DISSERTATION

Investigating HDL Function and Metabolism in Heart Failure, Liver Cirrhosis and Diabetes Mellitus

submitted by

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Graz, 28.08.2025 Anja Pammer

DISCLOSURES

The cumulative dissertation is based on the following publications:

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TABLE OF CONTENTS

ABBREVIATIONS

A ABCA1 ATP-binding cassette subfamily A member 1

ABCG1 ATP-binding cassette subfamily G member 1

AHF Acute heart failure

Apo Apolipoprotein

B BMI Body mass index

C CEC Cholesterol efflux capacity

CETP Cholesteryl-ester transfer protein

CRP C-reactive protein

CVD Cardiovascular disease

E EL Endothelial lipase

F FFAs Free fatty acids

H HDL High-density lipoprotein

HDL-C High-density lipoprotein cholesterol

HF Heart failure

HL Hepatic lipase

I IF Intermittent fasting

IL-6 Interleukin-6

L LCAT Lecithin-cholesterol acyltransferase

LDL Low-density lipoprotein

LDL-C Low-density lipoprotein cholesterol

LDL-R Low-density lipoprotein receptor

L-HDL Large HDL

LPL Lipoprotein lipase

LPS lipopolysaccharide

M MELD Model for end-stage liver disease

M-HDL Medium HDL

MPO Myeloperoxidase

N NAFLD Non-alcoholic fatty liver disease

NMR nuclear magnetic resonance

NT-proBNP N-terminal pro-B-type natriuretic peptide

P PLTP phospholipid transfer protein

PON1 Paraoxonase-1

R RCT Reverse cholesterol transport

ROS reactive oxygen species

S SAA Serum amyloid A

S-HDL Small HDL

SR-BI Scavenger Receptor B1

S1P Sphingosine-1-phosphate

T T2DM Type 2 diabetes mellitus

TNF-α Tumour necrosis factor α

V VLDL Very-low-density lipoprotein

X XS-HDL Extra small HDL

ZUSAMMENFASSUNG

Jenseits der traditionellen Bewertung des Low-Density-Lipoprotein-Cholesterins (LDL-C) rückt zunehmend die funktionelle Beschaffenheit des High-Density-Lipoproteins (HDL) als zentraler Modulator in der Pathophysiologie lebensstilassoziierter chronischer Erkrankungen in den Fokus. Adipositas begünstigt häufig eine Reihe metabolischer Störungen, darunter die Insulinresistenz, welche wiederum zur Entwicklung eines Typ-2-Diabetes mellitus (T2DM) sowie einer metabolisch assoziierten Steatosehepatitis führen kann. Sowohl isoliert als auch in Kombination tragen diese Krankheitsbilder in erheblichem Maße zur Entstehung und zum Fortschreiten einer akuten Herzinsuffizienz sowie einer Leberzirrhose oder eines Leberversagens bei.

Traditionell wurde HDL primär über statische Messungen des HDL-Cholesterins (HDL-C) beurteilt. Zunehmende wissenschaftliche Evidenz weist jedoch darauf hin, dass funktionelle Eigenschaften des HDL, darunter die Cholesterin-Efflux-Kapazität, antioxidative Aktivität und antiinflammatorische Effekte, eine präzisere und klinisch relevantere Abbildung des kardiovaskulären und metabolischen Risikos ermöglichen.

Im Rahmen dieser Dissertation wurde die Funktionalität von HDL in drei miteinander verbundenen Krankheitskontexten untersucht: akute Herzinsuffizienz, Leberzirrhose und Typ-2-Diabetes mellitus. Die Arbeit umfasst drei voneinander unabhängige Studien, die in ihrer Gesamtheit die klinische Relevanz der HDL-Funktion bei diesen hochprävalenten Erkrankungen verdeutlichen.

Bei hospitalisierten Patient:innen mit akuter Herzinsuffizienz war eine beeinträchtigte HDL-Funktion, assoziiert mit einer reduzierten Cholesterin-Efflux-Kapazität, einer verminderten Aktivität von Paraoxonase 1 (PON1) und Lecithin-Cholesterin-Acyltransferase (LCAT) sowie einem Verlust kleiner HDL-Partikel, signifikant mit einer erhöhten 3-Monats-Mortalität assoziiert. Diese Ergebnisse deuten darauf hin, dass funktionelle HDL-Parameter prädiktiver für kurzfristige klinische Verläufe sind als HDL-C-Spiegel allein.

In ähnlicher Weise zeigte sich bei Patient:innen mit Leberzirrhose mittels Kernspinresonanzspektroskopie eine deutliche Reduktion mittlerer, kleiner und sehr kleiner HDL-Subklassen, insbesondere in dekompensierten Stadien. Diese kleineren HDL-Partikel,

bekannt für ihre antiinflammatorischen und antioxidativen Eigenschaften, korrelierten invers mit systemischen Entzündungsmarkern und oxidativem Stress. Ihre Verminderung war ein unabhängiger Prädiktor sowohl für die 90-Tage- als auch für die 12-Monats-Mortalität.

In einer randomisierten kontrollierten Studie an adipösen Personen mit T2DM führten sowohl Ernährungsberatung als auch alternierendes Intervallfasten zu einer Verbesserung der HDL-Cholesterin-Efflux-Kapazität. Allerdings konnte ausschließlich durch die diätetische Intervention eine signifikante Steigerung der PON1- und LCAT-Aktivität erzielt werden, während intermittierendes Fasten spezifisch den Serumspiegel von Apolipoprotein M erhöhte, welches mit einer verbesserten Insulinsensitivität und Sphingosine-1-Phosphat-Signaltransduktion assoziiert ist. Diese Ergebnisse belegen, dass unterschiedliche diätologische Strategien differenzielle Effekte auf die HDL-Funktionalität ausüben können.

Insgesamt unterstreicht diese Dissertation, dass die HDL-Subklassenverteilung und -Funktion, insbesondere hinsichtlich der Cholesterin-Efflux-Kapazität sowie antioxidativer und antiinflammatorischer Eigenschaften, klinisch relevante Biomarker darstellen und potenzielle therapeutische Zielstrukturen bieten. Die konsistente Assoziation zwischen eingeschränkter HDL-Funktion und ungünstigen klinischen Verläufen in unterschiedlichen Krankheitskontexten unterstützt einen Paradigmenwechsel: Weg von der alleinigen Beurteilung des HDL-C hin zu einer funktionellen HDL-Charakterisierung im Rahmen der Risikostratifizierung und personalisierten Therapieansätze.

ABSTRACT

Beyond traditional assessments of low-density lipoprotein cholesterol (LDL-C), there is an increasing focus on the functional properties of high-density lipoprotein (HDL) as critical modulators in the pathophysiology of lifestyle-driven chronic diseases. Obesity often triggers a series of metabolic disturbances, such as insulin resistance, which can lead to type 2 diabetes mellitus (T2DM) and metabolic dysfunction-associated steatotic liver disease. Whether occurring independently or in combination, these conditions significantly contribute to the development and progression of acute heart failure and liver cirrhosis or failure. HDL has traditionally been evaluated through static measurements of HDL cholesterol (HDL-C). However, growing evidence suggests that HDL functionality, including cholesterol efflux capacity, antioxidant activity, and anti-inflammatory effects, offers a more precise and clinically meaningful reflection of cardiovascular and metabolic risk.

In this thesis, I investigated HDL functionality across three interconnected disease states: acute heart failure, liver cirrhosis, and type 2 diabetes mellitus (T2DM). This work comprises three independent studies that collectively highlight the clinical relevance of HDL function in the context of these high-burden conditions.

In patients hospitalized with AHF, impaired HDL function, as indicated by reduced cholesterol efflux capacity, paraoxonase 1 (PON1) and lecithin–cholesterol acyltransferase (LCAT) activity, and depletion of small HDL particles, was significantly associated with increased 3-month mortality. These findings suggest that HDL functionality is a stronger predictor of short-term outcomes than HDL-C levels alone.

Similarly, in liver cirrhosis, nuclear magnetic resonance spectroscopy revealed a profound depletion of medium, small, and extra-small HDL subclasses, particularly in decompensated stages. These smaller HDL particles, known for their anti-inflammatory and antioxidative properties, were inversely correlated with markers of systemic inflammation and oxidative stress, and their reduction independently predicted both 90-day and 12-month mortality.

In a randomized controlled trial involving obese individuals with T2DM, both dietary counseling and alternate-day intermittent fasting improved HDL cholesterol efflux capacity. However, only dietary counseling significantly enhanced PON1 and LCAT activity, while

intermittent fasting uniquely increased serum apolipoprotein M, which is associated with improved insulin sensitivity and spingosine-1-phosphate-signaling. These findings indicate that specific dietary strategies can differentially modulate HDL functionality.

Overall, this thesis highlights that HDL subclass distribution and function, particularly its cholesterol efflux capacity, antioxidative and anti-inflammatory properties, represent clinically relevant biomarkers and potential therapeutic targets. The consistent association between impaired HDL function and adverse outcomes across different disease contexts supports a paradigm shift from evaluating HDL-C levels alone to incorporating functional HDL profiling into risk stratification and personalized treatment strategies.

1 INTRODUCTION

1.1 Obesity: A Gateway to Chronic Disease, Including Type 2 Diabetes Mellitus, Liver Failure and Heart Failure

The global prevalence of overweight and obesity is increasing at an alarming rate, posing one of the most significant public health challenges of the 21st century. Obesity is defined as excessive fat accumulation that impairs health, typically defined by a body mass index (BMI) cut-off of 30 kg/m² in the Western world (9). Far from being a cosmetic issue, obesity is a chronic and multifactorial disease that significantly increases the risk of developing numerous non-communicable diseases. A 2022 report estimated that over half of Europe's population was overweight or obese, emphasizing the serious scale of this epidemic (10). Obesity has been associated with a notable rise in mortality, potentially reducing life expectancy by 5 to 10 years (11–13). This burden is compounded by obesity's role in promoting chronic low-grade inflammation, marked by elevated circulating cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor α (TNF- α), which contribute to insulin resistance and endothelial dysfunction (14–19). Obesity is a central risk factor in the development of multiple chronic diseases, acting as a common upstream driver of metabolic and organ dysfunction. Among the most critical obesity-related complications are type 2 diabetes mellitus (T2DM), liver failure, often stemming from non-alcoholic fatty liver disease (NAFLD), cardiovascular diseases (CVD) and subsequent heart failure (HF) and certain cancers due to its metabolic, inflammatory, and endocrine consequences (14,20–24). These conditions are not only prevalent but frequently coexist, creating a complex clinical picture that significantly worsens patient outcomes.

1.2 Dyslipidaemia in obesity, insulin resistance and heart failure

Obesity is closely associated with a distinct form of dyslipidaemia, characterized by elevated triglycerides (TG), reduced high-density lipoprotein cholesterol (HDL-C), and increased levels of small, dense low-density lipoprotein (LDL) particles. This atherogenic lipid profile significantly heightens cardiovascular risk and contributes to the pathogenesis of multiple

chronic diseases, including T2DM, HF, and liver cirrhosis (25,26). The development of this dyslipidaemic state reflects a broader metabolic disturbance driven by insulin resistance, excess visceral adiposity, and chronic low-grade inflammation (27–29).

The pathogenesis of obesity-induced dyslipidaemia is multifactorial. Visceral adiposity promotes increased lipolysis, releasing free fatty acids (FFAs) into the circulation, which are taken up by the liver in excess (30,31). This influx of FFAs contributes to hepatic overproduction of very-low-density lipoprotein (VLDL) particles, resulting in hypertriglyceridaemia (26,31–34). Concurrently, insulin resistance impairs insulin-mediated inhibition of adipose tissue lipolysis and enhances hepatic de novo lipogenesis through activation of sterol regulatory element-binding protein-1c (SREBP-1c), further driving hepatic lipid accumulation (32,34,35).

Moreover, the clearance of triglyceride-rich lipoproteins is compromised due to reduced expression and activity of lipoprotein lipase (LPL), especially in adipose and muscle tissues, as well as competition between VLDL and chylomicrons for LPL-mediated hydrolysis (26,36). Elevated apolipoprotein C-III levels, a natural inhibitor of LPL, further impair lipid metabolism (37). Hypertriglyceridaemia also promotes the action of cholesteryl ester transfer protein (CETP), which facilitates the exchange of triglycerides and cholesteryl esters between lipoprotein particles. This results in triglyceride-rich and cholesterol-depleted LDL and HDL particles that are further remodeled by hepatic lipase into small, dense LDL and HDL3 subtypes, both of which are more atherogenic and more rapidly catabolized (26,38–45).

The interplay between dyslipidaemia and insulin resistance is bidirectional and synergistic. Lipid accumulation in non-adipose tissues, such as liver and skeletal muscle, impairs insulin signaling via the accumulation of fatty acid intermediates like diacylglycerol, which disrupt insulin receptor pathways (46,47). As insulin resistance worsens, hepatic lipogenesis and VLDL production are further upregulated, perpetuating the dyslipidaemic state (48). FFAs also stimulate inflammatory pathways, including nuclear factor kappa B (NF-κB), leading to increased cytokine release (e.g., TNF-α, IL-6) that further suppress insulin sensitivity (49–51). These cytokines promote oxidative stress, generating reactive oxygen species (ROS) that damage endothelial cells and worsen vascular function (52). Modified LDL particles resulting from oxidative stress become increasingly atherogenic and pro-inflammatory (51).

This metabolic cascade directly contributes to the development of T2DM. Chronic lipid accumulation and inflammation in pancreatic β-cells impair insulin secretion and promote apoptosis, leading to progressive β-cell failure (46,47,52). Epidemiological studies have shown that an elevated LDL-C/HDL-C ratio is an independent predictor of incident diabetes, suggesting a causal role for dyslipidaemia in T2DM onset beyond shared risk factors (53). Diabetic patients typically present with an atherogenic lipid triad: elevated TGs, reduced HDL-C, and increased levels of small dense LDL, largely due to insulin resistance (54). These abnormalities contribute to chronic systemic inflammation, endothelial dysfunction, and impaired glucose metabolism (55).

Dyslipidaemia is also a key contributor to the development of HF, both as a downstream effect of atherosclerosis and through direct myocardial toxicity. While myocardial infarction is a well-established cause of HF, evidence indicates that dyslipidaemia can impair myocardial structure and function independently of ischemic events (56). Longitudinal data, including findings from the Framingham Heart Study, demonstrate a graded relationship between elevated non-HDL-C, low HDL-C, and increased HF risk (57). Elevated TGs and high total cholesterol/HDL ratios are especially predictive of HF in individuals with diabetes (58). Mechanistically, lipid accumulation in cardiomyocytes leads to lipotoxicity, mitochondrial dysfunction, oxidative stress, and disrupted calcium handling, which in turn contribute to cardiac fibrosis, ventricular stiffness, and contractile impairment (46,47,52,59). HF with preserved ejection fraction (HFpEF) is frequently observed in obese, insulin-resistant individuals and is closely linked to metabolic dysregulation. Low HDL-C further exacerbates cardiovascular dysfunction by impairing endothelial repair and reducing anti-inflammatory capacity (50,60,61). Thus, dyslipidaemia is not only a marker but an active driver of myocardial injury and functional decline.

Liver pathology represents another major consequence of dyslipidaemia and metabolic dysfunction. Excess FFAs are stored in the liver, leading to hepatic steatosis and the development of non-alcoholic steatohepatitis (NASH), which can progress to fibrosis and ultimately cirrhosis (48). NASH is strongly associated with insulin resistance and dyslipidaemia and is now recognized as a leading cause of cryptogenic cirrhosis (62). Moreover, liver disease and HF often coexist due to shared risk factors such as inflammation, alcohol use, and metabolic

stress. HF can cause hepatic congestion and dysfunction, while liver disease can independently impair cardiac function, even in the absence of overt cardiovascular disease (63,64).

Concluding, visceral adiposity fosters insulin resistance, dyslipidaemia, inflammation, and oxidative stress (15,65–68). Together, these mechanisms create a vicious cycle of metabolic dysfunction that fuels the onset and progression of multiple chronic diseases.

The metabolic syndrome framework encapsulates this systemic disturbance, illustrating how interconnected abnormalities in lipid metabolism, glucose homeostasis, and inflammatory pathways act in concert to promote cardiovascular, hepatic, and endocrine complications (47). Within this model, dyslipidaemia emerges as a critical node that links and amplifies the pathological processes underlying these chronic conditions (Figure 1).

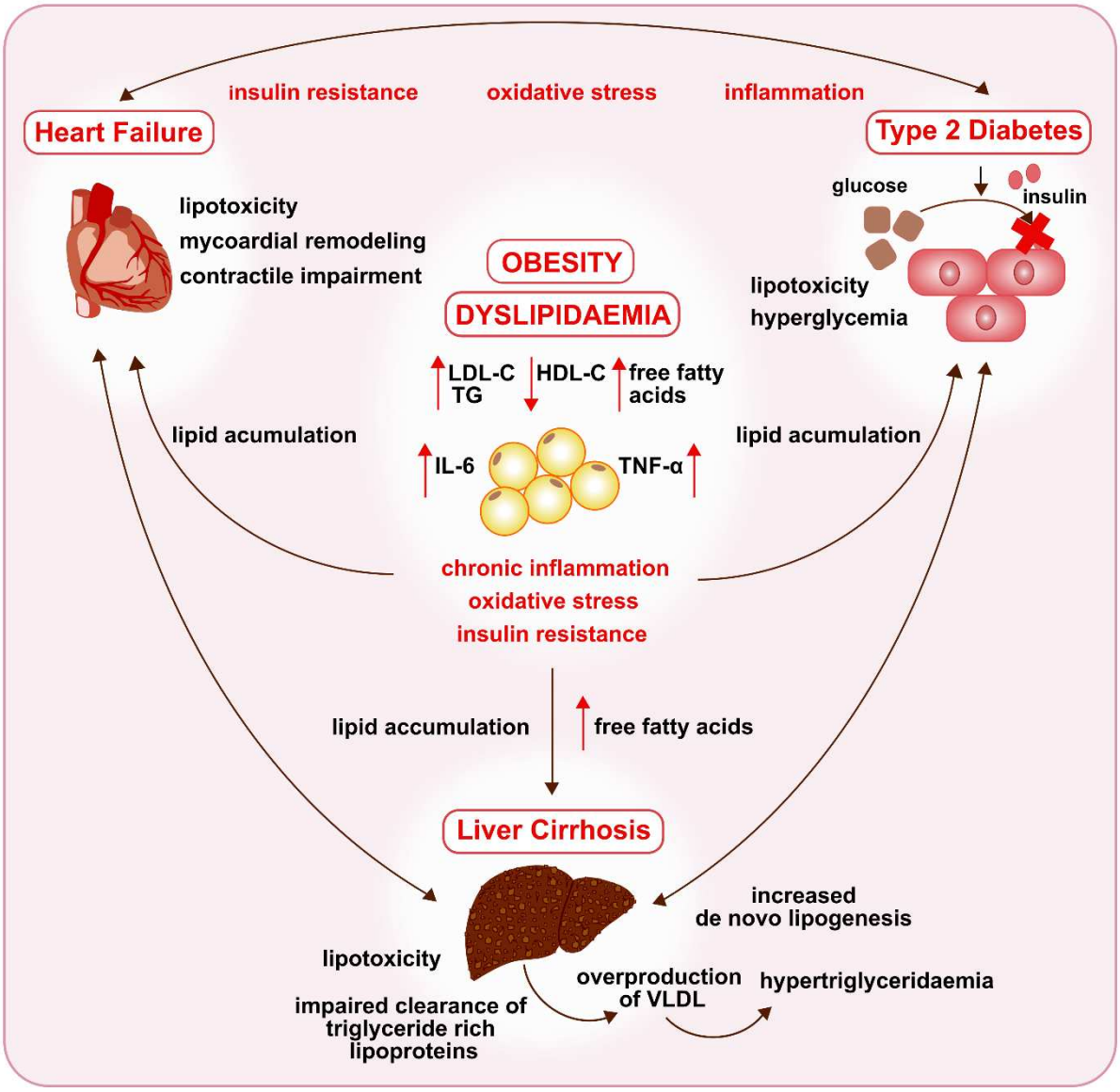

Figure 1. **Pathogenic pathways linking obesity-induced dyslipidaemia to chronic disease.** Obesity, particularly visceral adiposity, promotes insulin resistance and chronic low-grade inflammation, leading to dyslipidaemia characterized by elevated triglycerides, low HDL-C, and small dense LDL. Excess free fatty acids fuel hepatic VLDL overproduction and impair lipoprotein clearance. Insulin resistance exacerbates hepatic lipogenesis, while inflammation and oxidative stress further impair insulin signaling. This metabolic dysfunction promotes lipotoxicity, β-cell failure, endothelial damage, and myocardial and hepatic injury. Together, these interrelated processes drive the development of type 2 diabetes, heart failure, and liver cirrhosis.

