
Transthyretin	0.1	0.1	0.0
Ig γ-1 chain C region	0.2	0.3	2.1
ApoB-100	0.1	0.2	0.1
α-2-HS-glycoprotein	0.1	0.1	0.1
Haptoglobin-related protein	0.1	0.1	0.0
SAA2	0.2	0.3	9.3
Ig λ-2 chain C region	0.1	0.1	0.3
Ig κ chain C region	0.1	0.2	1.0
Haptoglobin	0.1	0.1	0.3
α-1-acid glycoprotein 1	0.1	0.1	0.8

Ig γ-2 chain C region	0.1	0.1	1.5
Ig α-1 chain C region	0.0	0.3	0.5
Ig λ-3 chain C region	0.1	0.2	0.0
Platelet-activating factor acetylhydrolase	0.0	0.1	0.0
Complement C3	0.2	0.2	0.0
Serotransferrin	0.1	0.2	0.1
Ig γ-3 chain C region	0.1	0.2	0.0
Platelet basic protein	0.1	0.0	0.0
Clusterin	0.1	0.2	0.7
Pulmonary surfactant-associated protein B	0.1	0.1	0.1
Prothrombin	0.1	0.0	0.1
Hemopexin	0.0	0.1	0.1
Phospholipid transfer protein	0.0	0.1	0.0
Complement C4-A	0.0	0.1	0.0
Flotillin-1	0.0	0.1	0.0
Cathelicidin antimicrobial peptide	0.0	0.1	0.0

HDL was isolated from healthy subjects (control, n = 22), patients with compensated (n = 20) and acutely decompensated (n = 22) cirrhosis by ultracentrifugation. The HDL proteome was analyzed by LC-MS/MS, and data were analyzed by searching the human NCBI database with Mascot 2.2 (MatrixScience). Values represent the percentage of peptides of the total peptide count per analyzed subject. Apo, apolipoprotein; SAA, serum amyloid A. Adopted from (119).

3.12. Cirrhosis is associated with a decreased paraoxonase activity of HDL

PON, an important HDL-associated enzyme, has been implicated in anti-oxidant functions (139), and its activity has been related to cardiovascular risk (62). Therefore, we determined the PON activity of isolated HDL and of serum HDL (Figure 14). The arylesterase activity of PON was measured using phenylacetate

as substrate. In line with the reduced levels of HDL-associated PON1 identified by mass spectrometry (Figure 13I), we observed a significantly decreased arylesterase activity of isolated HDL in patients with decompensated cirrhosis when compared with controls (Figure 14A). In good agreement with the low circulating levels of HDL observed in cirrhotic patients, especially in those with decompensation, arylesterase activity of serum HDL was even more profoundly suppressed (Figure 14B). These findings have been published in a similar fashion in an original article (119).

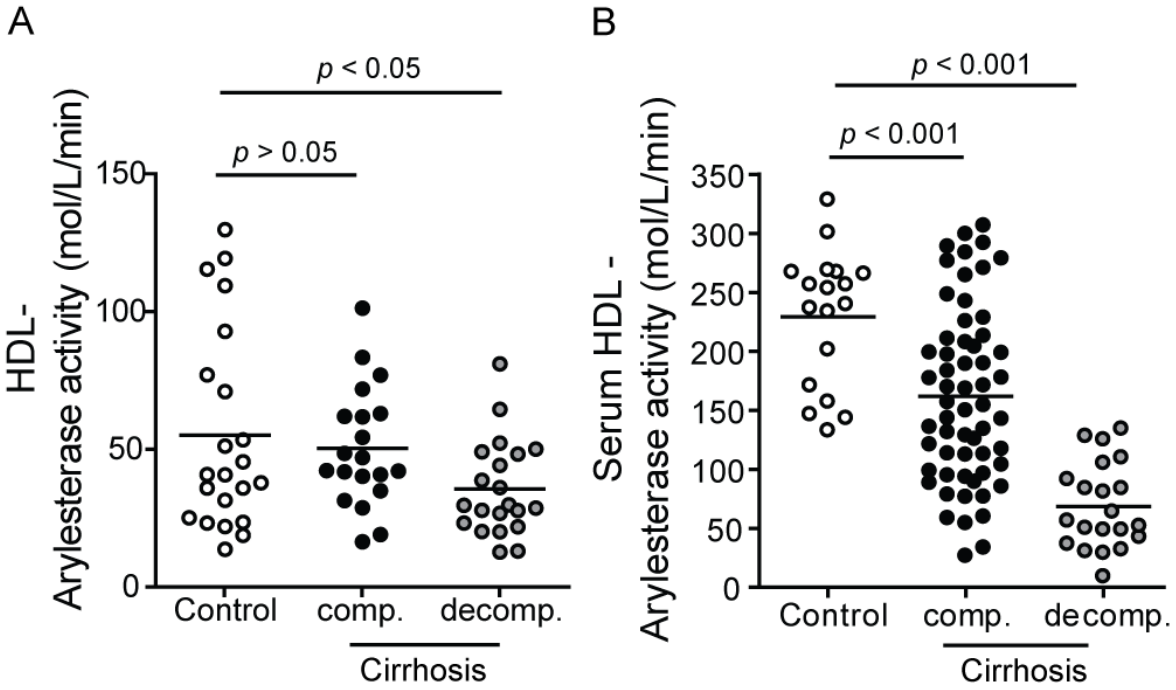


Figure 14. Cirrhosis is associated with a reduced paraoxonase activity of isolated HDL and of serum HDL. (A) HDL was isolated from healthy subjects (control, n = 22), patients with compensated (comp., n = 20) and acutely decompensated (decomp., n = 22) cirrhosis and HDL-associated PON activity was assessed. (B) PON activity of serum HDL of healthy subjects (control, n = 20), patients with compensated (comp., n = 59) and acutely decompensated (decomp., n = 21) cirrhosis. PON activity was measured using phenylacetate as a substrate and was calculated from the slope of the kinetic chart of two independent experiments. Adopted from (119).

3.13. HDL composition and distribution are linked to metrics of serum HDL functionality

To gain further insight into the relationship between HDL protein composition and serum HDL-mediated functions, a detailed correlation analysis was performed (Table 8). We observed that those proteins that were significantly altered in cirrhotic patients, such as apoA-I, apoE, apoC-III and SAA, were the strongest predictors of functionality of serum HDL, suggesting that HDL composition is linked to functionality of serum HDL. We found that HDL associated apoA-I and PON1 strongly associated with the functionality of serum HDL, whereas HDL associated SAA1, SAA2 and apoE were linked to impaired functionality. Interestingly, we found a negative correlation between the HDL_2/HDL_3 ratio and PON activity as well as anti-inflammatory capacity of serum HDL. In line with a previous study showing that small HDL_3 particles are major acceptors of ABCA1 released cholesterol (99), we found that the small HDL_3 particle content correlated strongly with the cholesterol efflux potential, but also with PON activity and the anti-inflammatory capacity of serum HDL. As expected, levels of HDL (apoA-I and cholesterol) strongly correlated with metrics of function of serum HDL. These findings have been published in a similar fashion in an original article (119).

Table 8. Correlation of HDL-composition, -distribution and -levels with metrics of function of serum HDL