1.3 High-density lipoproteins (HDL)

HDL, frequently designated as "good cholesterol", performs a pivotal function in lipid metabolism and cardiovascular health (69,70). HDL was initially understood primarily through its pivotal role in reverse cholesterol transport (RCT). This process is essential for removing excess cholesterol from peripheral tissues and transporting it back to the liver for elimination,

ultimately contributing to a reduced risk of atherosclerosis (71,72). Nevertheless, research findings have demonstrated that HDL has functions beyond its role in cholesterol transport, that include the regulation of inflammation, the provision of antioxidant properties, and the modulation of the immune system (60,61,73). These multifunctional properties of HDL have been demonstrated to position it as a key player in overall health, encompassing significant areas such as neurodegeneration, immune defence, and metabolic regulation (74–76).

1.3.1 HDL metabolism

The formation of HDL is initiated in the liver and the small intestine, where apolipoprotein A-I (apoA-I) is synthesised (77,78) (Figure 2). Following its secretion, lipid-poor apoA-I interacts with the ATP-binding cassette transporter A1 (ABCA1), an integral membrane protein highly expressed in hepatocytes and enterocytes (79). This interaction enables apoA-I to acquire lipids from the cellular lipid pool, leading to the formation of nascent HDL particles (80). These particles subsequently incorporate additional lipids and apolipoproteins, many of which are derived from the hydrolysis of triglyceride-rich lipoproteins (81).

The cholesterol taken up by HDL is esterified by lecithin-cholesterol acyltransferase (LCAT), an enzyme synthesized primarily in the liver and intestine, leading to the formation of mature HDL particles. (82,83). This reaction occurs on the surface of HDL and requires apoA-I as a crucial activator of LCAT (82). The cholesteryl esters thus generated are subsequently transferred to apoB-containing lipoproteins, such as VLDL through the action of CETP, typically in exchange for triglycerides. Alternatively, cholesteryl esters in HDL can be directly taken up by the liver via the scavenger receptor class B type 1 (SR-BI) (84). Upon binding of SR-BI to large, cholesterol-rich HDL particles, both cholesteryl esters and free cholesterol are internalised, facilitating cholesterol excretion via the bile, while apoA-I dissociates from the particle (85).

CETP also facilitates the enrichment of HDL particles with triglycerides, rendering them more susceptible to lipolysis by endothelial lipase (EL) and hepatic lipase (HL). EL primarily targets phospholipids, whereas HL hydrolyzes both phospholipids and triglycerides, albeit with differing specificities for phospholipid substrates (86). The process of lipolysis of triglycerides leads to the generation of smaller HDL particles, which are more rapidly catabolized (87). Another key regulator of HDL metabolism is phospholipid transfer protein (PLTP), which

facilitates the transfer of phospholipids among HDL particles and mediates lipid exchange between HDL and triglyceride-rich lipoproteins (88).

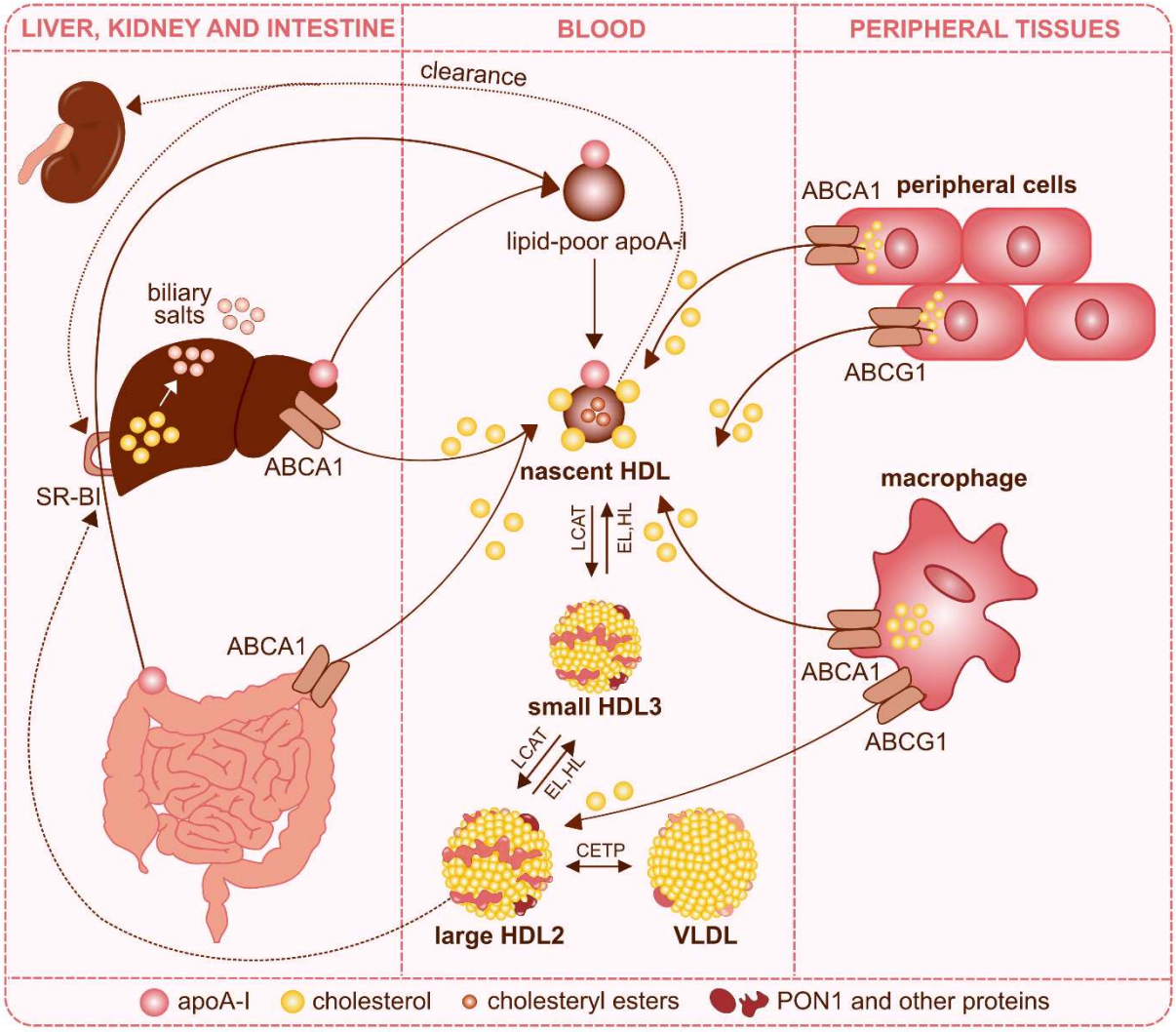

Figure 2. HDL metabolism. The figure illustrates the key steps in HDL metabolism, beginning with the hepatic and intestinal synthesis and secretion of lipid-poor apolipoprotein A-I (apoA-I). ApoA-I interacts with ATP-binding cassette transporter A1 (ABCA1), facilitating the acquisition of phospholipids and free cholesterol to form nascent pre-β-HDL particles. Lecithin-cholesterol acyltransferase (LCAT) then esterifies free cholesterol on the surface of HDL, promoting the formation of larger, spherical HDL particles. ABCA1 preferentially interacts with nascent and small HDL3 particles, whereas ATP-binding cassette transporter G1 (ABCG1) mediates cholesterol efflux to larger HDL2 particles. Cholesterol carried by HDL can be selectively delivered to the liver via scavenger receptor class B type I (SR-BI) or transferred to very low-density lipoproteins (VLDL) through the action of cholesteryl ester transfer protein (CETP). HDL-associated triglycerides and phospholipids are hydrolyzed primarily by endothelial lipase (EL) and hepatic lipase (HL), contributing to HDL remodeling and clearance.

1.3.2 HDL structure and composition

HDL particles are highly heterogeneous, differing in size, density, and composition (Figure 3) depending on their site of origin, maturation stage, and proteomic and lipidomic profiles (89–91). This structural diversity is central to their multifaceted biological functions, particularly in lipid metabolism, anti-inflammatory processes, and cardiovascular protection.

1.3.2.1 Protein composition of HDL

The HDL proteome is dominated by apoA-I, which constitutes approximately 70% of the total protein mass and is present in nearly all HDL particles (92–94). ApoA-I is synthesized primarily in the liver and intestine, and its amphipathic structure allows it to bind lipids efficiently, enabling its detergent-like behavior (95). Functionally, apoA-I is essential for HDL biogenesis, cholesterol efflux via interaction with cellular transporters, and activation of LCAT, which is critical for HDL maturation (95,96). ApoA-II is the second most abundant protein in HDL, comprising 15–20% of its protein content, and is found in approximately 50% of HDL particles (97). Synthesized predominantly in the liver and to a lesser extent in the intestine (98), apoA-II exists as a dimer with greater hydrophobicity than apoA-I. It modulates HDL metabolism and interacts with other apolipoproteins, such as apoA-I and apoE (99–101). Less abundant but functionally significant proteins include apolipoproteins C-II and C-III. These exchangeable apolipoproteins, synthesized in the liver, are present on HDL and triglyceride-rich lipoproteins (95,102). ApoC-II activates LPL, an enzyme critical for triglyceride hydrolysis on triglyceride-rich lipoproteins (103), whereas apoC-III inhibits LPL and interferes with triglyceride-rich lipoproteins binding to the endothelium, indirectly modulating apoC-II function (102,104,105). Apolipoprotein E (apoE), found on a subset of HDL particles, is synthesized in diverse tissues including liver, brain, endocrine organs, and macrophages (95). It facilitates receptor-mediated clearance of remnant lipoproteins through interactions with LDL-receptor family members (106–108), thereby contributing to plasma cholesterol regulation and cardiovascular risk reduction. Apolipoprotein M (apoM), primarily associated with HDL but also present in VLDL, LDL, and chylomicrons, is a lipocalin synthesized in the liver and kidney (109–114). Although only about 5% of HDL particles carry apoM, these particles exhibit enhanced cholesterol efflux capability, superior antioxidant activity, and endothelium-protective properties (110,112,114–116). ApoM is notable for binding bioactive lipids such as sphingosine-1-phosphate (S1P), further amplifying HDL's biological functions (111,112). Serum amyloid A (SAA), especially

SAA1, is an acute-phase protein synthesized in the liver during inflammation. Its expression can increase up to 1,000-fold, leading to HDL particles enriched with SAA at the expense of apoA-I (117–119). This remodeling results in a loss of HDL's atheroprotective and anti-inflammatory properties. To date, over 500 different proteins have been identified in HDL (120), although only a subset is consistently found across various studies, depending on isolation techniques and analytical methods (120,121). Importantly, most proteins are present on only a fraction of HDL particles, reflecting their compositional heterogeneity (122).

1.3.2.2 HDL-associated enzymes and lipid transfer proteins

Enzymatic components of HDL further contribute to its dynamic functionality. LCAT, predominantly associated with HDL, catalyzes the esterification of free cholesterol, facilitating the maturation of HDL particles (95,123). PON1, another HDL-associated enzyme primarily synthesized in the liver, degrades oxidized lipids and homocysteine thiolactones, thereby contributing to HDL's antioxidative capacity (95,124–126). CETP, expressed in the liver and adipose tissue, mediates the exchange of triglycerides and cholesteryl esters between HDL and apoB-containing lipoproteins such as VLDL and LDL (95,127,128). This lipid exchange significantly influences the composition and functional profile of circulating HDL particles.

1.3.2.3 Lipid composition of HDL

HDL particles exhibit a diverse lipidome, consisting of over 200 identified lipid species (129–131). Structurally, the surface monolayer comprises phospholipids, sphingolipids, lyso-phospholipids, and free cholesterol, while the hydrophobic core contains triglycerides and cholesteryl esters (131–134). Phospholipids represent the most abundant lipid class, accounting for 35–50 wt% of HDL lipids (131). Phosphatidylcholine, constituting 33–45 wt% of total HDL lipids, is the predominant species. Other notable phospholipids include lysophosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and plasmalogens, with minor contributions from phosphatidylglycerol, phosphatidylserine, phosphatidic acid, and cardiolipin (129–131,133,135–137). Sphingolipids contribute 5–10 wt% of total HDL lipids, with sphingomyelin being the most prevalent. Primarily derived from triglyceride-rich lipoproteins, sphingomyelin enhances HDL surface rigidity and affects the activity of embedded proteins (131,138–140). S1P is a lysosphingolipid that has been identified as a critical regulator of numerous physiological and pathophysiological processes, including

cancer, atherosclerosis, diabetes and osteoporosis. (141) . Around 65–80% of circulating S1P is associated with HDL via apoM binding (112,142). HDL-bound S1P exhibits greater stability and facilitates critical processes such as endothelial cell protection, angiogenesis, immune modulation, and apoptosis regulation (142–146).

Triglycerides constitute 5–12 wt% of HDL-associated lipids and are predominantly derived from VLDL via CETP-mediated exchange (131,147). These hydrophobic lipids contribute to the fluidity of the HDL core and are mainly composed of palmitic, oleic, and linoleic acid moieties (130,131,148,149). Free cholesterol accounts for 5–10 wt% of HDL lipid content and is localized on the particle surface, contributing to membrane fluidity and HDL's capacity to mobilize cholesterol (95,131). HDL also carries small amounts of other sterols, including oxysterols, estrogens, and phytosterols (131). Cholesteryl esters, formed by the action of LCAT, represent 30–40 wt% of HDL lipids and reside in the hydrophobic core. Approximately 80% of these esters are generated through the esterification of free cholesterol, with subsequent exchange for triglycerides facilitated by CETP (130,131).

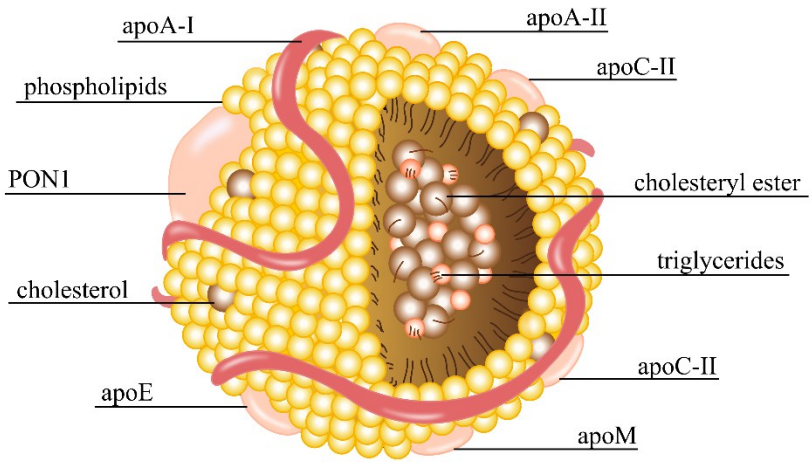

Figure 3. **Schematic representation of the structure and composition of HDL particles.** Not all associated proteins and lipids are displayed. Abbreviations: apo, apolipoprotein; PON1, paraoxonase1.

1.3.3 HDL subclasses and their characteristics

HDL exists in a variety of subclasses that differ in maturation state, composition, and origin, each with distinct functional properties (150,151). The smallest HDL form in plasma, known as pre-β HDL, consists of one or two apoA-I molecules, a phospholipid layer, and a small amount of unesterified cholesterol (152). Unlike the spherical mature HDL, pre-β HDL is discoidal in shape and has a molecular weight of about 67 kDa (152). It constitutes approximately 5% of the circulating apoA-I (153) and plays a key role in lipid uptake by interacting with the ABCA1 transporter, which initiates the formation of nascent HDL particles (81). Due to its high efficiency in absorbing cholesterol and phospholipids, pre-β HDL is considered important in preventing atherosclerosis (154).

As nascent HDL accumulates lipids from peripheral cells and the hydrolysis of triglyceride-rich lipoproteins, additional apolipoproteins bind to it. This process leads to the formation of small, dense HDL3 particles, which contain about 60% protein. In contrast, the larger HDL2 particles have a lower protein content (~43%) and are richer in lipids (155). The protein composition of HDL varies by particle size: HDL3 is enriched with apoJ, apoF, and enzymes such as PON1 and PLTP, while proteins like apoD, SAA1/2, and apoM are also preferentially associated with this subclass (150,156). ApoM, in particular, has been found to carry S1P in a hydrophobic binding pocket, and HDL3 has been shown to contain higher S1P levels than other subclasses (112,157,158). The complex formed between apoM and S1P has been shown to have potent anti-inflammatory and endothelial protective effects, thus contributing significantly to the atheroprotective properties of HDL (159,160). Furthermore, recent studies have demonstrated that intestine-derived HDL3 can neutralise bacterial lipopolysaccharide (LPS), thereby protecting against liver inflammation (161). This function is facilitated by enrichment of HDL3 with LPS-binding protein, which shields LPS from Toll-like receptor 4 recognition (161). Concluding,, they are smaller in size (1.125–1.21 g/ml, ~175 kDa), and have been proposed as the more anti-atherogenic form due to their superior cholesterol efflux capacity, antioxidant activity, and anti-inflammatory effects (162). Conversely, larger HDL2 (1.063–1.125 g/ml, ~350 kDa) particles exhibit higher lipid content and are more enriched in apoE, apoC-II, and apoC-III (156,162). The functional differences between HDL2 and HDL3 are partly attributed to their distinct proteomic and lipidomic compositions (157).

Although traditionally larger HDL particles were thought to be more cardioprotective due to their greater cholesterol content, emerging evidence suggests that smaller, lipid-poor HDL particles may exhibit superior atheroprotective functions, including enhanced cholesterol efflux capacity and antioxidative activity (163–165). Conversely, larger HDL particles enriched in cholesterol and triglycerides have been associated with an increased risk of coronary artery disease and may not reliably confer cardiovascular protection (166,167). Notably, in chronic inflammatory states, structural and compositional alterations in HDL can impair its functional properties, diminishing its anti-inflammatory capacity and potentially rendering it pro-inflammatory (168).

1.3.3.1 The multifaceted functions of HDL

HDL particles are complex particles whose functions vary depending on their size and composition. The functionality of HDL encompasses various functional metrics, including cholesterol efflux, its antioxidant and anti-inflammatory activities.