	Cholesterol efflux		Paraoxonase activity		NF-κB expression		Barrier regenerative activity	
HDL-composition								
	r	***p***	**r**	***p***	**r**	***p***	**r**	***p***
apoA-I	0.629	<0.001	0.565	<0.001	-0.240	0.070	0.381	<0.01
apoE	-0.519	<0.001	-0.484	<0.001	0.362	<0.01	-0.142	0.310
apoC-III	0.337	0.010	0.323	0.015	-0.565	<0.001	0.145	0.301
SAA1	-0.495	<0.001	-0.480	<0.001	0.122	0.363	-0.218	0.117
SAA2	-0.524	<0.001	-0.508	<0.001	0.156	0.243	-0.240	0.083
PON1	0.395	<0.01	0.468	<0.001	-0.089	0.507	0.211	0.129
HDL-distribution								
	r	***p***	**r**	***p***	**r**	***p***	**r**	***p***
HDL_2/ HDL_3 ratio	-0.160	0.114	-0.329	0.001	-0.352	<0.001	-0.100	0.358
small HDL_3 particles	0.371	<0.001	0.432	<0.001	-0.449	<0.001	0.106	0.329
HDL-levels								
	r	***p***	**r**	***p***	**r**	***p***	**r**	***p***
HDL-C	0.874	<0.001	0.590	<0.001	-0.506	<0.001	0.293	<0.01
apoA-I	0.678	<0.001	0.569	<0.001	-0.597	<0.001	0.219	0.073

apo, apolipoprotein; SAA, serum amyloid A; PON1, paraoxonase 1. Adopted from (119).

3.14. Metrics of HDL function and one year mortality

Based on the marked alterations in HDL function, we analyzed whether those alterations were associated with one year mortality (Table 9). We observed that all

metrics of HDL function correlate with one year mortality. When adjusted for levels of total cholesterol, LDL-cholesterol, triglycerides, age and sex, we still observed a correlation between one year mortality and cholesterol efflux potential, PON activity and barrier regenerative activity of serum HDL. Strikingly, after further adjustment for levels of HDL-cholesterol, the cholesterol efflux potential of serum HDL was still associated with one year mortality. We performed a receiver operating characteristics (ROC) analysis, a statistical method to assess a test's diagnostic performance (Figure 15). With an area under the curve (AUROC) of 0.89, the ROC analysis revealed excellent diagnostic accuracy of cholesterol efflux capacity of serum HDL to predict one-year mortality in patients with cirrhosis. These findings have been published in a similar fashion in an original article (119).

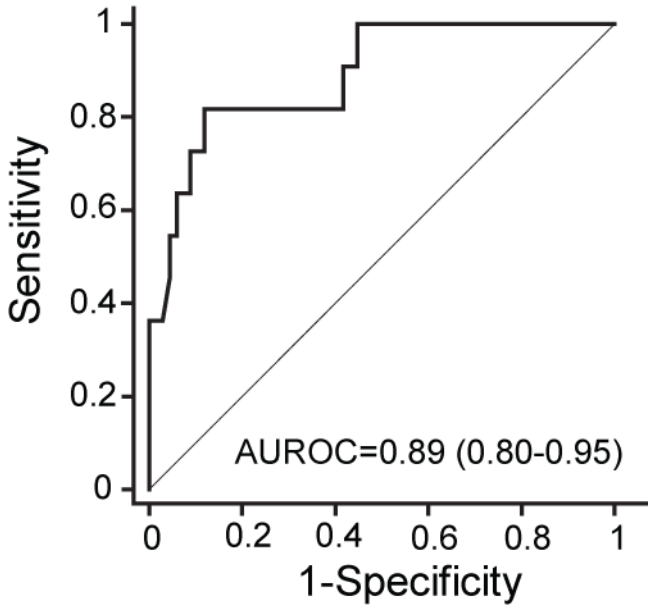


Figure 15. Cholesterol efflux potential predicts one year mortality. Receiver operating characteristics (ROC) curves illustrating the performances of [^{3}H]-cholesterol efflux capability of serum HDL of patients with compensated (n = 59, 5 deceased) and acutely decompensated cirrhosis (n = 21, 11 deceased) for prediction of one-year mortality. AUROC, area under ROC curve. Adopted from (119).

Table 9. Correlation of one-year mortality and metrics of HDL function

	One-year mortality					
			adjusted*		adjusted†	
	r	***p***	**r**	***p***	**r**	***p***
Cholesterol efflux	-0.458	<0.001	-0.456	<0.001	-0.321	0.010
Paraoxonase activity	-0.382	<0.001	-0.273	0.020	-0.210	0.076
NF-κB expression	0.290	0.009	0.161	0.204	0.107	0.403
Barrier regenerative activity	-0.344	0.004	-0.268	0.032	-0.242	0.056

*adjusted for LDL-cholesterol, total-cholesterol, triglycerides, age and sex. †adjusted for LDL-cholesterol, total-cholesterol, triglycerides, age, sex and HDL-cholesterol. Adopted from (119).

4. Discussion

In the present study, we provide compelling evidence that HDL composition, metabolism and function are altered in patients with compensated and decompensated cirrhosis. We observed that serum HDL from patients with liver disease showed (i) a markedly reduced anti-inflammatory and (ii) anti-oxidative capacity, (iii) a decreased cholesterol efflux potential which predicted one year mortality and a diminished ability to (iv) stimulate endothelial repair and (v) eNOS activation. Additionally, we identified several proteins to be altered on HDL of patients with cirrhosis. Furthermore, the activities of serum enzymes involved in HDL metabolism were significantly changed in cirrhosis leading to alterations in HDL particle distribution.

4.1. HDL metabolism

Alterations in serum lipids and lipoproteins in cirrhotic patients are secondary to complex abnormalities in lipoprotein synthesis, secretion and catabolism (112,140). Of note, we found that several enzyme activities involved in lipoprotein metabolism were profoundly reduced in cirrhotic patients, resulting in the formation of large HDL_2-like particles. LCAT mediates the conversion of phosphatidylcholine and free cholesterol into lysophosphatidylcholine and cholesteryl ester (a more hydrophobic form of cholesterol), which is then sequestered into the core of HDL, promoting the formation of spherical HDL (141). CETP mediates the equimolar transfer of cholesteryl ester from HDL for triglyceride from LDL, VLDL or CMs. Three lipases are known to play roles in remodeling HDL in humans, LPL, HL and EL (56). LCAT, CETP, LPL and HL are mainly secreted by the liver; hence low activities of these enzymes in cirrhosis may be expected. However, PLTP is secreted by a wide variety of tissues and the cause for decreased PLTP activity in cirrhotic patients is unclear. Whereas CETP mediates the transfer of cholesteryl esters from and triglycerides to HDL, PLTP is involved in the transfer of phospholipids from apoB-containing lipoproteins to HDL in a process that is essential for HDL maturation and maintenance. Of particular interest, PLTP is a member of the LPS-binding protein gene family, and PLTP deficiency in mice is associated with a significant increase in LPS-induced mortality (142). Given that a strong inverse relationship between PLTP serum activity and HDL particle size was reported (143), one might conclude that large HDL particles observed in liver

disease alter PLTP activity. However, further studies will have to show whether functionality of isolated HDL particles of cirrhotic subjects is altered, as observed in other inflammatory disease states (111,129). CETP mediates the transfer of cholesteryl esters from HDL for triglycerides from LDL, thereby influencing the lipid composition of HDL. It was shown that pharmacological inhibition of CETP increased the phospholipid and cholesteryl ester content of HDL with a concurrent decrease of the triglyceride content (144) thereby increasing the mean HDL particle diameter (145). In line with these observations, our data indicate that the very low CETP activity observed in cirrhotic patients might be crucial for increasing the average HDL particle size. However, several other enzyme activities involved in HDL metabolism are also altered, and it is likely that the decreased serum *sn*1 lipolytic activity of cirrhotic patients may delay the formation of pre-β-HDL (146). Hepatic lipase and endothelial lipase decrease the triglyceride and the phospholipid content of HDL thereby generating small HDL particles. Hepatic lipase catalyzes the hydrolysis of phospholipids and triglycerides thus facilitating the conversion of HDL_2 to the denser HDL_3 (147). A hepatic lipase deficiency in humans results in increased levels of HDL_2 particles (148). Endothelial lipase was also shown to contribute to the remodeling of HDL_2 to smaller HDL particles (149).