1.3.3.2 Cholesterol efflux capacity

The assessment of CVD risk has traditionally relied on the use of HDL-C levels as a biomarker, based on epidemiological evidence showing a negative correlation between higher HDL-C concentrations and the incidence of CVD events (169). Nevertheless, efforts to therapeutically augment HDL-C have not yielded the anticipated reduction in CVD outcomes, thus prompting a re-examination of the relationship between HDL quantity and quality (170). Recent studies have indicated that the function of HDL, as opposed to the concentration of HDL-C, offers a more precise reflection of its atheroprotective role (171). Among the various functional properties of HDL, its cholesterol efflux capacity (CEC), defined as the ability to accept cholesterol from macrophages and initiate RCT, is the most well-studied and a key anti-atherosclerotic mechanism (172). Contrary to the multifaceted influence of HDL-C levels on CVD risk, which is modulated by numerous metabolic and lifestyle factors, CEC has demonstrated a robust, independent inverse correlation with CVD risk across diverse populations (173,174). The process of cholesterol efflux is initiated when HDL particles interact with membrane cholesterol transporters, including ABCA1, ABCG1, and SR-BI, on macrophages. This interaction results in the extraction of cholesterol for subsequent transport back to the liver for excretion (175). In clinical practice, the assessment of CEC is commonly

performed using ex vivo assays. In these assays, radiolabeled or fluorescent cholesterol-loaded macrophages (typically the mouse macrophage cell line J774) are incubated with apoB-depleted human serum. The result of this incubation is the quantification of the capacity of HDL to extract cholesterol (176). The results of the assays have shown that individuals with higher cholesterol efflux capacity have a significantly lower incidence of atherosclerotic events. This is independent of HDL-C levels and other traditional risk factors (169).

1.3.3.3 Antioxidative functions of HDL

HDL has been demonstrated to play a crucial antioxidative role by protecting cells and lipoproteins from oxidative damage. This process has been implicated in the pathogenesis of cardiovascular, metabolic and inflammatory diseases (177). It has been established that HDL can inhibit the process of oxidative modification of LDL, a major instigator of atherosclerosis and vascular inflammation. This process is primarily facilitated by mechanisms involving apoA-I, PON1, and redox-active methionine residues (178). The HDL3 particles, which are characterised by their small size and high density, have been shown to exhibit a high degree of efficacy in the acceptance of lipid hydroperoxides from LDL, with subsequent neutralisation of these molecules. This process serves to prevent the onset of inflammatory responses (151). It is important to note that the antioxidative function of HDL extends beyond vascular protection. Indeed, this function is impaired in chronic metabolic conditions such as type 2 diabetes and non-alcoholic fatty liver disease. In such cases, dysfunctional HDL exacerbates oxidative stress and tissue damage (179,180). Inflammatory diseases have been shown to have a detrimental effect on the quality of HDL by altering its protein and lipid composition. This, in turn, has been demonstrated to impair its capacity to neutralise oxidative molecules and reduce monocyte recruitment to sites of inflammation (181,182). Multiple researchers demonstrated and asserted that the functional capacity of HDL, as opposed to its circulating concentration, is the critical determinant of its protective effects in both health and disease. (60,61,119,183–185). They highlight that the antioxidative capacity of HDL, particularly HDL3, is preserved even in cases of altered HDL composition, such as in cases of CETP deficiency (183). Therefore, it can be concluded that HDL's antioxidative function is a dynamic and clinically meaningful trait that defends against cardiovascular disease and modulates the inflammatory and metabolic stress that underpins many chronic conditions.

1.3.3.4 Anti-inflammatory functions of HDL

HDL plays a vital role in protecting against inflammation through several interconnected mechanisms. It has been demonstrated that HDL has the capacity to reduce the expression of adhesion molecules, such as vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule (ICAM-1), on endothelial cells. This, in turn, serves to limit monocyte recruitment to inflamed vascular sites (186). The anti-inflammatory action of HDL is partly attributable to its primary protein, apoA-I, which exerts its suppressive effect on monocyte activation via the process of cholesterol efflux, facilitated by ABCA1 transporters (182). Other molecules, such as saturated lysophosphatidylcholines and sphingosine-1-phosphate, also play a role in the suppression of inflammation (187–190). It has been demonstrated that HDL interferes with pro-inflammatory signalling by blocking interactions between monocytes and T-cell microparticles. This process has been shown to reduce the production of cytokines such as IL-1β, TNF, and IL-6 (191). In the absence of any underlying pathologies, HDL has been demonstrated to enhance endothelial nitric oxide synthase activity, thereby contributing to a reduction in inflammation and the promotion of vascular protection (192). Furthermore, the enzymes associated with HDL, including PON1, possess antioxidant capabilities that serve to further mitigate inflammation by means of the neutralisation of oxidised lipids (193). In conclusion, HDL exerts anti-inflammatory effects through modulation of monocyte activity, suppression of cytokines, endothelial protection, and oxidative defence. HDL has been demonstrated to play a pivotal role in regulating the immune response by affecting signalling pathways such as NF-κB and peroxisome proliferator-activated receptor gamma. This, in turn, results in a reduction in the production of chemokines and their receptors, as observed in both live animal models and cell cultures (194).

1.3.3.5 Anti-infectious functions of HDL

HDL has emerged as a multifaceted component of the immune system with notable antiviral and anti-bacterial properties. Infection is a prevalent cause of hospitalisation in HF, and its presence is associated with an increased risk of re-hospitalisation and mortality (195). Liver cirrhosis is particularly associated with impaired immune responses and gut barrier dysfunction, predisposing patients to spontaneous bacterial peritonitis and life-threatening sepsis, with infection rates up to ten times higher than in non-cirrhotic individuals (196). In addition, individuals suffering from diabetes mellitus have elevated infection rates, with the most

significant incidence rate ratios observed for bone and joint infections, sepsis, and cellulitis. They are twice as likely to be hospitalized with infection and to die of infection-related death, compared with healthy individuals (197).

It was demonstrated in the early studies that HDL has the capacity to neutralise bacterial LPS and lipoteichoic acid. These are the key triggers of inflammatory responses in Gram-negative and Gram-positive infections, respectively (198). HDL achieves this through sequestration and clearance via SR-BI, preventing Toll-like receptor activation and the subsequent cytokine storm (199). Furthermore, it is evident that HDL and its primary protein constituent, apoA-I, possess inherent antiviral properties that manifest through hindering virus binding and subsequent penetration into host cells (200,201). Viruses such as hepatitis C, dengue, and Zika utilise lipoprotein receptors, including SR-BI, to gain entry into cells. The ability of HDL to prevent these interactions is attributable to two mechanisms: firstly, competitive binding to the receptors, and secondly, reduction of receptor availability through cholesterol depletion in membrane lipid rafts (202,203). In the context of the SARS-CoV-2 virus, HDL has been observed to modulate the activity of angiotensin-converting enzyme 2 and SR-BI through the process of cholesterol efflux. This, in turn, has been shown to impede the fusion and subsequent entry of the virus into host cells (204). Furthermore, the antioxidant enzymes present in HDL, including PON1, glutathione peroxidase-3, and catalase, neutralise reactive oxygen species produced during infection. This process serves to protect immune cells and tissues from oxidative damage (205,206). The immune system is significantly modulated by HDL, with its function being to control cholesterol levels in lipid rafts. This process influences immune cell signalling and fosters anti-inflammatory responses. Furthermore, it has been demonstrated that this process also boosts the production of pro-resolving lipid mediators, such as resolvins and lipoxins, in macrophages (207). In endothelial cells, HDL has been shown to enhance barrier strength and decrease the expression of leukocyte adhesion molecules, which is essential for limiting viral spread and preserving vascular balance during infections (208).

1.4 Beyond HDL-C: clinical perspectives on HDL functionality

HDL-C has historically been utilised as a fundamental biomarker in the assessment of cardiovascular risk, attributable to its robust inverse correlation with coronary heart disease in observational studies (69). Its prognostic value, however, is not limited to CVD. A number of

studies have demonstrated a correlation between low concentrations of HDL-C and unfavourable outcomes in a range of conditions, including chronic liver failure, sepsis, and pneumonia (209–211). Despite these associations, HDL-C has failed to deliver as a therapeutic target. Clinical trials aiming to raise HDL-C levels pharmacologically have not yielded reductions in cardiovascular events, and Mendelian randomization studies have not established a causal link between genetically elevated HDL-C and reduced cardiovascular risk (212,213). These limitations have prompted a critical re-evaluation of HDL's role, shifting attention from HDL-C levels to the qualitative and functional characteristics of HDL particles. In this context, HDL subclass analysis provides additional insight into HDL functionality. As previously mentioned, HDL exists as a spectrum of subclasses, ranging from large, cholesterol-rich HDL2 to small, dense HDL3 particles, each with distinct structural and functional properties. Emerging evidence indicates that smaller HDL particles, such as HDL3 and medium-sized HDL, may possess superior antioxidant and anti-inflammatory capabilities and are more strongly associated with reduced atherosclerotic burden and coronary calcification than total HDL-C (165,214). These findings support the idea that HDL subclass profiling may better capture the functional heterogeneity of HDL particles compared to simple HDL-C quantification. Looking ahead, the integration of HDL subclass measurement into clinical practice holds significant promise. Advanced lipoprotein profiling techniques, such as nuclear magnetic resonance (NMR) spectroscopy, are increasingly accessible and can quantify HDL particle size and number with high precision (215). These technologies may enable clinicians to identify patients with dysfunctional HDL profiles, those with low concentrations of protective HDL3 or medium-sized HDL, even in the presence of normal HDL-C levels (216). In parallel, HDL functionality assays, such as CEC and PON1 activity, are under development and may be standardized for routine diagnostic use in the coming years (217).

In conclusion, while HDL-C remains a widely used biomarker, its limitations have catalyzed a paradigm shift toward understanding HDL quality over quantity. Incorporating HDL subclass profiling and functionality assessments into clinical workflows could significantly improve disease risk prediction, stratification, and therapeutic targeting in cardiovascular and non-cardiovascular conditions.

1.5 Pathophysiological processes driving HDL dysfunction

HDL plays a crucial role in health and disease due to its antioxidant, anti-inflammatory, and cholesterol efflux-promoting functions. However, in various chronic diseases, including obesity, T2DM, HF, and liver cirrhosis, HDL can be altered in composition and function. This dysfunction alters its protective capacities and contributes to disease progression.

1.5.1 HDL function in HF: oxidative stress, inflammation and co-morbidities

In HF, HDL undergoes significant structural and functional remodelling, thereby compromising its cardioprotective properties (Figure 4). Dysfunction of HDL in HF is not simply a reflection of lipid levels, rather it is closely linked to alterations in its protein and lipid composition (218). Comorbidities that are frequently associated with HF including diabetes (219,220), obesity (221,222) and the process of aging (223), have been demonstrated to contribute to alterations in the composition and function of HDL. Diabetes mellitus induces glycation of HDL and its associated apolipoproteins. Glycation of HDL diminishes its atheroprotective properties and cholesterol efflux capacity, thereby accelerating atherosclerosis progression. HDL derived from diabetic patients exhibits reduced efficacy in maintaining endothelial protective functions. Many pleiotropic effects of HDL are mediated by apoM, which is significantly reduced in diabetic individuals primarily due to apoM-S1P glycation, impairing its S1P-binding capacity (224–226). In HF apoA-I and apoM, which play a critical role in cholesterol efflux and anti-inflammatory defence, have been shown to be reduced (227–230). Obesity lowers HDL-cholesterol levels and alters HDL subclass distribution, notably reducing small, dense HDL3 particles, which are most strongly linked to favorable HF outcomes (221,222,231). Aging similarly impacts HDL by enhancing oxidative modifications (232) and decreasing apoM secretion (233), impairing endothelial repair and increasing cardiac fibrosis risk (233). Systemic inflammation in HF is driven by elevated levels of pro-inflammatory cytokines such as TNF-α, IL-6, and CRP (234). The alteration of HDL composition by these cytokines is evidenced by a reduction in apoA-I and an enrichment of particles with SAA. This process consequently converts HDL from an anti-inflammatory to a pro-inflammatory molecule (235). Chronic inflammation upregulates enzymes such as endothelial lipase and secretory phospholipase A2 (236,237), accelerating HDL catabolism and depleting essential structural and functional components (238). This inflammatory environment also reduces LCAT and CETP activity, hindering HDL maturation and compromising its antioxidant capacity (239). Concurrently,

oxidative stress, defined by the overproduction of reactive oxygen species, results in HDL oxidation. This modification has been demonstrated to compromise the ability of HDL to activate key antioxidative enzymes, such as PON1. In addition, it has been shown to reduce the capacity of HDL to prevent LDL oxidation and support nitric oxide bioavailability (1,240). One key mechanism of HDL oxidation involves the enzyme myeloperoxidase (MPO), which catalyzes the chlorination and nitration of apoA-I (241). In patients diagnosed with acute heart failure, both total and small HDL particle concentrations were found to be significantly reduced, and their lower levels were strongly and independently associated with increased 3-month mortality. This finding indicates that small HDL particles may have a protective role, which is likely attributable to their antioxidant, anti-inflammatory, and cholesterol efflux capacities (242). In chronic HF, patients who died from cardiovascular causes exhibited a lower proportion of small HDL particles, leading to an overall increase in average HDL particle size. This shift in HDL particle distribution has been shown to be independently associated with higher cardiovascular mortality, thus underscoring the protective role of small HDL species (243).

In summary, comorbidities such as obesity, diabetes and aging impair liver synthesis of HDL-associated proteins, increase oxidative stress and SAA incorporation, and promote apoM clearance via the kidneys. Together, these lead to protein loss and HDL oxidation. These dysfunctional HDL particles fail to transition properly between large and small subclasses due to reduced CETP and HL activity, ultimately compromising the particle's beneficial roles (Figure 4). These pathophysiological alterations transform HDL from a protective entity into a dysfunctional particle, unable to attenuate myocardial stress, fibrosis, or vascular injury.

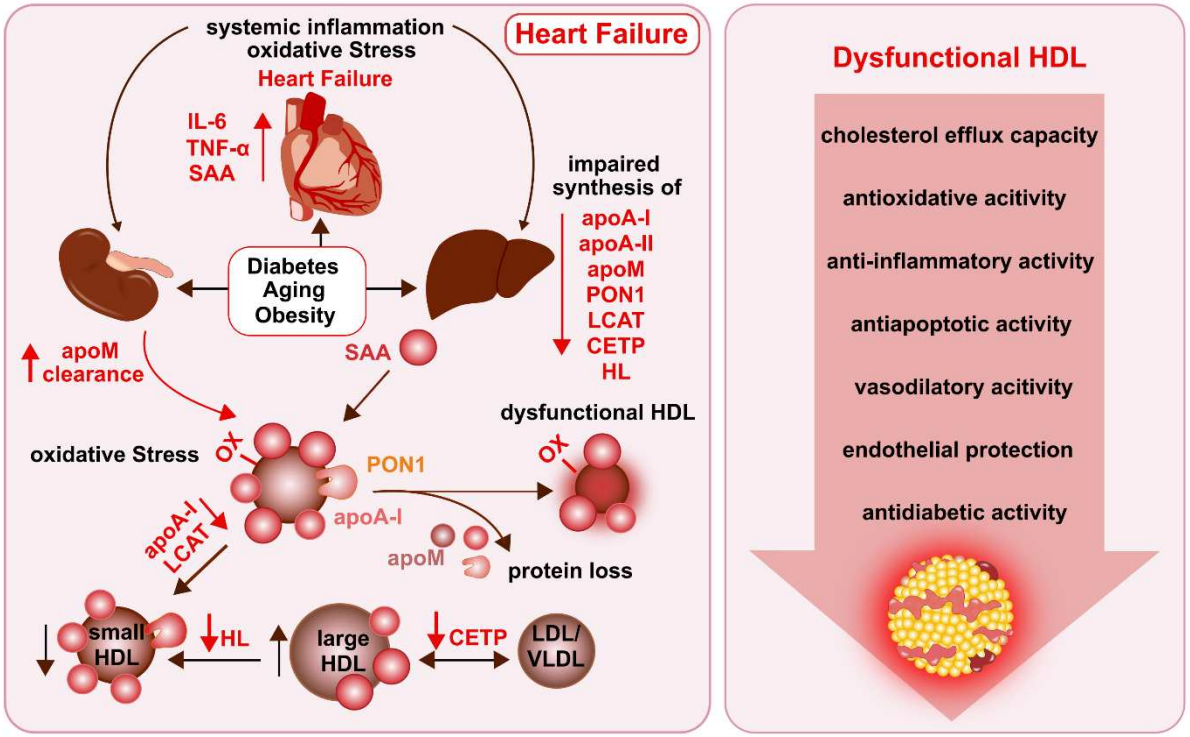

Figure 4. Impaired HDL metabolism and function in heart failure. Heart failure leads to a decreased cardiac index and elevated filling pressures, which in turn can cause liver damage and impair apolipoprotein synthesis. Additionally, heart failure-related kidney injury may enhance the renal loss of apoM. Co-existing conditions like diabetes, obesity or aging are also associated with renal and hepatic injury as well as lower circulating levels of apolipoproteins, promoting inflammation that worsens both kidney and liver damage, thereby further aggravating cardiac dysfunction. Altered HDL composition due to heart failure, inflammation and existing co-morbidities results in impaired HDL function.

1.5.2 HDL function in liver cirrhosis: oxidative stress, inflammation and hepatic insulin resistance

Liver cirrhosis has been shown to substantially alter HDL metabolism due to impaired lipoprotein synthesis, systemic inflammation, oxidative stress, and hepatic insulin resistance (Figure 5). The aetiology of these pathological states is characterised by a reduction in circulating HDL-C levels and a disruption in the functionality of enzymes that are vital for the remodelling of HDL, including LCAT and PON1 (244,245). Cirrhosis is marked by systemic inflammation, with increased levels of pro-inflammatory mediators such as IL-6, TNF-α, and SAA, which impair the liver's ability to produce critical HDL components (244,246). In patients with cirrhosis, there is an alteration in the distribution of HDL subclasses, with a shift towards larger, less functional HDL2 particles. These particles are often characterised by an enrichment in inflammatory proteins and a depletion of protective molecules, such as apoA-I and PON1. This shift has been demonstrated to result in a reduction of HDL's capacity to support

cholesterol efflux, endothelial repair, and antioxidative defence, particularly in cases of acute decompensation (244). Furthermore, the process of cirrhosis instigates a cascade of inflammatory and oxidative stress responses, which in turn remodel the structure of HDL through a series of complex biochemical reactions, including glycation and oxidation of apolipoproteins. This process also involves the incorporation of acute-phase reactants, such as SAA, into the structure of HDL, further diminishing its anti-inflammatory and antioxidative properties (244,247). These alterations are particularly detrimental in the context of infection. The role of HDL in neutralising bacterial LPS and modulating immune responses is well-documented. In patients suffering from cirrhosis, low HDL levels and impaired HDL function results in a reduced capacity to bind and detoxify LPS and higher risk of infection (209,244,248). This results in a diminished ability of the host to defend against Gram-negative bacteria and an increased susceptibility to sepsis (249–251).