4.2. HDL functionality

4.2.1. Anti-inflammatory capacity

We observed that the anti-inflammatory activity of serum HDL was significantly reduced in patients with cirrhosis. This might be of relevance given that several studies have shown that cirrhosis is accompanied by an increased risk for infections and sepsis leading to an imbalanced cytokine release (9,10). The risk for individuals with cirrhosis to become septic is more than doubled compared with individuals without cirrhosis (11). In addition, a mortality rate between 84% and 88% can be observed in cirrhotic patients with sepsis (150,151). However, the molecular mechanisms for the increased susceptibility to infections are unclear (16). It was shown that low levels of apoA-I, the main constituent of HDL, are an independent factor to predict 90-day mortality in cirrhotic patients with severe sepsis (152) and that circulating lipoproteins protect from endotoxin toxicity (153).

Therefore, the results of the present study provide evidence that HDL is required for dampening inflammatory responses in patients with cirrhosis. There are several proposed mechanisms how HDL might reduce inflammatory signaling. HDL regulates the plasma membrane cholesterol content through the ability to remove cholesterol and other lipid species from cells, thereby dampening inflammatory receptor signaling (154). Successful signal transduction relies on proper membrane microdomains containing high concentrations of cholesterol and sphingolipids. An increased cholesterol efflux potential of serum is expected to perturb these microstructures, thereby promoting anti-inflammatory effects in cells. Therefore, functional HDL is thought to be an important endogenous inhibitor of inflammatory responses. In several *in vivo* studies it was shown that rHDL, an artificial HDL like particle containing apoA-I, phospholipids and cholesterol, can bind LPS and is thereby protective against mortality and reduces cytokine production in different animal models (155-157). In addition, the susceptibility to LPS-induced liver injury (assessed by the degree of hepatocyte apoptosis and necrosis) is higher in rats with cirrhosis than in normal animals (158,159) and decreases when HDL administration is used to neutralize LPS (160,161). In line with these studies we observed that rHDL dose-dependently suppressed NF-κB activation of monocytes *in vitro*. Of particular interest, we observed that cirrhosis markedly impaired the ability of serum HDL to suppress NF-κB activation and cytokine production. Furthermore it was shown in humans that intravenous infusion of rHDL prior to the induction of endotoxemia in healthy volunteers markedly reduced TNF-α, IL-6 and IL-8 release and reduced flu-like symptoms (71). In line with this notion, we observed a negative correlation between levels of pro-inflammatory cytokines in sera of patients with cirrhosis and HDL-cholesterol levels. Therefore, the reduced HDL-cholesterol levels observed in patients with cirrhosis might account for the increased levels of IL-6, IL-1β and IL-8 in serum of patients with liver disease. Of particular interest, an *ex vivo* study in patients with advanced cirrhosis revealed that a LPS-induced overproduction of pro-inflammatory cytokines by monocytes can be abolished by incubation of whole blood with rHDL (72). A recent study provided evidence that HDL induces the transcriptional regulator activating transcription factor 3 (ATF3), resulting in the dampening of Toll-like receptor-induced pro-inflammatory cytokines secretion in macrophages (162). Another study suggested that HDL prevents the induction of

LPS-induced genes by promoting TRIF-related adaptor molecule (TRAM) translocation, thereby impairing subsequent signaling by Toll-like receptor 4 (163).