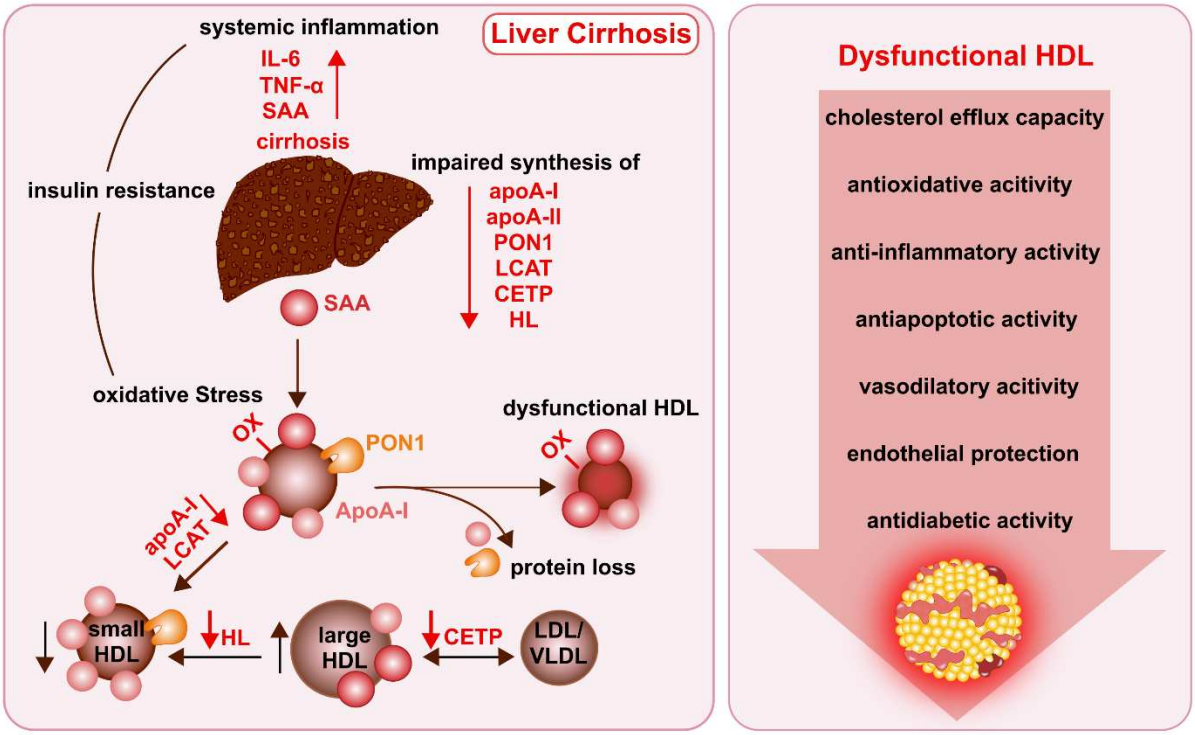

Figure 5. Altered HDL metabolism in patients with cirrhosis. Liver cirrhosis significantly disrupts HDL metabolism through mechanisms involving impaired hepatic lipoprotein synthesis, systemic inflammation, oxidative stress, and insulin resistance. These pathological changes lead to decreased circulating HDL-C levels and dysfunctional HDL remodeling, largely due to reduced activity of key enzymes such as lecithin-cholesterol acyltransferase (LCAT) and paraoxonase-1 (PON1). Cirrhosis-induced systemic inflammation, marked by elevated interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and serum amyloid A (SAA), further suppresses hepatic production of protective HDL components. A shift in HDL subclass distribution toward larger, less functional HDL2 particles is observed, accompanied by protein enrichment (e.g., SAA) and depletion (e.g., apoA-I, PON1). These alterations compromise HDL's capacity for cholesterol efflux, endothelial repair, and antioxidative defense.

1.5.3 HDL function in T2DM and obesity: oxidative stress, inflammation, and glycation

In T2DM and obesity, HDL particles undergo a series of structural and functional modifications driven by systemic oxidative stress, chronic inflammation, and glycation (Figure 6). These changes have shown to induce glycation of HDL apolipoproteins, with the greatest impact on apoA-I, which result in a reduction in the capacity of HDL to efflux cholesterol, as well as its ability to activate LCAT (252). Additionally, glycation has been demonstrated to compromise the interaction of HDL with ATP-binding cassette transporters, namely ABCA1 and ABCG1. This further impairs the process of cholesterol efflux from macrophages (253). The presence of chronic low-grade inflammation, a common occurrence in cases of obesity T2DM, has been observed to result in alterations to the composition of HDL. Specifically, this inflammation has been shown to enrich HDL with SAA and to reduce its antioxidative enzymes, such as PON1. Consequently, this shift in composition renders HDL pro-inflammatory and pro-oxidant, therefore dysfunctional (254,255). Oxidative stress has been demonstrated to lead to the oxidation of HDL lipids and proteins. This process results in the transformation of HDL into a dysfunctional particle that has the potential to become atherogenic (256) (Figure 6). The alterations described herein have been shown to contribute to impaired insulin secretion, reduced β-cell viability, and increased vascular inflammation. Consequently, this pathophysiological triad has been demonstrated to exacerbate the pathophysiology of type 2 diabetes mellitus (257). In cases of obesity, adipose tissue becomes inflamed and secretes pro-inflammatory cytokines, such as TNF-α and IL-6, which in turn impair the anti-inflammatory functions of HDL (258).

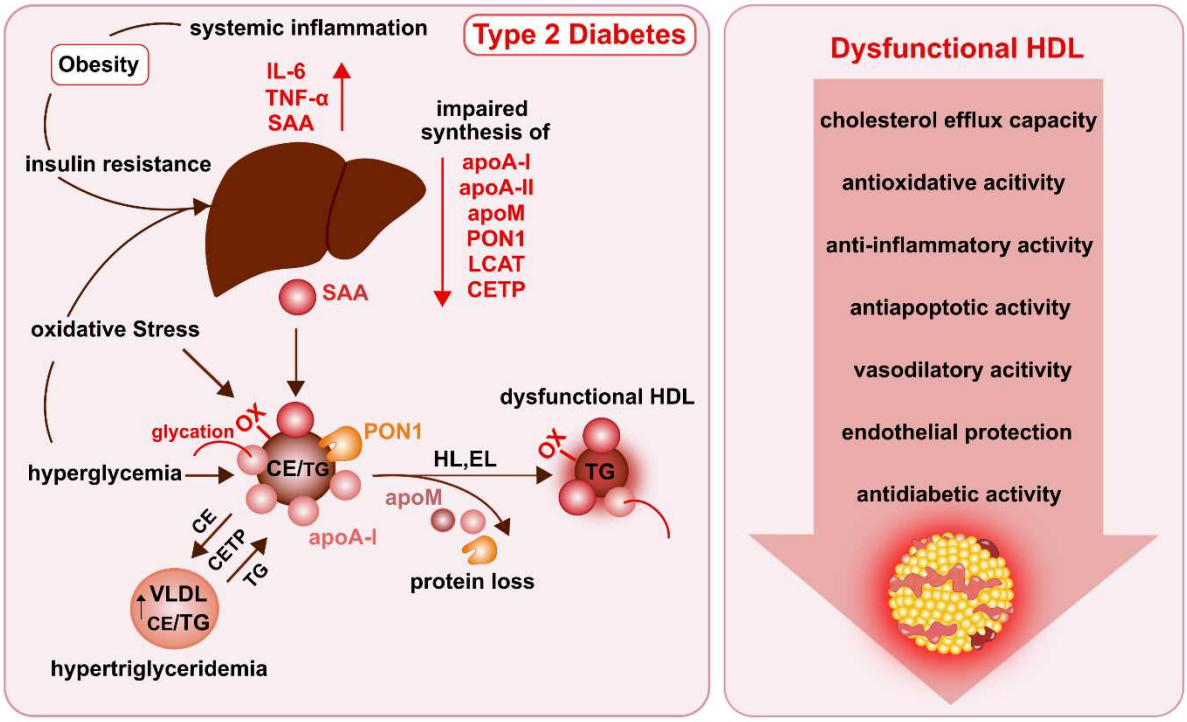

Figure 6. Altered HDL metabolism in T2DM leading to dysfunctional HDL. The constellation of metabolic disturbances, including hyperglycemia, insulin resistance, hypertriglyceridemia, and both acute and chronic systemic inflammation, interact synergistically to alter the lipidomic and proteomic composition of HDL particles, thereby impairing their functional capacity. Chronic inflammation is characterized by elevated circulating levels of interleukin-6 (IL-6), which in turn stimulates hepatic and extrahepatic synthesis of serum amyloid A (SAA). SAA incorporates into HDL particles, displacing essential protein components such as apolipoprotein A-I (apoA-I) and paraoxonase-1 (PON1), leading to diminished anti-inflammatory and antioxidative functions. Concomitantly, oxidative stress promotes the peroxidation of HDL-associated lipids and proteins, while persistent hyperglycemia facilitates the non-enzymatic glycation of HDL proteins, including apoA-I, via the formation of advanced glycation end products, further compromising HDL functionality. In the context of hypertriglyceridemia, elevated activity of cholesteryl ester transfer protein (CETP) enhances the exchange of triglycerides for cholesteryl esters within HDL particles, leading to triglyceride enrichment and depletion of cholesteryl esters. Additionally, type 2 diabetes is associated with impaired transfer of surface fragments from very-low-density lipoproteins (VLDL) to HDL; these fragments are primarily composed of free cholesterol and phospholipids.

2 RESULTS

The results section will provide a brief overview of the following publications:

1. Pammer A, Klobučar I, Stadler JT, Meissl S, Habisch H, Madl T, Frank S, Degoricija V, Marsche G. Impaired HDL antioxidant and anti-inflammatory functions are linked to increased mortality in acute heart failure patients. Redox Biology. DOI: 10.1016/j.redox.2024.103341

2. Pammer A, Madl T, Habisch H, Kerbl-Knapp J, Rainer F, Stadlbauer V, Horvath A, Douschan P, Stauber R, Marsche G. Depletion of Small HDL Subclasses Predicts Poor Survival in Liver Cirrhosis. Antioxidants. DOI: 10.3390/antiox14060664

3. Pammer A, Obermayer A, Stadler JT, Pferschy PN, Tripolt NJ, Habisch H, Madl T, Sourij H, Marsche G. Effects of dietary interventions and intermittent fasting on HDL function in obese individuals with T2DM: a randomized controlled trial. Cardiovascular Diabetology. DOI: 10.1186/s12933-024-02426-5

Ad 1.) In my first project, I explored the relationship between HDL dysfunction and mortality in patients with acute heart failure (AHF). Analyzing 315 patients, we found that those who died within three months after being hospitalized had significantly lower HDL cholesterol efflux capacity, reduced PON1 and LCAT activity, and fewer small HDL particles. These associations remained significant after adjusting for confounders (Figure 7). Our findings highlight that impaired HDL antioxidant and anti-inflammatory functions are strong predictors of short-term mortality in AHF, supporting HDL function metrics as valuable clinical risk indicators.

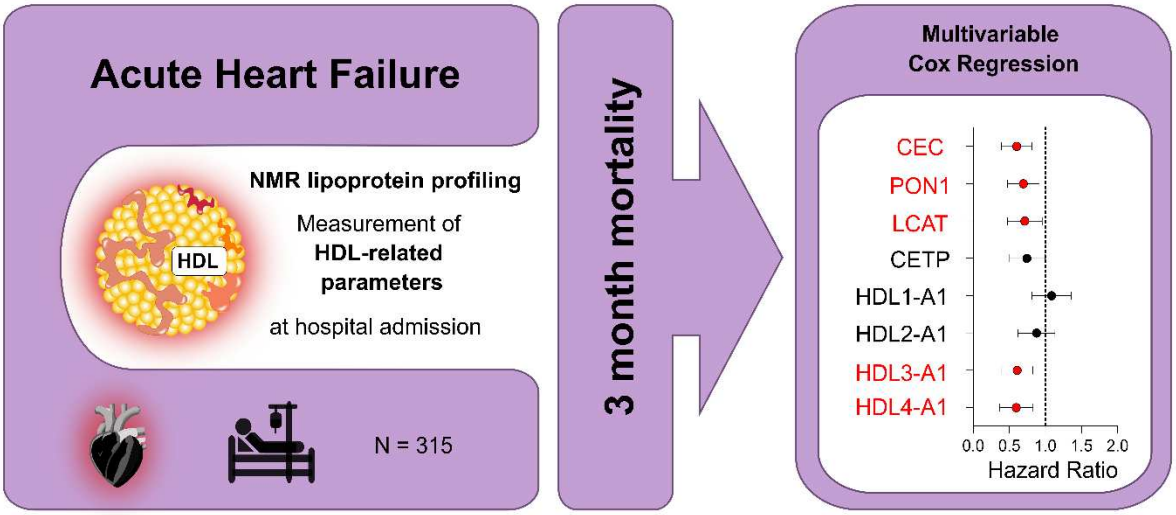

Figure 7. Graphical Abstract. Impaired HDL antioxidant and anti-inflammatory functions are linked to increased mortality in acute heart failure patients. HDL-related parameters indicated in red are inversely significantly associated with 3 months mortality risk of patients with acute heart failure. A1, apolipoprotein A1; CEC, cholesterol efflux capacity; CETP, cholesteryl-ester transferprotein; LCAT, lecithin cholesterol-acyltransferase; PON1, paraoxonase 1. Image reproduced from Pammer et al. *(1)* under Creative Commons Attributions License.

Ad 2.) In my second project, I investigated the prognostic relevance of HDL subclasses in patients with liver cirrhosis. Using NMR Technology, we analyzed HDL profiles in 363 cirrhotic patients (both compensated and decompensated) and compared them to healthy controls. We found a significant depletion of small and extra-small HDL particles in cirrhotic patients. These HDL subclasses, known for their antioxidant and anti-inflammatory functions, were inversely correlated with markers of oxidative stress and liver dysfunction. Importantly, reduced levels of small HDL particles independently predicted 12-month mortality in compensated cirrhosis patients, even after adjusting for the traditionally used MELD scores (Figure 8). This project underscored the critical role of small HDL particles in modulating disease outcomes and highlighted their potential as prognostic biomarkers or future therapeutic targets in chronic liver disease.

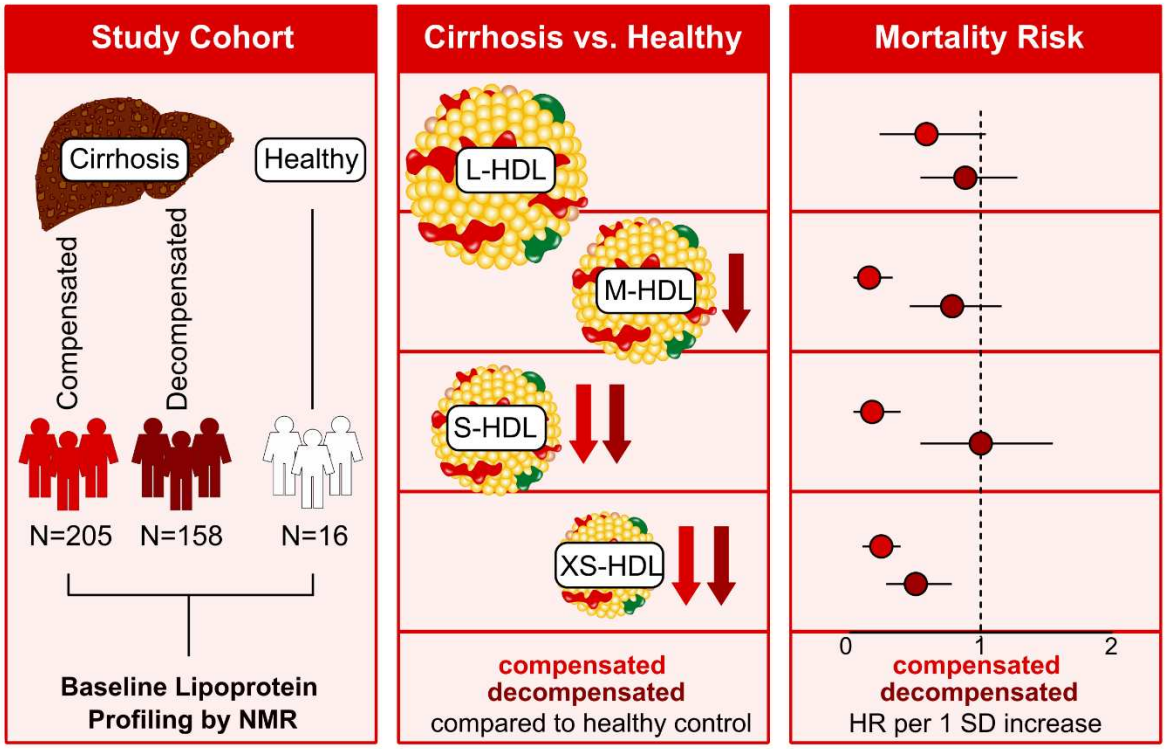

Figure 8. **Graphical Abstract.** Depletion of Small HDL Subclasses Predicts Poor Survival in Liver Cirrhosis. Results on patients with compensated cirrhosis are indicated in light red while those with decompensated are displayed in dark red. HDL, high-density lipoprotein; L-, large; M-, medium; S-, small; XS-, extra-small. Image reproduced from Pammer et al. (2) under Creative Commons Attributions License.

Ad 3.) In my third project, I evaluated the effects of dietary intervention and intermittent fasting (IF) on HDL functionality in obese individuals with T2DM. In this randomized controlled trial (INTERFAST-2), participants received either dietary counseling alone or in combination with alternate-day fasting over 12 weeks. Both groups showed improved HDL cholesterol efflux capacity, indicating enhanced reverse cholesterol transport. Notably, only the IF group demonstrated a significant increase in serum apoM, a component of HDL associated with improved insulin sensitivity. In contrast, improvements in PON1 and LCAT activity were significant only in the non-IF group (Figure 9). These findings suggest that while both interventions improve HDL function, IF specifically boosts apoM levels, which may have distinct metabolic benefits.

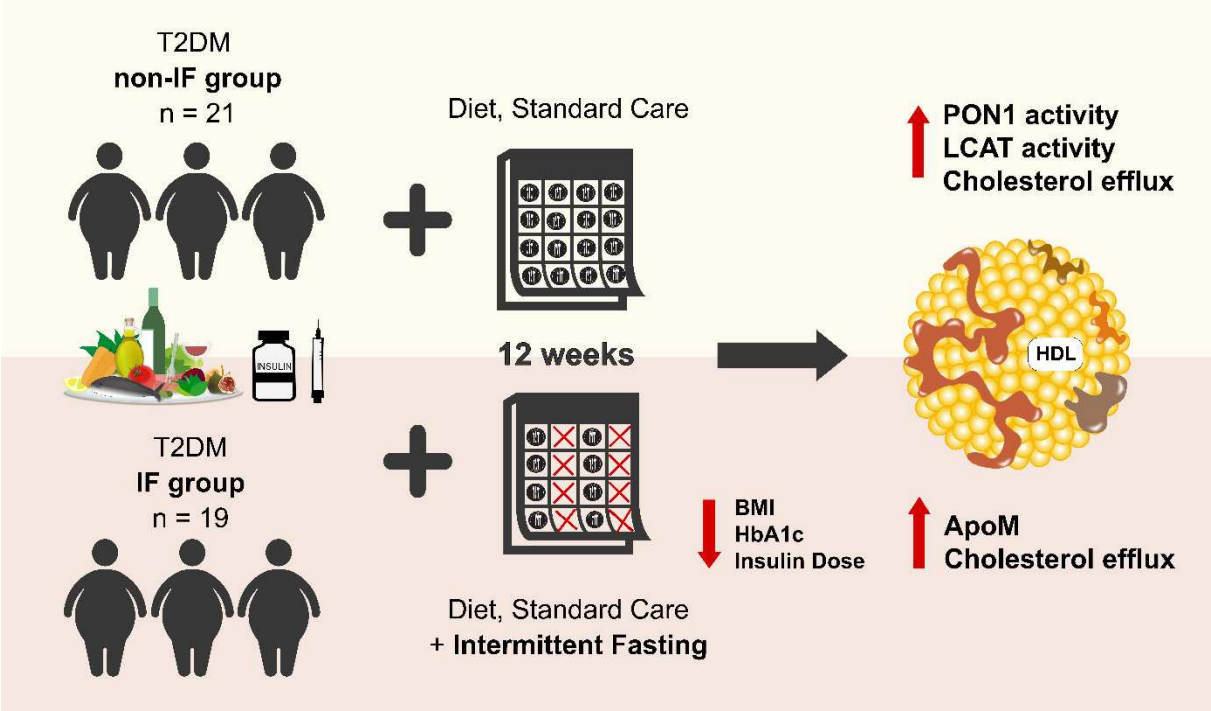

Figure 9. Graphical Abstract. Effects of dietary interventions and intermittent fasting on HDL function in obese individuals with T2DM: a randomized controlled trial. ApoM, apolipoprotein M; HbA1c, haemoglobin a1c; LCAT, lecithin cholesterol-acyltransferase;. PON1, paraoxonase. Image reproduced from Pammer et al. *(3)* under Creative Commons Attributions License.