4.2.2. Cholesterol efflux potential

A previous study in noncholestatic cirrhotics showed that HDL-cholesterol levels were significantly associated with death or need for liver transplantation at 6 or 12 months (118). However, the HDL cholesterol measurement targets only a single HDL component compromising 20% of HDL mass and HDL-lipid composition varies widely from a few percent up to 50% of particle mass. Thus assessing HDL cholesterol has an inherent bias toward the larger, more cholesterol/lipid rich particles and underestimates smaller, lipid-poor forms. This is critical, because recent evidence indicates that HDL is actually a collection of numerous subspecies that play distinct functional roles, not only in lipid homeostasis but also in inflammation and innate immunity (111,129). As an example, pre-β forms of HDL contain relatively little lipid/cholesterol, but are superior in mobilizing cellular cholesterol via cell surface transporters (99,164,165). Apo-B depleted serum cholesterol efflux, which is driven by pre-β forms of HDL, was recently demonstrated to strongly predict incident cardiovascular mortality, whereas HDL-cholesterol was not associated (59,136). These data support the emerging concept that HDL function may be a better marker of cardiovascular risk than HDL-cholesterol levels. The steady-state levels of HDL-cholesterol suffer from limitations intrinsic to its mass based and static measurement not reflecting the heterogeneity of HDL particles in terms of structure, size, lipidomic/proteomic composition, metabolism, and function (129,166). In the present study, we provide novel evidence that apo-B depleted serum cholesterol efflux capability is significantly decreased in patients with cirrhosis and predicts one year mortality. ApoB-depleted sera of controls showed the highest ABCA1 mediated efflux (44% of total efflux), whereas sera of compensated and decompensated showed suppressed ABCA1 dependent efflux capacity (26% and 23% of total efflux) In line with that finding, we observed that low levels of small HDL_3 particles in liver disease subjects (identified by native gel electrophoreses) were associated with decreased cholesterol efflux capacity and anti-inflammatory activities of serum.

Remarkably, in patients with cirrhosis the cholesterol efflux capacity of isolated HDL, which is largely ABCA1 independent (167), was unaltered.

Therefore, reduced production of apoA-I, the major apolipoprotein of HDL, as well as a shift to larger HDL particles, as observed in the present study, might be central underlying mechanisms linked to the markedly suppressed cholesterol efflux capability in cirrhotic patients. All together our data clearly suggest that the formation of lipid-poor HDL is profoundly suppressed in liver disease. A very interesting finding of our study is that cholesterol efflux capacity predicted 1-year mortality in cirrhotic patients, even after adjustment for LDL-cholesterol, total-cholesterol, triglycerides, age, sex, and HDL-cholesterol. Although our data suggest an inverse relationship between cholesterol efflux capacity and mortality, the causal nature of this relationship is uncertain. Nevertheless, our results suggest that HDL cholesterol efflux capability might be an important marker and perhaps even a protective factor in cirrhosis, in addition to its role in cardiovascular disease.

4.2.3. Anti-oxidative functions

In addition to its important role in the reverse cholesterol transport and to its anti-inflammatory capacity, HDL possesses also anti-oxidative properties. The major proteins carried on HDL, such as apoA-I and apoA-II, as well as other proteins like PON are known to have anti-oxidant potentials (138). Oxidized lipids such as oxidized LDL can produce reactive oxygen species leading to induction of monocyte migration, which represents an important event in atherosclerosis (168,169). Under normal conditions, HDL is able to prevent reactive oxygen species formation thereby reducing LDL oxidation and recruitment of inflammatory mediators (170,171). We measured the anti-oxidant capacity of serum HDL by measuring the inhibition of free radical-induced oxidation of dihydrorhodamine (124). We observed no changes in serum HDL's ability to inhibit the oxidation of dihydrorhodamine. The paraoxonase gene family consists of three genes, *PON1, PON2* and *PON3*. In circulation, PON1 and PON3 are associated with HDL particles while PON2 on the other hand appears to be associated with the endoplasmatic reticulum. Although all three paraoxonases exert lactonase

activities, only PON1 exhibit the activity to hydrolyze paraoxon (172). In experimental studies PON1 has been shown to inhibit oxidation of LDL *in vitro* (173,174). Additionally, polymorphisms of the *PON1* gene are related to heart disease (175) and decreased PON1 levels are associated with increased risk for cardiovascular disease (176). In *in vivo* studies in mice it was shown that a knockout of PON increased lipoprotein oxidation and that those mice are susceptible for atherosclerosis (60,61,177). A finding of particular interest is that HDL-associated PON1 levels and activity are reduced in patients with cirrhosis, as well as PON1 activity of serum HDL. There is strong evidence for a mechanistic link between the activity of PON1 with systemic oxidative stress and prospective cardiovascular risk, indicating a potential mechanism for the atheroprotective function of PON1 (62). A decrease of HDL-associated PON1 activity as observed in patients with cirrhosis is therefore expected to alter anti-oxidant and cardioprotective properties of HDL.