3 DISCUSSION

Emerging evidence suggests that HDL functionality is proving to be a more reliable biomarker for disease risk stratification when compared to traditional HDL-C levels. While HDL-C quantifies the cholesterol content within HDL particles, it fails to capture the diverse and dynamic roles HDL plays in processes such as cholesterol efflux, antioxidation, and inflammation modulation. Recent research emphasises that specific HDL subclasses, differentiated by size, density, and composition, serve not only as biomarkers but also as functional indicators of HDL's protective capabilities.

During my PhD, my research focused on investigating HDL functionality, composition and metabolism in metabolic and inflammatory settings. Our findings revealed that HDL functional, compositional and metabolic parameters are altered in pathologies such as acute heart failure as well as liver cirrhosis. We observed a decrease in the S- and XS-HDL subclasses (HDL3 and HDL4) at the time of hospital admission in acute heart failure patients who died within 3 months. Furthermore, low levels of small HDL particles, along with important HDL functional parameters such as CEC, PON1 activity and LCAT activity, could independently predict 3 months mortality risk. Correlations of HDL subclasses with HDL functional parameters revealed strong associations of S- and XS- HDL particles with PON1- and LCAT- activity, linking them to increased anti-inflammatory and antioxidative properties (1). In liver cirrhosis we observed similar results. HDL subclass distribution was significantly altered in individuals with cirrhosis compared to healthy controls, with S- and XS- particles being significantly reduced. Low levels of M- to XS-HDL subclasses could independently predict 12 months mortality in patients with compensated cirrhosis, while low concentrations of XS-HDL independently predicted 90 days mortality risk in the advanced decompensated cirrhosis stage. Additionally, we observed alterations in HDL composition in between subclasses. Total cholesterol content of M-HDL, S-HDL and XS-HDL subclasses was reduced in all patients with cirrhosis alongside a notable decrease in free cholesterol content within L-HDL particles. Triglyceride concentrations were increased across all HDL subclasses in patients with compensated and decompensated cirrhosis (2). I further investigated the impact of dietary strategies and intermittent fasting on HDL function in individuals with type 2 diabetes, which is known to be a common risk factor for heart failure as well as liver cirrhosis. A well-balanced

diet combined with meticulous insulin management improved HDL functional parameters such as cholesterol efflux capacity, PON1- and LCAT activity. Surprisingly, additional intermittent fasting mitigated the effect of the dietary intervention on PON1- and LCAT- activities, but significantly increased concentrations of the anti-inflammatory apoM. We additionally observed non-significant upward trends of S- and XS- HDL subclass concentrations upon 12 weeks dietary intervention.

3.1 Impaired HDL antioxidant and anti-inflammatory functions are linked to increased mortality in acute heart failure patients (Redox Biol. 2024 Oct:76:103341.doi: 10.1016/j.redox.2024.103341.)

Previous studies have indicated that the antioxidative and anti-inflammatory properties of HDL may be pivotal in the development of HF, as they help preserve the heart's structure and function, thereby lowering the risk of mortality (259–261). Previous research has shown that smaller HDL subclasses (231,242) and reduced HDL cholesterol efflux capacity (262) are linked to a higher risk of mortality after an index AHF hospitalization. However, these parameters have not been directly compared. Therefore, we performed the first comprehensive evaluation of multiple aspects of HDL function, structure, and metabolism in patients presenting to the emergency department with severe clinical manifestations of AHF requiring hospitalization.

In this study, I investigated the functional properties of HDL, focusing on its antioxidant and anti-inflammatory activities in the context AHF. While HDL-C levels have historically been viewed as cardioprotective, evidence increasingly suggests that the qualitative functionality of HDL is more important than its quantity, particularly during acute inflammatory states such as AHF (60,169).

Our findings revealed that impaired HDL function, but not HDL-C levels, was significantly associated with increased 3-month mortality, even after adjusting for traditional risk factors such as NT-proBNP and renal function.

These results are in line with previous work showing that HDL particles can become dysfunctional in inflammatory and oxidative environments (223,263–266). In patients who died

within the follow-up period, we observed significantly lower HDL-associated PON1 activity, a marker indicative for oxidative damage and reduced protective capacity of HDL (267).

The acute inflammatory milieu of AHF appears to modify HDL particles. Oxidation by MPO and other enzymes reduces HDL's capacity to neutralize reactive oxygen species and promote cholesterol efflux (268,269). In our study, decreased HDL antioxidative activity, as measured by reduced PON1 activity and low levels of small HDL subclasses, were strongly linked to mortality. These dysfunctional HDL particles may not only lose protective functions but gain harmful properties, thus worsening the patient's clinical course.

Importantly, the function of HDL in removing excess cholesterol from peripheral cells, the so-called cholesterol efflux capacity, was significantly compromised in non-survivors. This supports the concept that HDL dysfunction may not be a mere consequence of AHF but a contributor to its progression and poor outcomes (266).

The implications of these findings are substantial. Although HDL-C has been considered cardioprotective, recent large-scale intervention trials aimed at increasing HDL-C failed to show cardiovascular benefit (270). Our results support the hypothesis that merely increasing HDL-C may be ineffective if the particles are functionally impaired or exhibit pro-inflammatory properties. These findings underscore the importance of shifting the clinical focus from the quantity of HDL to its functional quality.

The concept that HDL can shift from an anti-atherogenic to a pro-inflammatory particle in disease states is supported by in vitro and clinical studies (271–274). This transformation may be particularly relevant in AHF, which is characterized by profound oxidative stress, systemic inflammation, and endothelial activation (275). HDL particles are highly sensitive to such changes, and their function may serve as an integrative marker of systemic metabolic and inflammatory status.

From a clinical perspective, these findings raise critical questions about the use of HDL metrics in prognostic assessment. Our data suggest that functional assays of HDL may provide superior prognostic information compared to HDL-C levels alone. Although currently limited to research settings, assessments of HDL subclasses by NMR could in the future, become valuable tools in stratifying patient risk and guiding therapy.

Another key question is whether HDL dysfunction in AHF is reversible. It remains unclear whether impaired HDL function in AHF patients is transient, driven by the acute phase response, or represents a chronic feature of those predisposed to worse outcomes.

Clarifying this distinction will be critical for guiding future interventional studies aimed at restoring HDL function through pharmacological or lifestyle-based approaches.

Despite its contributions, this study has limitations. The observational design precludes causal inference, and the generalizability of the findings may be limited without validation in diverse populations, ethnic groups, and earlier stages of disease. Nonetheless, the study provides novel and comprehensive insights into HDL's functional, structural, and metabolic properties in hospitalized AHF patients. In particular, the strong positive correlation observed between HDL functionality and its subclasses highlights the relevance of HDL quality in this clinical context.

In conclusion, we observed that impaired antioxidant and anti-inflammatory functions of HDL are strongly and independently associated with increased short-term mortality in patients hospitalized with acute heart failure. These functional impairments, particularly reduced cholesterol efflux capacity, PON1 activity and small HDL subclasses were more predictive of outcome than HDL-C levels themselves. Our findings support a paradigm shift toward assessing HDL function as a biomarker and potential therapeutic target in heart failure management.

3.2 Depletion of Small HDL Subclasses Predicts Poor Survival in Liver Cirrhosis (Antioxidants 2025, 14(6), 664; doi: 10.3390/antiox14060664)

Chronic liver disease remains a major global health burden, contributing to nearly two million deaths each year (276). Growing evidence underscores the central role of disrupted lipid metabolism in driving inflammatory pathways and worsening disease progression (244,277,278).

In the second part of my dissertation, I investigated the relationship between HDL subclasses and mortality in patients with liver cirrhosis, which is to my knowledge the first comprehensive analysis of HDL subclass composition in cirrhosis and its clinical implications. We observed that liver cirrhosis leads to a profound depletion of small HDL particles, which are known for

their antioxidant and anti-inflammatory properties (279,280). Through NMR spectroscopy, we aimed to characterize alterations in HDL subclass distribution and assess their prognostic value for survival in cirrhotic individuals.

We observed a striking reduction in M-HDL, S-HDL, and XS-HDL subclasses in cirrhotic patients, especially those with decompensated disease, whereas L-HDL remained unchanged. This pattern persisted, regardless of the etiology of cirrhosis, indicating a consistent alteration in HDL metabolism across different liver disease contexts.

We further evaluated the compositional changes of HDL subclasses. Cholesterol content was markedly reduced in M-, S-, and XS-HDL particles, while triglyceride content was elevated across all HDL subclasses, suggesting both quantitative and qualitative impairments of HDL in cirrhosis. These compositional shifts may reflect impaired lipid metabolism and remodeling, potentially linked to the reduced activity of enzymes such as LCAT and CETP which are known to be diminished in cirrhosis (244).

Notably, we observed strong inverse correlations between small HDL subclasses and markers of systemic inflammation and oxidative stress. XS-HDL levels negatively correlated with CRP, bilirubin, tyrosine, and phenylalanine, indicating a potential role of these particles in modulating inflammatory and redox balance. Elevated phenylalanine and tyrosine in liver disease have been linked to oxidative stress–induced inhibition of phenylalanine hydroxylase (281), suggesting a direct interaction between small HDL depletion and metabolic dysfunction. Likewise, higher bilirubin (impaired hepatic detoxification) and CRP (systemic inflammation) correlated negatively with all HDL subclasses. These findings align with the well-established biological functions of HDL subclasses, in which key protective activities, such as cholesterol efflux, antioxidative action, anti-inflammatory effects, and antiapoptotic signalling, are largely mediated by small, dense, protein-rich HDL particles (280).

Most importantly, in this cohort, reduced concentrations of small HDL subclasses independently predicted mortality. In patients with compensated cirrhosis, M-HDL, S-HDL, and XS-HDL were all significantly associated with lower 12-month survival, even after adjusting for age, sex, and MELD score. Among patients with decompensated cirrhosis, XS-HDL was independently inversely associated with 90-day mortality. After adjusting for CRP levels, the prognostic significance of HDL subclasses in compensated cirrhosis diminished, whereas in decompensated patients, low levels of XS-HDL retained strong predictive power.

This suggests that HDL's prognostic value may be partly mediated by systemic inflammation in early disease stages but remains independently valuable in advanced disease.

We believe that the loss of small HDL particles could exacerbate systemic inflammation and oxidative damage, fueling a vicious cycle in liver disease progression. This hypothesis is supported by studies demonstrating HDL's role in neutralizing bacterial LPS and modulating immune responses (278,282). In cirrhosis, this protective function may be compromised, leading to greater susceptibility to infections and inflammation-driven decompensation (209,244,283).

Furthermore, the reduced levels of HDL may impair reverse cholesterol transport, thereby contributing to lipid dysregulation and hepatocellular stress (284). The observed association of HDL depletion with mortality is consistent with similar observations in cardiovascular disease, Alzheimer's disease, and AHF, where small HDL particles were predictive of poor outcomes (1,285,286).

From a translational perspective, the ability of HDL subclass profiling, particularly through standardized NMR spectroscopy, to enhance mortality prediction represents a significant advance. The predictive performance of M-HDL in compensated cirrhosis was comparable to the MELD score and combining both improved prognostic accuracy.

However, we are aware that this study has limitations. The observational nature of the study precludes the establishment of causal relationships between alterations in HDL subclasses and mortality. Furthermore, reliance on plasma-based measurements imposes limitations on spatial resolution, as these systemic markers are unable to accurately delineate hepatic oxidative stress from oxidative activity originating in other organs or from generalized systemic inflammation. Future research incorporating direct assessments of intrahepatic oxidative stress, such as liver biopsies or hepatic-specific imaging and biomarkers, would enable more accurate, organ-specific evaluation.

In conclusion, we demonstrated that the depletion of small HDL subclasses is not merely a biochemical anomaly but a clinically relevant predictor of survival in cirrhosis. Our findings support the potential of small HDL particles as biomarkers and therapeutic targets in liver disease. Restoring or mimicking the function of small HDL, via pharmacological agents or

apoA-I mimetic peptides, could be an avenue for future interventions aiming to improve patient outcomes (282,287).

3.3 Effects of dietary interventions and intermittent fasting on HDL function in obese individuals with T2DM: a randomized controlled trial (Cardiovasc Diabetol. 2024 Sep 12;23(1):339. doi: 10.1186/s12933-024-02426-5.)

Type 2 diabetes represents nearly 90% of the estimated 537 million diabetes cases worldwide, and its prevalence is rising sharply, with concerning increases among children and young adults under 40 (288). Early detection and proactive management are essential to prevent or delay serious microvascular and macrovascular complications, as well as to reduce associated mortality (289). In patients with T2DM, previous studies suggest that IF may confer benefits such as weight loss, potentially reducing daily insulin requirements (290,291). Furthermore, when paired with a balanced diet and optimal insulin management, IF may enhance HDL metabolism and function.

In this study, we sought to evaluate the effects of dietary intervention combined with IF on the functionality HDL in obese individuals with T2DM. HDL not only protects against cardiovascular disease but also improves glycemic control by mechanisms such as increasing insulin secretion and activating AMP-activated protein kinase pathways in skeletal muscle (257).

To assess HDL function, we measured HDL cholesterol efflux capacity, PON1 activity, LCAT activity, CETP activity, and apoM levels in individuals with T2DM that performed IF versus a non-fasting T2DM group. Following 12 weeks, we observed that apoM levels significantly increased in the IF group, but not in the non-IF group. In the IF group, apoM levels negatively correlated with both body mass index and fasting glucose, suggesting an association between weight loss, glycemic control, and apoM concentration. Given that apoM plays a critical role in carrying sphingosine-1-phosphate, which supports insulin secretion and promotes formation of preβ-HDL particles, these findings suggest a unique mechanistic advantage of IF (292,293).

We also examined HDL cholesterol efflux capacity, the ability of HDL to remove cholesterol from macrophages. Interestingly, both groups demonstrated significant improvements in efflux

capacity, regardless of fasting regimen. This metric is strongly predictive of reduced cardiovascular risk and has been shown to be inversely associated with T2DM prevalence (169,173).

When we assessed antioxidant and anti-inflammatory properties of HDL via PON1 activity, we observed that only the non-IF group experienced a significant increase in activity, while changes in the IF group were not significant. Similarly, LCAT activity, crucial for HDL maturation and with recognized antioxidant effects, also increased significantly in the non-IF group, but not in the IF group. CETP activity remained unchanged across both groups.

These results prompted us to consider potential explanations for the divergent responses observed. One plausible factor is the reduced intake of polyphenol-rich foods such as fruits and vegetables on fasting days in the IF group. These dietary components are known to enhance HDL function. For example, the PREDIMED trial demonstrated improved cholesterol efflux capacity following a Mediterranean diet rich in extra virgin olive oil (294). Similarly, increased PON1 activity has been linked to consumption of antioxidant-rich foods like nuts and fish (295), while lycopene from tomatoes may upregulate LCAT activity (296).

It is possible that the IF group's restricted intake on fasting days compromised their intake of these beneficial nutrients, limiting gains in PON1 and LCAT activity despite overall improvements in HDL function. However, we note that apoM increases unique to the IF group may still offer metabolic benefits, as caloric restriction is known to upregulate apoM expression via hepatic glucose and lipid metabolic pathways (113,297).

The selective increase in apoM levels in the IF group could also partially explain improved glucose regulation. ApoM-S1P complexes have been shown to stimulate insulin secretion, thus contributing to glycemic control (292).

In interpreting our findings, we acknowledge the limitations of a small sample size and an open-label study design. Additionally, subtle baseline differences in HDL and triglyceride levels between groups may have impacted outcomes. Nonetheless, improvements in HDL cholesterol efflux across both groups suggest that our interventions had genuine physiological effects independent of starting metrics.

Reflecting on the broader context, previous studies caution that alternate-day fasting might have adverse effects in animal models with genetic dyslipidemia. In LDL receptor-deficient mice,

such fasting increased atherosclerosis risk (298,299). These findings underline the importance of personalizing dietary strategies based on individual risk profiles.

In conclusion, we found that a Mediterranean-style diet enhances HDL function, particularly cholesterol efflux capacity, in people with T2DM. Adding intermittent fasting increased apoM levels, a potentially important mechanism for glycemic improvement, but did not enhance other HDL functions beyond what was achieved through dietary changes alone. This suggests that while intermittent fasting may provide metabolic benefits via apoM, its added value for HDL functionality is nuanced and potentially diet dependent.

4 CONCLUSION

In this thesis, I explored the multifaceted role of HDL functionality across three distinct pathological conditions: AHF, liver cirrhosis, and T2DM. The combined findings from these studies reinforce a growing consensus that HDL function, rather than HDL-C quantity alone, holds greater clinical relevance in predicting outcomes and informing therapeutic strategies across diverse disease states.

In the study on AHF, we demonstrated that impaired antioxidant and anti-inflammatory functions of HDL, rather than reduced HDL-C levels, were significantly and independently associated with increased 3-month mortality. Key functional markers, particularly PON1 activity and cholesterol efflux capacity, were compromised in non-survivors, highlighting HDL dysfunction as a potential contributor to poor clinical outcomes. Furthermore, we found a marked reduction in small HDL subclasses, reinforcing the notion that these particles are not only more functionally potent but also more vulnerable to modification in inflammatory and oxidative environments, such as those, characteristic of AHF. These findings suggest that in the acute setting, HDL particles may lose their vasoprotective functions and potentially acquire pro-inflammatory properties, exacerbating disease progression.

A similar pattern emerged in our study on liver cirrhosis, where we investigated HDL subclass distribution and its prognostic implications. We observed a profound depletion of small HDL subclasses, especially XS-HDL particles in patients with decompensated cirrhosis. These particles showed strong inverse correlations with markers of inflammation and oxidative stress, and their reduction was independently predictive of both short- and long-term mortality. Importantly, the depletion of small HDL particles appeared consistent across patients with cirrhosis and remained prognostically relevant even after adjusting for conventional severity scores such as MELD. These results support the hypothesis that HDL dysfunction is not simply a downstream consequence of liver impairment, but an active participant in systemic inflammation and metabolic dysregulation, further exacerbating disease severity and increasing the risk of mortality.

The interventional study in obese individuals with T2DM added another layer to this understanding by demonstrating that HDL function is modifiable through lifestyle interventions. While both dietary counseling and IF improved HDL cholesterol efflux capacity,

an essential anti-atherogenic function, only the non-IF group showed significant improvements in PON1 and LCAT activities, suggesting a nuanced interaction between diet composition and HDL function. Conversely, the IF group uniquely exhibited an increase in apoM levels, which correlated with improved glycemic control. These results highlight the differential impact of dietary components on various aspects of HDL functionality and emphasize that enhancing HDL quality may require not only caloric restriction but also the inclusion of specific nutrient-rich foods.

Taken together, these studies converge on several critical insights. First, HDL function is a more sensitive and clinically relevant marker of disease progression and prognosis than HDL-C levels alone. Second, the depletion of small HDL subclasses emerges as a consistent and powerful predictor of poor outcomes across conditions characterized by systemic inflammation and metabolic dysregulation, including AHF and liver cirrhosis. Third, HDL function is not static; it can be modulated through targeted interventions, although the type and composition of such interventions significantly influence specific functional outcomes.

Despite the valuable contributions and insights provided by these studies, some limitations should be acknowledged. The observational nature of the research restricts the ability to draw causal inferences, and generalizability remains limited due to factors such as small sample sizes, lack of population diversity, and reliance on systemic rather than organ-specific measurements. Additionally, open-label designs may introduce behavioural biases, and baseline differences between study groups could influence outcomes. These limitations highlight the need for future research employing more rigorous designs and diverse cohorts to validate and expand upon the current findings.

In conclusion, this dissertation establishes HDL functionality as a central, dynamic mediator of systemic health across diverse clinical settings. The consistent association between impaired HDL function and increased mortality in both acute and chronic disease contexts support a paradigm shift from evaluating HDL quantity to assessing HDL quality. These findings pave the way for future research into therapeutic strategies aimed at restoring HDL function, whether through pharmacological agents, dietary interventions, or lifestyle modification. Ultimately, functional HDL profiling could become a valuable tool for risk stratification and personalized treatment in cardiovascular, hepatic, and metabolic diseases.

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Impaired HDL antioxidant and anti-inflammatory functions are linked to increased mortality in acute heart failure patients

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ABSTRACT

Aims: Acute heart failure (AHF) is typified by inflammatory and oxidative stress responses, which are associated with unfavorable patient outcomes. Given the anti-inflammatory and antioxidant properties of high-density lipoprotein (HDL), this study sought to examine the relationship between impaired HDL function and mortality in AHF patients. The complex interplay between various HDL-related biomarkers and clinical outcomes remains poorly understood.

Methods: HDL subclass distribution was quantified by nuclear magnetic resonance spectroscopy. Lecithin–cholesterol acyltransferase (LCAT) activity, cholesterol ester transfer protein (CETP) activity, and paraoxonase (PON-1) activity were assessed using fluorometric assays. HDL-cholesterol efflux capacity (CEC) was assessed in a validated assay using [3H]-cholesterol-labeled J774 macrophages.

Results: Among the study participants, 74 (23.5 %) out of 315 died within three months after hospitalization due to AHF. These patients exhibited lower activities of the anti-oxidant enzymes PON1 and LCAT, impaired CEC, and lower concentration of small HDL subclasses, which remained significant after accounting for potential confounding factors. Smaller HDL particles, particularly HDL3 and HDL4, exhibited a strong association with CEC, PON1 activity, and LCAT activity.

Conclusions: In patients with AHF, impaired HDL CEC, HDL antioxidant and anti-inflammatory function, and impaired HDL metabolism are associated with increased mortality. Assessment of HDL function and subclass distribution could provide valuable clinical information and help identify patients at high risk.

1. Introduction

Previous research has demonstrated that low levels of HDL particles are independent risk factors for both mortality and hospital readmissions due to recurrent symptoms of AHF [1–3]. Studies in animal models have demonstrated HDL-mediated favorable cardiac remodeling in pre-heart failure settings such as diabetic cardiomyopathy [4], post-myocardial infarction [5], and chronic pressure overload [6]. A single injection of reconstituted HDL given to mice shortly after myocardial infarction has shown therapeutic potential by increasing cardiac glucose uptake, protecting heart cells from death, and restoring cardiac function, effectively halting the progression to heart failure [7].

Oxidative stress is a fundamental pathophysiological mechanism closely associated with the aging process and is involved in heart failure's development and progression [8,9]. Various constituents of HDL, in particular its major protein constituent apolipoprotein A-I (apoA-I),

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the antioxidant enzyme paraoxonase 1 (PON1) and lecithin–cholesterol acyltransferase (LCAT) play a crucial role in the anti-oxidative and cardioprotective effects triggered by HDL [10–13]. PON1 is an essential antioxidant component in HDL that is important in hydrolyzing oxidized lipids within lipoproteins [14–17]. LCAT plays a vital role in the maturation of HDL particles and has a unique ability to break down oxidized short-chain phospholipids [18]. LCAT-targeted therapies have emerged as a promising approach for treating LCAT deficiency and potentially other cardiovascular diseases [19]. Small, dense HDL particles show the most potent antioxidative, anti-inflammatory, and anti-apoptotic activities [20]. The ability of HDL to remove cholesterol and oxysterols from cells, better known as cholesterol efflux capacity (CEC), is a potent anti-atherogenic function of HDL [21]. By facilitating the removal of excess cholesterol from cardiac cells, HDL may help prevent lipid accumulation and cellular damage. By promoting the efflux of cholesterol and 7-oxysterols, HDL particles maintain endothelial function and preserve active eNOS dimer levels [22].

Despite previous research, the relationship between different HDL-related biomarkers and clinical outcomes remains incompletely understood. Furthermore, a direct comparison of their association with incident events is lacking. In this study, we investigated the relationship between various HDL parameters and the prognosis of patients who presented to the emergency department with severe signs and symptoms of AHF that required hospital treatment. We aimed to identify HDL markers indicative of quality and quantity associated with 3-month mortality following the index AHF hospitalization.

2. Methods

2.1. Study cohort

The AHF study is a prospective, observational, single-center study conducted over 36 months. It recruited consecutive adult patients who presented to the emergency department of the university hospital center with severe signs and symptoms of acute heart failure (AHF) requiring hospitalization. Patients with mild clinical signs and symptoms of heart failure who were appointed to ambulatory treatment, as well as hospitalized AHF patients with severe non-cardiovascular comorbidities and those who refused to participate were excluded (Supplemental Fig. 1).

At the time of presentation to the emergency department, a comprehensive patient history was documented for all participants. Physical and echocardiography examinations were conducted, and venous blood samples were collected for analysis before treatment. The follow-up period relevant to this substudy was three months post-index AHF hospitalization, with the primary endpoint being the participants' survival status at that time.

All patients who participated in the study provided written informed consent following the guidelines set forth by Good Clinical Practice. Furthermore, the study adhered to the principles outlined in the Declaration of Helsinki. Ethical approval was obtained from the local ethics committees of the Sisters of Charity University Hospital Centre in Zagreb, Croatia (EP 2258/18-10) and the Medical University of Graz in Austria (EC 33–258 ex 20/21).

2.2. ApoB-Depletion of serum

To analyze the composition and function of HDL, we utilized serum HDL (apoB-depleted serum) [23]. To prepare apoB-depleted serum, 40 μL polyethylene glycol (20 % in 200 mmol/L glycine buffer) (Sigma-Aldrich, Darmstadt, Germany) was gently mixed with 100 μL serum. The mixture was incubated at room temperature for 20 min, followed by centrifugation at 10,000×*g* for 30 min at 4 °C. The supernatant was then collected and samples were stored at −70 °C until required.

2.3. NMR spectroscopy measurements

Serum levels of HDL subclasses were measured on a Bruker 600 MHz Avance Neo NMR spectrometer using the Bruker IVDr lipoprotein subclass analysis protocol [24]. NMR spectra were recorded at a constant temperature of 310 K using various pulse sequences for proton spectra acquisition and water suppression. Data analysis for lipoprotein quantification was performed using the Bruker IVDr Lipoprotein Subclass Analysis (B.I.LISA™) method.

2.4. Cholesterol efflux capacity

The cholesterol efflux capacity was assessed as previously published [25,26] with a cell-based method. J774.2 cells (Sigma Aldrich, Darmstadt, Germany) were seeded at a density of 300,000 cells per well in 48-well plates and incubated for 24 h. The cells were labeled with 0.5 μCi/mL radiolabelled [3H]-cholesterol (Hartmann Analytic, Braunschweig, Germany) in DMEM media containing 2 % FBS, 1 % penicillin/streptomycin, and 8(4-chlorophenylthio)-cyclic adenosine monophosphate (0.3 mM) (Sigma-Aldrich, Darmstadt, Germany) overnight. After rinsing, cells were equilibrated for 2 h with serum-free DMEM containing 2 % bovine serum albumin (Sigma-Aldrich, Darmstadt, Germany), rinsed again, and incubated with 2.8 % apoB-depleted serum for 3 h. CEC was quantified by calculating the ratio of radioactivity in the media to the total radioactivity of the media and lysed cells.

2.5. LCAT activity

According to the manufacturer's protocol, serum LCAT activity was assessed using a commercial kit (MAK107, Merck, Darmstadt, Germany). Samples were incubated with the LCAT substrate for 4 h at 37 °C. The fluorescent substrate emits at 470 nm, and upon LCAT-mediated hydrolysis, a monomer with fluorescence at 390 nm is released. LCAT activity was measured by monitoring the change in the ratio of emission intensities at 470 nm and 390 nm over time.

2.6. Arylesterase activity of paraoxonase 1

The arylesterase activity of PON1 was evaluated utilizing a photometric assay with phenylacetate substrate, as outlined in the specified reference [27]. ApoB-depleted serum was added to a 200 μL buffer solution containing 100 mM Tris, 2 mM $CaCl_2$ (pH 8.0), and 1 mM phenylacetate. Phenylacetate hydrolysis was monitored at 270 nm. Enzymatic activity was determined using the Beer-Lambert law with a molar extinction coefficient of 1310 L mol^{-1} cm^{-1}.

2.7. CETP activity

CETP activity was determined using a commercial assay kit (MAK106-1 KT, Merck, Darmstadt, Germany) following the manufacturer's protocol. Diluted serum samples were incubated with donor and acceptor molecules in a buffer at 37 °C for 3 h. The donor molecule contains a self-quenched fluorescent lipid, which exhibits increased fluorescence upon CETP-mediated transfer to the acceptor molecule. Fluorescence intensity was measured at an excitation wavelength of 465 nm and an emission wavelength of 535 nm.

2.8. Statistical analysis

Statistical analyses were conducted using SPSS (Version 29.0.0.0) (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 8.0. A p-value of less than 0.05 was considered statistically significant. Participant characteristics are represented as the median and interquartile range (Q1, Q3) or count and proportion. Mann-Whitney *U* Test or Fischer Exact Test were used to examine differences in clinical and laboratory characteristics, HDL composition, metabolism, and function between AHF

Table 1
Baseline characteristics of the study cohort.

	Alive	Deceased	All	p-Value
	(n = 241)	(n = 74)	(n = 315)	
Demographics				
Age (years)	74.0 (66.0, 81.0)	79.0 (69.5, 85.8)	76.0 (67.0, 82.0)	**0.004**
Sex, Female	106 (44.0 %)	30 (40.5 %)	136 (43.2 %)	0.688
Comorbidities				
Hypertension	227 (94.2 %)	67 (90.5 %)	294 (93.3 %)	0.289
T1DM	2 (0.8 %)	2 (2.7 %)	4 (1.3 %)	0.236
T2DM	100 (41.5 %)	32 (43.2 %)	132 (41.9 %)	0.789
CAD	123 (51.0 %)	33 (44.6 %)	156 (49.5 %)	0.354
CMP	217 (90.0 %)	71 (95.9 %)	288 (91.4 %)	0.154
AF	122 (50.6 %)	48 (64.9 %)	170 (54.0 %)	**0.034**
CKD	94 (39.0 %)	49 (66.2 %)	143 (45.4 %)	**< 0.001**
COPD	57 (23.7 %)	27 (36.5 %)	84 (26.7 %)	**0.035**
MetS	164 (68.0 %)	53 (71.6 %)	217 (68.9 %)	0.667
Physical examination at the time of presentation to the emergency department				
BMI (kg/m2)	27.5 (24.8, 31.2)	29.4 (26.3, 33.0)	28.0 (25.0, 31.6)	**0.018**
MAP (mmHg)	103.3 (90.0, 120.0)	90.0 (82.1, 106.7)	100.0 (88.3, 118.3)	**< 0.001**
Heart rate (beats/min)	100.0 (80.0, 115.0)	95.5 (76.0, 118.8)	100.0 (80.0, 116.0)	0.185
Respiratory rate (breaths/min)	28.0 (24.0, 32.0)	28.0 (25.0, 34.0)	28.0 (24.0, 33.0)	0.336
Laboratory parameters at the time of presentation to the emergency department				
Total cholesterol (mmol/L)	3.7 (2.9, 4.7)	3.3 (2.8, 4.0)	3.5 (2.9, 4.5)	**0.008**
HDL-C (mmol/L)	1.1 (0.9, 1.4)	1.0 (0.8, 1.2)	1.1 (0.9, 1.3)	**0.027**
LDL-C (mmol/L)	1.9 (1.4, 2.8)	1.8 (1.4, 2.3)	1.9 (1.4, 2.7)	0.052
Albumin (g/L)	38.0 (35.2, 41.8)	36.6 (34.0, 39.2)	37.8 (34.8, 41.3)	**0.011**
Creatinine (µmol/L)	111.0 (87.0, 148.0)	133.5 (107.8, 168.0)	117.0 (90.5, 152.5)	**0.001**
eGFR (mL/min/1.73 m2)	51.0 (33.4, 68.8)	38.4 (29.3, 50.2)	46.6 (32.3, 65.0)	**< 0.001**
CRP (mg/L)	10.3 (4.7, 25.5)	31.2 (10.0, 55.4)	12.2 (5.5, 33.1)	**< 0.001**
IL-6 (pg/mL)	22.9 (11.6, 45.0)	58.3 (20.1, 104.8)	25.1 (12.9, 60.1)	**< 0.001**
NT-proBNP (pg/mL)	5796.0 (3315.0, 12323.0)	10568.0 (5855.0, 20537.8)	6692.0 (3531.0, 14395.5)	**< 0.001**
AHF type				0.154
New onset AHF	24 (10.0 %)	3 (4.1 %)	27 (8.6 %)	
AHF following CHF	217 (90.0 %)	71 (95.9 %)	288 (91.4 %)	
NYHA class at the time of presentation to the emergency department				0.378
3	15 (6.2 %)	2 (2.7 %)	17 (5.4 %)	
4	226 (93.8 %)	72 (97.3 %)	298 (94.6 %)	
Echocardiography				
LVEDd/BSA (mm/m2)	29.1 (18.0, 44.8)	27.4 (18.3–38.8)	28.5 (18.0, 44.8)	**0.043**
IVS (mm)	13.0 (2.0, 19.0)	13.0 (8.0, 22.0)	13.0 (2.0, 22.0)	0.189
PW (mm)	13.0 (8.0, 19.0)	13.0 (8.0, 16.0)	13.0 (8.0, 19.0)	0.313
LVEF (%)	40.0 (15.0, 66.0)	40.0 (15.0, 65.0)	40.0 (15.0, 66.0)	0.972
SPAP (mmHg)	50.0 (30.0, 90.0)	52.5 (30.0, 102.0)	50.0 (30.0–102.0)	**0.010**
AHF class				0.648
HFrEF, EF < 40 %	110 (46.6 %)	33 (49.3 %)	143 (47.2 %)	
HFmrEF, EF 41–49 %	66 (28.0 %)	15 (22.4 %)	81 (26.7 %)	
HFpEF, EF ≥ 50 %	60 (25.4 %)	19 (28.4 %)	79 (26.1 %)	

Participant characteristics are reported as median and interquartile range (Q1, Q3), as well as counts and frequencies. Differences between AHF patients who were alive and those who died within 3 months after index AHF hospitalization were tested using the Mann–Whitney *U* test or Fisher's exact test. P values < 0.05 are considered significant and are depicted in bold. AHF, acute heart failure; BMI, body mass index; CAD, coronary artery disease; CHF, chronic heart failure; CMP, cardiomyopathy; COPD, chronic obstructive lung disease; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; IL-6, interleukin-6; IVS, interventricular septum thickness; MAP, mean arterial pressure; HFrEF, heart failure with reduced ejection fraction; HFmrEF, heart failure with mildly reduced ejection fraction; HFpEF, heart failure with preserved ejection fraction; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; LVEDd, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; MetS, metabolic syndrome; NT-proBNP, N-terminal B-type natriuretic peptide; PW, left ventricular posterior wall thickness; SPAP, systolic pulmonary artery pressure; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus.

patients who survived and those who died within 3 months after index AHF hospitalization. The prognostic value of HDL parameters for 3-month mortality was examined using univariable and multivariable Cox regression analyses. Kaplan-Meier survival analyses were performed to compare the tertiles of the HDL parameters using the log-rank test. The Spearman correlation coefficient was used to assess correlations between the measured indicators of HDL function and metabolism and HDL subclasses.

3. Results

3.1. Clinical characteristics of the study cohort

A total of 315 patients with severe clinical signs and symptoms of AHF requiring hospitalization were enrolled in the study over 36 months. The median age of the cohort was 76 years and 43 % of patients were female. More than 90 % of the enrolled patients had a prior diagnosis of cardiomyopathy and the index presentation was an acute worsening of chronic heart failure. At the time of presentation to the emergency department, all enrolled patients were in New York Heart Association (NYHA) functional class III (5.4 %) or IV (94.6 %). The demographic characteristics, comorbidities, vital signs, other physical measurements, and laboratory test results obtained at the time of presentation to the emergency department, as well as the classification of participants into different AHF groups, are presented in detail in Table 1 and Supplementary Table 1. Chronic medications of the AHF cohort are listed in Supplementary Table 2. Within three months after the index AHF hospitalization, 74 patients (23.5 %) died. Table 1 and

Supplementary Table 1 also illustrate the differences between those who survived and those who succumbed within three months after the index AHF hospitalization. Patients who died within three months after the index AHF hospitalization were significantly older than those who survived the same period, and more likely to have concomitant chronic kidney disease and chronic pulmonary disease. The deceased patients had significantly higher body mass index (BMI), lower systemic blood pressure, higher pulmonary artery pressure, and greater left ventricular dilation. However, there was no significant difference in left-ventricular ejection fraction at the time of presentation to the emergency department due to AHF. In addition, patients who died within 3 months of the index hospitalization for AHF had significantly more elevated serum levels of NT-proBNP, more markedly impaired renal function, more reduced hepatic biosynthetic capacity (decreased cholesterol and albumin levels), and more elevated levels of inflammatory markers, including C-reactive protein and interleukin-6 (acute infectious disease was an exclusion criterion for inclusion). These measurements were obtained at the time of emergency department presentation.

3.2. Comparative analysis of baseline functionality, metabolism of HDL, and serum levels of HDL subclasses in AHF survivors versus non-survivors after 3-month follow-up

In a comparative analysis, we observed that patients who died within three months after index AHF hospitalization had significantly altered functional, structural, and metabolic characteristics of HDL compared to survivors. Specifically, markers such as CEC, PON1 activity, and LCAT and CETP enzymatic activities were significantly reduced (Fig. 1). In addition, we observed a marked reduction in levels of the small HDL subclasses (HDL3-apoA-I and HDL4-apoA-I) in non-surviving patients, whereas levels of larger HDL subclasses (HDL1-apoA-I and HDL2-apoA-I) were not altered.