4.2.4. Endothelial regenerative activity

The endothelium forms an interface between vessel wall and the circulating blood and plays a key role in vascular homeostasis. Alterations in endothelial cell functions, including increased cell apoptosis, reduced NO availability and increased expression of adhesion molecules contribute to the development of atherosclerosis. Notably, vascular leak of fluid from plasma to interstitial space increases during severe inflammation. At this time, no therapeutics sufficiently addresses the loss of endothelial barrier function, which is the underlying cause of vascular leak. Interestingly, HDL exerts direct protective effects on the vascular endothelium. There is evidence that the cholesterol mobilizing activity of HDL maintains endothelial function in an ABCG1-dependent manner, preventing active eNOS dimer disruption and cell adhesion molecule expression (178). Artery re-endothelialization after perivascular electric injury is blunted in apoA-I deficient mice, and reconstitution of apoA-I rescues normal re-endothelialization (179). HDL was found to promote endothelial cell motility, a process that relates to endothelial barrier function in the context of a vascular injury (180). In line with these previous reports, we observed that low HDL-cholesterol levels in patients with decompensated liver disease were linked to impaired endothelial regenerative and

barrier promoting activities of serum and might, therefore, increase the permeability to inflammatory cells. Interestingly, low PON activity was recently shown to be linked to a loss of endothelial repair-stimulating effects of HDL (74). We observed that sera of cirrhotic patients showed a marked decrease in PON activity, raising the possibility that both, low serum content of HDL and PON activity impede efficient endothelial repair.

4.2.5. eNOS stimulating activity

Endothelial derived NO, produced by eNOS from the precursor L-arginine, exerts a variety of atheroprotective effects and plays a pivotal role in angiogenesis, in regulating vascular tone, leukocyte adhesion, cellular proliferation and platelet aggregation (181). In experimental studies it was shown that pharmacological inhibition of NO synthesis resulted in enhanced monocyte binding to the endothelium and this effect was attenuated by supplementation with L-arginine (182). Furthermore an increased leukocyte-endothelial cell interaction was observed, in eNOS-deficient mice (183). Moreover, atherosclerotic lesion formation was accelerated in LDL-receptor knockout mice, when eNOS was inhibited while treatment with L-arginine decreased lesion formation (184). Specific removal of eNOS led to an enhanced atherosclerotic lesion formation in apoE knockout mice which are prone to development of atherosclerosis (185). HDL was shown to stimulate eNOS-mediated NO production via different mechanisms. HDL binding to SR-BI activates the PI-3K/Akt signaling pathway leading to the phosphorylation of eNOS at serine residue 1177 thereby activating eNOS (78,186). Activation of eNOS can also be induced by HDL-associated S1P. HDL-associated apoM is considered to be a S1P binding protein. Hence, HDL is the main carrier of S1P in plasma. Upon binding to $S1P_1$ and $S1P_3$ receptors, intracellular Ca^{2+} is mobilized and the PI-3K/Akt signaling pathway gets activated (187,188). PON1, another HDL-associated protein was recently identified to participate in HDL's ability to stimulate eNOS (74). The authors demonstrated that an impaired HDL-associated PON1 activity led to a diminished ability to stimulate eNOS activating pathways. They further observed that HDL isolated from PON1-deficient mice failed to stimulate eNOS activation and that supplementation with purified PON1 partially restored HDL's capacity to stimulate eNOS (74). It was