3.3. Associations of HDL subclasses, function, and metabolism with 3-month mortality after index AHF hospitalization

Univariable Cox regression analyses showed that lower CEC, activities of PON1, LCAT, and CETP, as well as lower serum levels of small HDL subclasses were all significantly associated with an increased risk of 3-month mortality after an index AHF hospitalization (Fig. 2). These associations (with exception of HDL2-apoA-I and CETP activity)

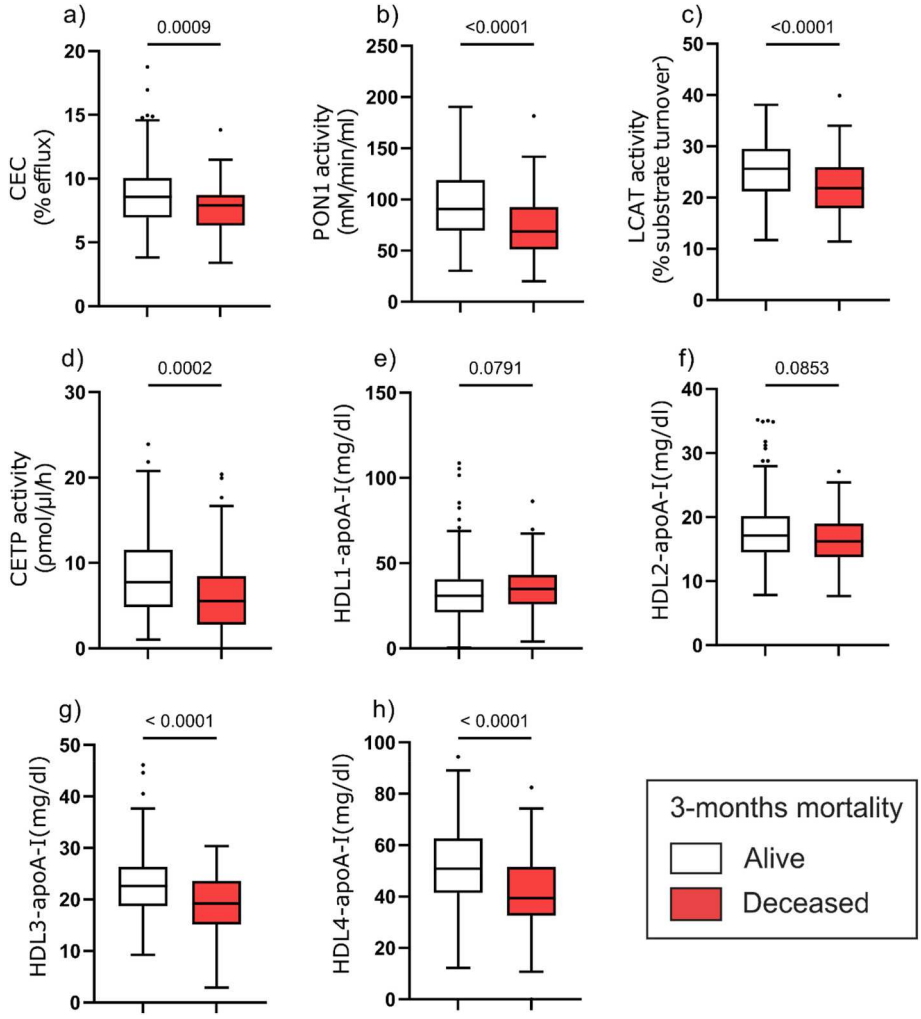

Fig. 1. Differences in indicators of HDL serum levels, function, and metabolism between AHF patients who died compared to those who were alive within 3 months after index AHF hospitalization. CEC of HDL (a), HDL-associated PON1-activity (b), serum LCAT-activity (c), serum CETP-activity (d), HDL1-apoA-I (e), HDL2-apoA-I (f), HDL3-apoA-I (g), and HDL4-apoA-I (h), were assessed. Data are presented as Tukey box plots with median and interquartile range, as well as minimum, maximum, and outliers. Differences between groups were analyzed using the Mann-Whitney *U* test. P values < 0.05 were considered significant. ApoA-I, apolipoprotein A-I; CEC, cholesterol efflux capacity; CETP, cholesteryl ester transfer protein; HDL, high-density lipoprotein; LCAT, lecithin-cholesterol acyltransferase; PON1, paraoxonase 1.

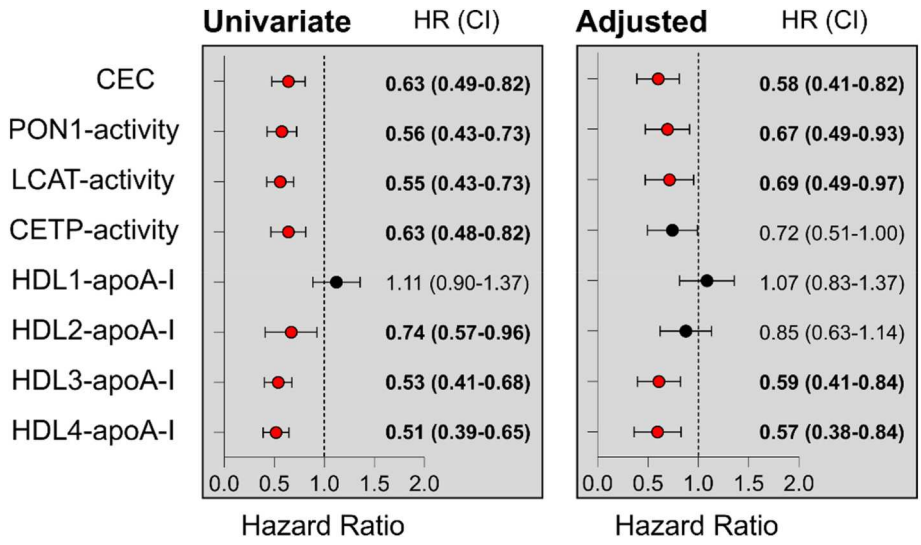

Fig. 2. Cox regression analysis was used to assess the association between standardized HDL-related parameters and HDL subclasses and the risk of 3-month mortality following index AHF hospitalization. Hazard ratios (HRs) per 1 standard deviation increase and 95 % confidence intervals (CIs) were calculated. Covariates included age, sex, body mass index (BMI), smoking status, diabetes mellitus, total cholesterol, creatinine, interleukin-6 (IL-6), albumin, systolic blood pressure, diastolic blood pressure, and N-terminal pro-B-type natriuretic peptide (NT-proBNP). Significant associations ($p < 0.05$) are highlighted in bold. Abbreviations: ApoA-I, apolipoprotein A-I; CEC, cholesterol efflux capacity; CETP, cholesteryl ester transfer protein; HDL, high-density lipoprotein; LCAT, lecithin-cholesterol acyltransferase; PON1, paraoxonase 1.

remained significant after adjustment for age, sex, BMI, smoking status, presence of diabetes, total cholesterol, creatinine level, interleukin-6 (IL-6), albumin, systolic and diastolic blood pressure, and NT-proBNP (Fig. 2).

For Kaplan-Meier survival analysis (Fig. 3), patients were categorized into tertiles based on HDL parameters. Patients with the highest levels of CEC, PON1, and LCAT activities, as well as small HDL subclasses (HDL3-apoA-I and HDL4-apoA-I), demonstrated significantly better 3-month survival following index AHF hospitalization compared to those in the lowest tertile.

We performed receiver operating characteristics curve analyses to assess the accuracy of CEC, PON1 activity, LCAT activity, HDL3-apoA-I, and HDL4-apoA-I to predict mortality within 3 months after index AHF hospitalization (Supplemental Fig. 2). The best predictive value with an area under the curve (AUC) of 0.69 was observed for HDL3-apoA-I, followed closely by LCAT activity (AUC of 0.68), HDL4-apoA-I (AUC of 0.68) and PON1 activity (AUC of 0.67) and CEC (AUC of 0.63). All HDL parameters showed a comparable predictive value to NT-proBNP (AUC of 0.66).

3.4. Correlation between HDL function/metabolism and composition of HDL subclasses

Correlations between serum levels of HDL particles/subclasses and the indicators of HDL function and metabolism were examined by Spearman correlation analyses (Fig. 4). We found that CEC was significantly positively correlated with all HDL subclasses, however, most profoundly with parameters of medium-size HDL subclasses 2 and 3. In contrast, PON1-activity was most profoundly positively correlated with parameters of HDL subclass 3 followed by subclasses 4 and 2, and only weakly with large-buoyant HDL subclass 1. Similarly, as found for PON1-activity, the LCAT-activity was most profoundly positively correlated with small HDL subclasses 3 and 4.

4. Discussion

Previous research suggested that the antioxidative and anti-inflammatory properties of HDL may play a crucial role in the pathogenesis of HF by protecting the structure and function of the heart and thus reducing the risk of death [28]. Previous findings demonstrated that smaller HDL subclasses [2,29] and impaired HDL cholesterol efflux capacity [30] are associated with increased mortality risk following index AHF hospitalization. However, no studies have directly compared these metrics. Our study is the first comprehensive assessment of multiple measures of HDL function, structure, and metabolism in a cohort of patients with severe clinical signs and symptoms of AHF who presented to the emergency department and required hospitalization. Multivariable Cox regression analysis demonstrated that CEC, LCAT, and PON1 activity, as well as small HDL subclasses, remained significantly inversely associated with 3-month mortality after adjusting for multiple clinical and laboratory risk factors.

Interestingly, using ROC analysis we identified three promising predictors of mortality after hospitalization due to severe clinical signs and symptoms of AHF: PON1 activity, LCAT activity, and levels of small-density HDL subclasses. These markers performed similarly to the current gold standard for HF diagnosis and prognosis, NT-proBNP. Notably, small HDL subclasses can be conveniently measured by NMR, making them potentially suitable for routine clinical use [31].

While total HDL cholesterol levels have traditionally been used to assess cardiovascular risk, this approach may not capture the relevant information. NMR technology offers a more nuanced approach by allowing the analysis of HDL subclasses. This provides a deeper understanding of a patient's HDL particle function, as we observed a strong correlation of CEC, PON-1, and LCAT activities with small HDL subclasses. Biomarkers related to HDL function could provide complementary information and enhance risk stratification for mortality of the patients hospitalized due to severe signs and symptoms of AHF [32].

Our study has limitations. Due to the observational design, this study cannot establish causality between the observed associations. Reproducing our results across disparate cohorts, ethnicities, and earlier disease stages would enhance the overall external validity of these findings.

The present study offers several key strengths. First, it provides comprehensive insights into HDL's functional, structural, and metabolic properties and their relationship to outcomes in AHF patients. Unlike prior studies focusing on HDL structure, we comprehensively assessed HDL function, structure, and metabolism in hospitalized AHF patients, offering novel insights into HDL's role in this population. Notably, we observed a strong positive correlation between the functionality of HDL

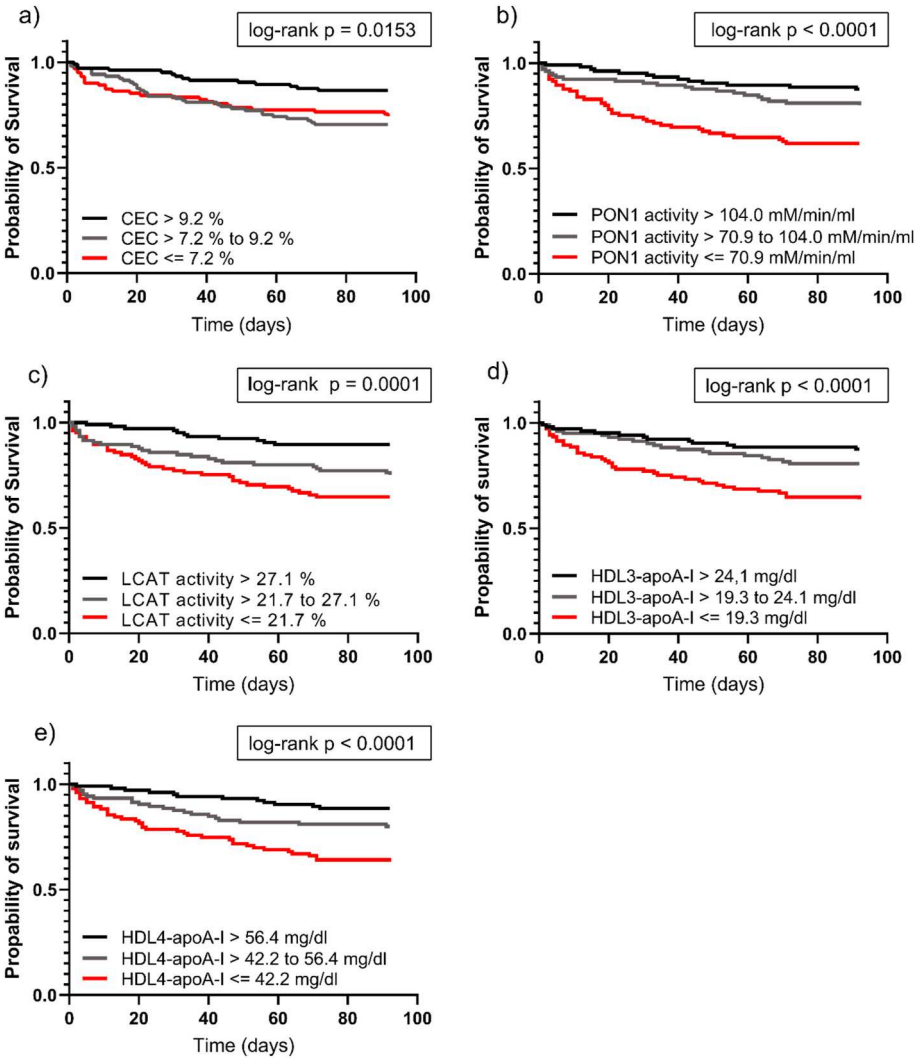

Fig. 3. Kaplan-Meier 3-month survival estimates with subsequent log-rank test of HDL-related parameters. For each variable assessed, AHF patients (n = 315) were categorized into tertiles for CEC, (a), PON1-activity (b), LCAT-activity (c), HDL3-apoA-I (d), HDL4-apoA-I (e). Patients were divided into tertiles having high, middle, or low activities of enzymes or serum concentrations of apolipoproteins. Number of patients per tertile is 105. ApoA-I, apolipoprotein A-I; CEC, cholesterol efflux capacity; HDL, high-density lipoprotein; LCAT, lecithin-cholesterol acyltransferase; PON1, paraoxonase 1.

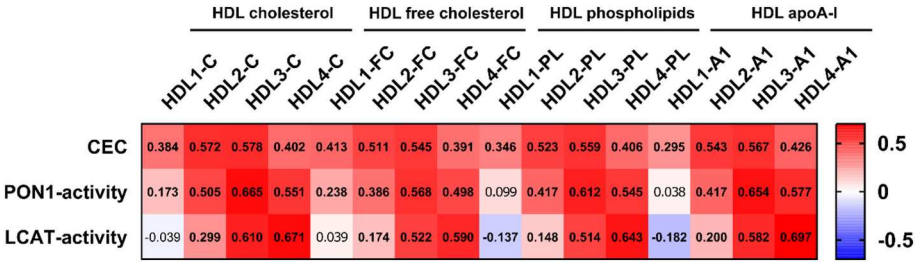

Fig. 4. Heatmap for correlation analyses between indicators of HDL function and metabolism and serum levels of HDL subclasses. Values are presented as Spearman correlation coefficient. P-values <0.05 are considered significant after a Bonferroni correction for multiple testing and significant correlations are depicted in bold. A1, apolipoprotein A-1; C, cholesterol; CEC, cholesterol efflux capacity; FC, free cholesterol; HDL, high-density lipoprotein; LCAT, lecithin cholesterol acyltransferase; PL, phospholipid; PON1, paraoxonase 1.

and its subclasses. Smaller HDL particles, particularly HDL3 and HDL4, exhibited a strong association with CEC, PON1 activity, and LCAT activity. These findings are consistent with previous research suggesting that the protective effect of higher HDL particle concentration on heart failure mortality is likely driven primarily by these smaller HDL subclasses [1,33]. By determining a patient's HDL subclasses, clinicians may be able to tailor treatment plans to target these specific HDL functions. This personalized approach could lead to more effective treatment of HF, especially in patients with cardiomyopathies in the early stages. HDL-mediated cholesterol efflux capacity could be a crucial protective mechanism in heart failure due to its potential to mitigate oxidative stress, inflammation, and apoptosis, all of which contribute to

cardiac dysfunction. Large population studies have consistently linked reduced HDL cholesterol efflux capacity to increased cardiovascular risk. This reflects the anti-inflammatory and anti-atherogenic properties of cholesterol efflux pathways, which inhibit hematopoietic stem cell proliferation, macrophage inflammation, and foam cell formation [34]. By facilitating the removal of excess cholesterol from cardiac cells, HDL may help prevent lipid accumulation and cellular damage. In addition, HDL maintains endothelial function by promoting efflux of cholesterol and 7-oxysterols and preserving active eNOS dimer levels [22]. Supporting this notion, prior research has shown that administering a single bolus of reconstituted HDL after ischemia significantly enhanced myocardial glucose uptake and improved cardiac structural remodeling, leading to better functional recovery in mice [7]. It is important to highlight that sepsis is a significant contributor to mortality in individuals with heart failure, accounting for approximately 25 % of deaths in this population [35]. HDL exerts pleiotropic effects in host defense against pathogens, capable of sequestering and neutralizing potentially harmful substances like bacterial lipopolysaccharides and preventing viruses from entering or fusing with host cells [36].

Improving our understanding of HDL function and developing strategies to enhance its beneficial effects could lead to significant advancements in HF and AHF treatment.

5. Conclusions

Our findings suggest that specific HDL parameters could serve as valuable biomarkers for predicting severe AHF outcomes. This insight paves the way for the development of targeted therapies aimed at improving patient prognosis in the future.

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CRediT authorship contribution statement

Anja Pammer: Data curation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Iva Klobučar:** Data curation, Validation, Writing – review & editing. **Julia T. Stadler:** Formal analysis, Validation, Writing – review & editing. **Sabine Meissl:** Methodology, Writing – review & editing. **Hansjörg Habisch:** Data curation, Methodology, Writing – review & editing. **Tobias Madl:** Data curation, Funding acquisition, Writing – review & editing. **Saša Frank:** Data curation, Investigation, Writing – review & editing. **Vesna Degoricija:** Formal analysis, Resources, Writing – review & editing. **Gunther Marsche:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2024.103341.

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Depletion of Small HDL Subclasses Predicts Poor Survival in Liver Cirrhosis

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Abstract: Liver cirrhosis is a complex condition characterized by oxidative stress, inflammation, and metabolic dysfunction, contributing to systemic complications and high mortality. High-density lipoprotein (HDL), particularly its small subclasses, is known for its antioxidant, anti-inflammatory, and cholesterol efflux capacities. This study examined changes in HDL subclass distribution and composition in cirrhosis and assessed their prognostic relevance for mortality. We analyzed HDL profiles using nuclear magnetic resonance spectroscopy in patients with compensated (n = 205) and decompensated (n = 158) cirrhosis, compared to healthy controls (n = 16). Across all HDL subclasses in cirrhotic patients, cholesterol content was decreased, and triglyceride levels were elevated. In particular, compensated cirrhosis was associated with a marked reduction in small and extra-small HDL particles, while large HDL levels remained unchanged. This reduction was even more pronounced in decompensated disease. Small HDL particle levels were inversely correlated with oxidative stress markers and liver dysfunction and independently predicted 12-month mortality in patients with compensated cirrhosis, even after adjusting for MELD score. In conclusion, our findings highlight a substantial depletion of small and extra-small HDL particles as a key feature of cirrhosis, linked to oxidative stress and mortality in the compensated stage.

Keywords: HDL subclasses; oxidative stress marker; NMR; liver failure; cirrhosis; mortality

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1. Introduction

Chronic liver disease constitutes a significant global health challenge, accounting for approximately two million deaths annually [1]. Emerging evidence highlights the pivotal role of dysregulated lipid metabolism in orchestrating inflammatory pathways and exacerbating disease severity [2–4]. In particular, decreased levels of high-density lipoprotein (HDL) cholesterol and its primary apolipoprotein, apolipoprotein A-I (ApoA-I),

have been consistently associated with heightened systemic inflammation, greater disease severity, and reduced survival in individuals with cirrhosis [5].