recently shown in patients with chronic heart failure that an altered HDL composition or a post-translational modification of the HDL particle can lead to activation of the PKC-βII pathway with a subsequent activation of p70S6K leading to an inhibition of PKB-dependent regulation of eNOS (77,189). The activation of this pathway leads mainly to phosphorylation of the inhibitory site of eNOS at threonine residue 495 (190). An interesting finding of this study was that serum HDL of patients with compensated cirrhosis was significantly less effective in stimulating the phosphorylation of the activating site of eNOS at Ser^{1177}. The observed shift towards large HDL_2 particles with the concurrent reduction in the amount of small HDL_3 particles in cirrhosis could account for the reduced ability to induce phosphorylation at Ser^{1177}. It was shown that small HDL_3 particles are enriched in S1P and that those particles decreased apoptosis in endothelial cells (191). In line with this finding, we observed a strong correlation between the amount of small HDL_3 particles and serum HDLs ability to stimulate phosphorylation of eNOS at Ser^{1177} (p = 0.004). Furthermore, cholesterol efflux might be required for eNOS activation since cholesterol free cyclodextrin, a cyclic oligosaccharide which can form complexes with hydrophobic compounds such as cholesterol, was able to mimic the eNOS stimulating effect while cholesterol loaded cyclodextrin was not (192). This is in line with our finding that the cholesterol efflux potential of serum HDL was decreased in patients with cirrhosis.

4.3. HDL composition

Prompted by the observations of recent studies that inflammation can alter HDL composition thereby rendering it dysfunctional (103,106,129,193), we used shotgun proteomics to determine possible changes in the protein composition of isolated HDL of patients with cirrhosis. We observed marked alterations in the HDL proteome of cirrhotic patients, especially from patients with decompensated cirrhosis with significantly decreased levels of apoA-I, apoA-II, apoC-I, apoC-II and PON1 while apoE and the pro-inflammatory acute phase proteins SAA1 and SAA2 were significantly increased. By performing a detailed correlation analysis, we identified HDL-associated proteins to be significantly related to metrics of serum HDL functionality. It was shown that the pro-inflammatory mediators SAA1 and SAA2 associate in circulation with HDL with a concurrent displacement of apoA-I

and a subsequent remodeling of HDL (194-197). As a consequence, SAA1 and SAA2 render HDL pro-inflammatory and impede its cholesterol efflux capability (110,111,195,198). In line with these findings, we observed a strong negative correlation between SAA1 and SAA2 and the cholesterol efflux potential of serum HDL whereas apoA-I was strongly positively associated. Of particular interest, we detected that apoE was associated with serum HDLs ability to suppress NF-κB expression in monocytes. In addition, we found a strong negative correlation between apoE and the cholesterol efflux potential of serum HDL. These findings are in line with a very recent study demonstrating that apoE regulates HDL's ABCA1-specific cholesterol efflux capacity, independently of particle size (199).

4.4. Limitations

Limitations of our explorative study are the cross-sectional design and the small sample sizes of patient cohorts, which were from a single institution. Therefore, larger studies are needed to validate our findings. Moreover, our study is limited by the correlative nature, not permitting causal inference.

4.5. Conclusion

In summary, the results of our study provide the novel evidence that HDL composition and several metrics of HDL function, including HDL cholesterol efflux capacity, anti-inflammatory and paraoxonase activity and the ability to induce activation of eNOS are markedly suppressed in cirrhotic patients. Moreover, our data indicate that cirrhosis reduces the ability of serum HDL to promote endothelial regeneration. These are novel and important observations and might explain, at least in part, why cirrhotic patients are highly susceptible to bacterial infections. Given that cholesterol efflux appears to predict mortality of cirrhotic patients as observed in the present study, our results might provide valuable information to develop rational HDL-raising therapeutic strategies. Consequently, such alterations in HDL levels, composition, distribution and function may be clinically relevant and improve our ability to monitor cirrhotic patients at high risk and may provide a link between altered HDL function, severe inflammation and mortality.

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