Multiple mechanistic pathways support the protective role of HDL in liver disease. First, HDL exhibits antioxidative properties that counteract oxidative stress, a central driver of hepatocellular injury and vascular remodeling [6,7]. Second, HDL participates in reverse cholesterol transport, thereby reducing lipid accumulation contributing to hepatic damage [8]. Third, in cirrhotic patients, impaired hepatic function and portal hypertension lead to increased intestinal permeability, facilitating the translocation of microbial products, such as lipopolysaccharides (LPS), into the systemic circulation. HDL may mitigate these effects by neutralizing circulating endotoxins and promoting tissue repair, thus potentially attenuating disease progression. These multifaceted functions position HDL as a promising therapeutic target for the management of cirrhosis-related complications [4,9,10]. However, the reduced concentrations and impaired functionality of HDL in cirrhosis are likely to compromise these protective mechanisms, thereby exacerbating systemic inflammation and underscoring the dual role of HDL as a biomarker and potential therapeutic target. The functional heterogeneity of HDL is reflected in its diverse subclasses, which differ in size, density, and compositional characteristics. Among these, small, dense HDL subpopulations—characterized by distinct protein and lipid profiles—have attracted particular clinical interest due to their pronounced atheroprotective and anti-inflammatory properties. These subclasses exhibit enhanced cholesterol efflux capacity, potent antioxidant and anti-inflammatory effects, and a superior ability to inhibit endothelial cell apoptosis [7,11]. Notably, recent evidence suggests that these small HDL subclasses offer improved prognostic value in assessing mortality risk in various pathological conditions, surpassing the predictive accuracy of conventional total HDL cholesterol measurements [12–15]. In the present study, we used proton nuclear magnetic resonance (NMR) spectroscopy to analyze the distribution and compositional changes in HDL subclasses in patients with cirrhosis. Our study investigates the link between small HDL subclasses and markers of oxidative stress and liver dysfunction and whether specific subclass shifts are associated with mortality.

2. Materials and Methods

2.1. Study Cohort

The plasma samples of the study cohort were obtained from a previously published study of 508 patients with cirrhosis that investigated HDL-related biomarkers in chronic liver failure [5]. Of these, 363 plasma samples were available for further analysis. The current study included two distinct groups: (i) 205 consecutive patients with stable cirrhosis recruited between 2011 and 2016 at the Medical University of Graz (from the Hepatology Outpatient Clinic or the Gastroenterology/Hepatology Ward, including stable patients undergoing evaluation for liver transplantation), and (ii) 158 patients with decompensated cirrhosis, with or without acute-on-chronic liver failure (ACLF), enrolled between February and September 2011 as part of the multicenter CANONIC study in 12 European countries (see Moreau et al., 2013 [16] for details). In both cohorts, cirrhosis was diagnosed based on liver histology or a combination of clinical, biochemical, and imaging criteria. Patients with a history of solid organ transplantation or hepatocellular carcinoma with advanced Barcelona Clinic Liver Cancer stages C and D were excluded. In addition, individuals with cholestatic liver disease were not included because of the effect of cholestasis on lipid profiles. Hospitalized patients with cirrhosis were evaluated for acute decompensation or ACLF, as defined by Moreau et al. in 2013 [16]. In addition to the collection of baseline data, including medical history, physical examination, and laboratory measurements,

information on liver transplantation and mortality at 90 days and 12 months after the start of the study was collected.

Additionally, 16 age- and sex-matched healthy controls, who did not meet the following exclusion criteria, were included: any history of cardiovascular disease, pregnancy, obesity, dyslipidemia, liver disease, renal disease, diabetes, or clinical signs of inflammation. The control participants were not taking any medication that lowers cholesterol or reduces inflammation.

Blood samples were obtained from patients and healthy controls at the outset of the study. The study was approved by the local Institutional Review Board (Medical University of Graz, 23-056 ex 10/11, 23-096 ex 10/11, 23-285 ex 10/11) in accordance with the Declaration of Helsinki. Each patient was required to provide written informed consent unless the requirement for this had been waived by the local Institutional Review Board.

2.2. NMR Spectroscopy Measurements

Serum levels of HDL-ApoA-I levels within each subclass were quantified using a Bruker 600 MHz Avance Neo NMR (Bruker, Rheinstetten, Germany) spectrometer and are reported in mg/dL, reflecting the mass concentration of apolipoprotein A-I in plasma. These values represent protein mass associated with each HDL subclass, not particle number. NMR spectra were recorded at a constant temperature of 310 K using various pulse sequences for proton spectra acquisition and water suppression. ApoA-I is the primary structural protein of HDL, and its subclass-specific distribution provides an indirect measure of HDL particle remodeling and potential functional capacity. The Bruker IVDr lipoprotein subclass analysis protocol (B.I.LISATM) was used for assessing subclass concentrations [17].

2.3. Statistical Analysis

Statistical analyses were conducted using SPSS (Version 29.0.0.0) (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 10.4.1. A p-value of less than 0.05 was considered statistically significant. Participant characteristics are represented as the median and interquartile range (Q1–Q3) or count and proportion. Mann–Whitney U Test or Fisher's Exact Test were used to examine differences in clinical and laboratory characteristics and HDL subclass distribution between individuals with compensated and decompensated cirrhosis. A post hoc power analysis was conducted to assess the adequacy of the control group size (n = 16) in detecting differences in HDL parameters. Using $\alpha = 0.05$ and assuming a two-sided t-test, comparisons with patient groups (n = 205 or n = 158) showed statistical power ranging from 0.76 to 0.87 to detect effect sizes of 0.7–0.8 (Cohen's d), which correspond to the magnitude of group differences observed in key HDL metrics. Statistical significance between healthy controls and individuals with cirrhosis was assessed using the Kruskal–Wallis test with Dunn's multiple comparisons post hoc analysis. The associations between HDL subclasses and inflammatory/oxidative markers were evaluated using Spearman's correlation. The prognostic value of HDL parameters for 90-day or 12-month mortality was examined using multivariable Cox regression analyses, as well as receiver operating characteristic (ROC) analysis.

3. Results

3.1. Baseline Characteristics

In this study, we analyzed 363 plasma samples from two distinct patient cohorts: 205 individuals with compensated cirrhosis from a single-center cohort in Austria and 158 individuals with decompensated cirrhosis from a European multicenter cohort, including 41 cases of ACLF. Additionally, 16 age- and sex-matched healthy controls were included

for comparison (Table 1). The mean age across both cirrhosis cohorts was 58 years. Among individuals with decompensated cirrhosis, 38.6% were female, compared to 25.4% in the compensated cirrhosis group. Compared to patients with compensated cirrhosis, patients with decompensated cirrhosis had a significantly different clinical profile, characterized by lower serum albumin ($p < 0.001$) levels and higher bilirubin ($p < 0.001$) and creatinine ($p = 0.004$) concentrations. Inflammatory markers, including white blood cell count ($p = 0.007$) and C-reactive protein ($p < 0.001$), were markedly elevated in the decompensated cirrhosis group, while lipid parameters, specifically total cholesterol and HDL cholesterol, were significantly lower ($p < 0.001$). The primary cause of cirrhosis differed between cohorts: alcohol-related cirrhosis was most common in the compensated group (55.4%), whereas the decompensated group showed a more even distribution between alcohol-related (39.3%) and viral (32.3%) etiologies. Mortality rates also varied substantially, with 90-day mortality at 3.9% for patients with compensated cirrhosis and 20.3% for those with decompensated cirrhosis, while 12-month mortality rates were 7.3% and 34.8%, respectively (Table 1).

Table 1. Baseline characteristics of the study cohort.

	Compensated Cirrhosis (n = 205)	**Decompensated Cirrhosis (n = 158)**	***p*-Value**
Age (years)	58 (52–63)	58 (51–66)	0.092
Gender (female)	52 (25.4)	61 (38.6)	0.007
MELD Score	11.60 (8.82–16.12)	18.00 (13.00–22.25)	<0.001
Albumin	3.90 (3.30–4.40)	3.00 (2.60–3.40)	<0.001
Bilirubin [mg/dL]	1.39 (0.80–2.87)	2.83 (1.40–6.79)	<0.001
Creatinine [mg/dL]	0.83 (0.72–1.02)	0.90 (0.73–1.55)	0.004
INR	1.29 (1.17–1.49)	1.46 (1.27–1.85)	<0.001
WBC	5.14 (3.90–6.63)	5.70 (4.20–8.25)	0.007
CRP	3.05 (1.20–8.03)	15.50 (4.6–38.10)	<0.001
Total Cholesterol	175.36 (43.45–336.91)	119.79 (92.32–151.17)	<0.001
HDL-Cholesterol	43.37 (0.06–85.13)	28.30 (20.04–40.44)	<0.001
Etiology			<0.001
Alcohol	123 (55.40)	62 (39.20)	
Virus	36 (16.20)	51 (32.30)	
Other	54 (24.3)	28 (17.70)	
90-day mortality	8 (3.90)	32 (20.30)	<0.001
12 months mortality	15 (7.30)	55 (34.80)	<0.001

Participant characteristics are reported as median and interquartile range (Q1–Q3), as well as counts and frequencies (%). Mann–Whitney U Test or Fisher's Exact Test were used to examine differences in clinical and laboratory characteristics. Abbreviations: CRP, c-reactive protein; INR, international normalized ratio; MELD, model for end-stage liver disease; WBC, white blood cell count.

3.2. HDL Subclass Distribution in Cirrhosis

Proton NMR spectroscopy was used to analyze HDL subclass composition, encompassing a range from large to extra-small particles. In patients with compensated cirrhosis, a significant reduction in small to extra-small HDL (S-HD to XS-HDL) particle concentrations was observed ($p < 0.001$), with no significant change in large HDL (L-HDL) ($p = 0.404$) and medium-sized HDL (M-HDL) ($p = 0.095$). Decompensated cirrhosis exhibited a more pronounced reduction in M-HDL, S-HDL and XS-HDL subclasses ($p < 0.001$) (Figure 1).

3.3. Composition of HDL Subclasses in Patients with Cirrhosis

The distribution of HDL subclasses in patients with compensated and decompensated cirrhosis differed markedly from that in healthy controls, prompting a detailed analysis of HDL particle composition. Lipid levels were normalized to ApoA-I subclass concentrations to evaluate the lipid content of L- to XS-HDL particles (Figure 2). This analysis revealed

significantly reduced HDL-cholesterol levels in M-HDL, S-HDL and XS-HDL subclass concentrations in all patients with cirrhosis ($p < 0.001$), (Figure 2A), alongside a notable decrease in free cholesterol content within L-HDL particles ($p < 0.001$) (Figure 2B). Phospholipid levels showed only subtle variations, with a modest increase observed in S-HDL particles ($p = 0.015$) among individuals with decompensated cirrhosis. In contrast, triglyceride concentrations were elevated across all HDL subclasses in patients with compensated and decompensated cirrhosis ($p < 0.001$) (Figure 2D).

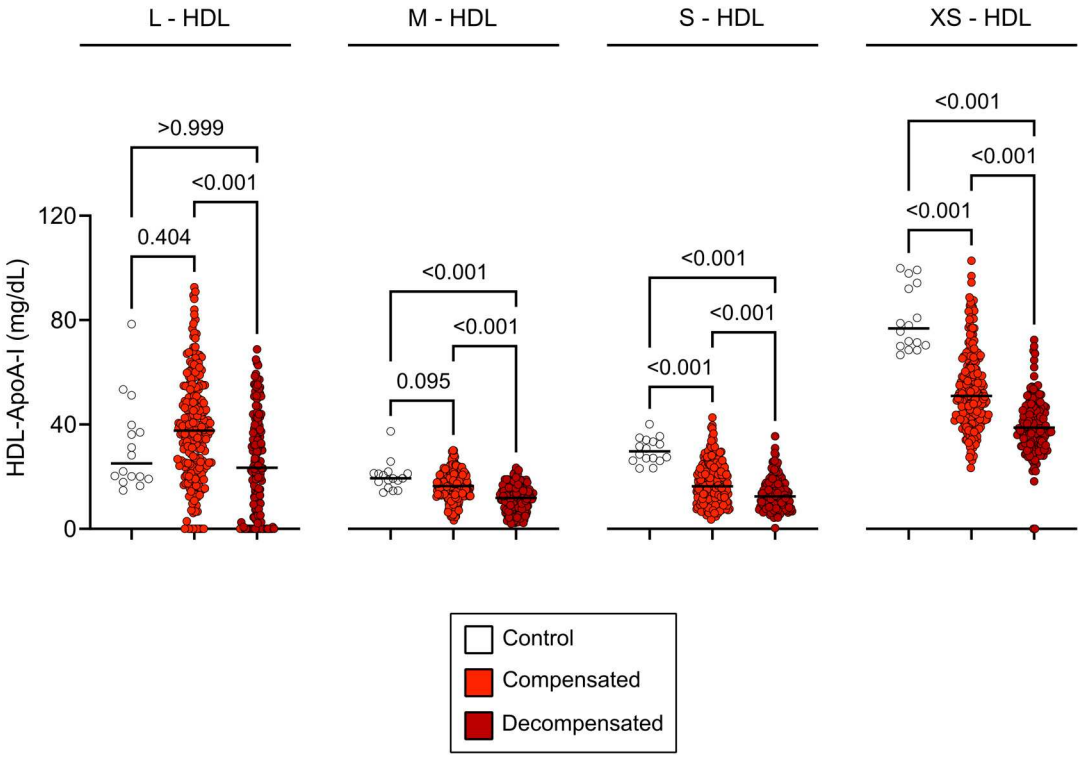

Figure 1. HDL subclass concentrations across study groups. Baseline concentrations of HDL-ApoA-I subclasses in healthy controls, compensated cirrhosis, and decompensated cirrhosis. Concentrations in compensated cirrhosis are represented in light red, decompensated cirrhosis in dark red, and healthy controls in white. The graphs show all data points, with the line representing the median of each data set. Statistical significance was assessed using the Kruskal–Wallis test with Dunn's multiple comparisons post hoc analysis. Abbreviations: ApoA-I, apolipoprotein A-I; HDL, high-density lipoprotein; L-, large; M-, medium; S-, small; XS-, extra-small.

3.4. Inflammation, Etiology of Liver Failure, and HDL Subclass Distribution

We examined the association between inflammation (C-reactive protein levels) and HDL subclass distribution in cirrhosis. In patients with compensated cirrhosis, elevated CRP $\geq$ 5 mg/L was strongly linked to a significant reduction in M-HDL ($p = 0.038$), S-HDL ($p < 0.001$), and XS-HDL ($p = 0.013$) subclass concentrations, while L-HDL ($p = 0.052$) concentrations showed only a non-significant trend. Conversely, in patients with decompensated cirrhosis, HDL subclass distribution exhibited no association with inflammation status (all $p > 0.999$) (Figure 3). Irrespective of underlying etiology, encompassing predominant causes such as alcohol-related liver disease and viral hepatitis, compensated liver cirrhosis was associated with a moderate reduction in M-HDL, S-HDL and XS-HDL subclasses (Figure S1A). This observation remained consistent across both alcohol-related and hepatitis-related cirrhosis when compared to other etiologies. In patients with decompensated cirrhosis, the distribution of HDL subclasses was not significantly influenced by disease etiology (Figure S1B).

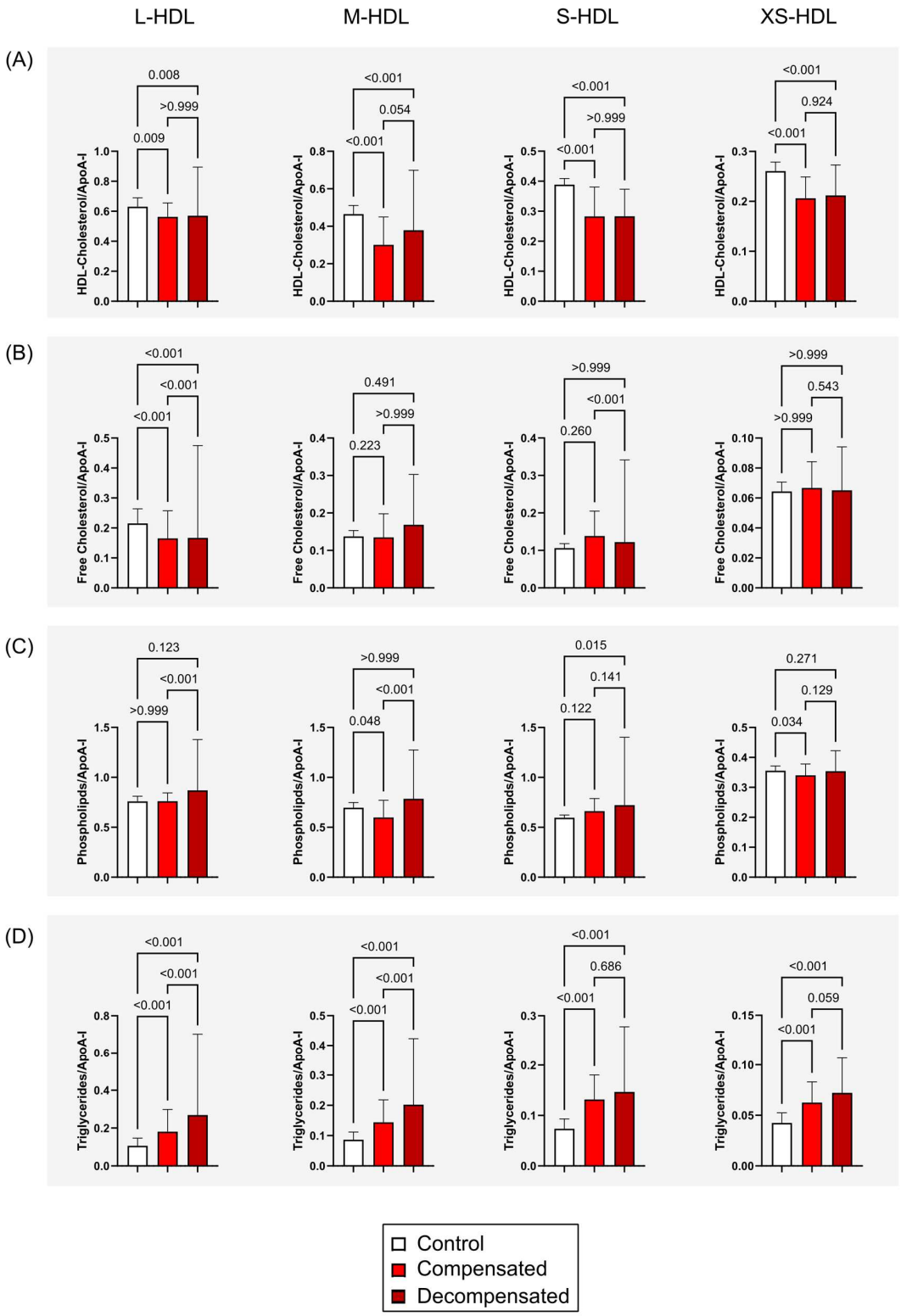

Figure 2. HDL Subclass composition across study groups. HDL lipid subclass measurements were normalized to corresponding ApoA-I subclass levels to assess compositional differences in lipid content across L- to XS-HDL particles. (**A**) HDL-total cholesterol (free and esterified) content in L-HDL to XS-HDL particles in healthy controls and patients with compensated or decompensated cirrhosis. (**B**) HDL-free cholesterol levels across HDL subclasses. (**C**) HDL-phospholipid content in HDL particles. (**D**) HDL-triglyceride levels in L-HDL to XS-HDL particles. Error bars indicate the standard deviation of the mean. Statistical significance was evaluated using the Kruskal–Wallis test followed by Dunn's multiple comparisons post hoc analysis. Abbreviations: ApoA-I, apolipoprotein A-I; HDL, high-density lipoprotein; L-, large; M-, medium; S-, small; XS-, extra-small.

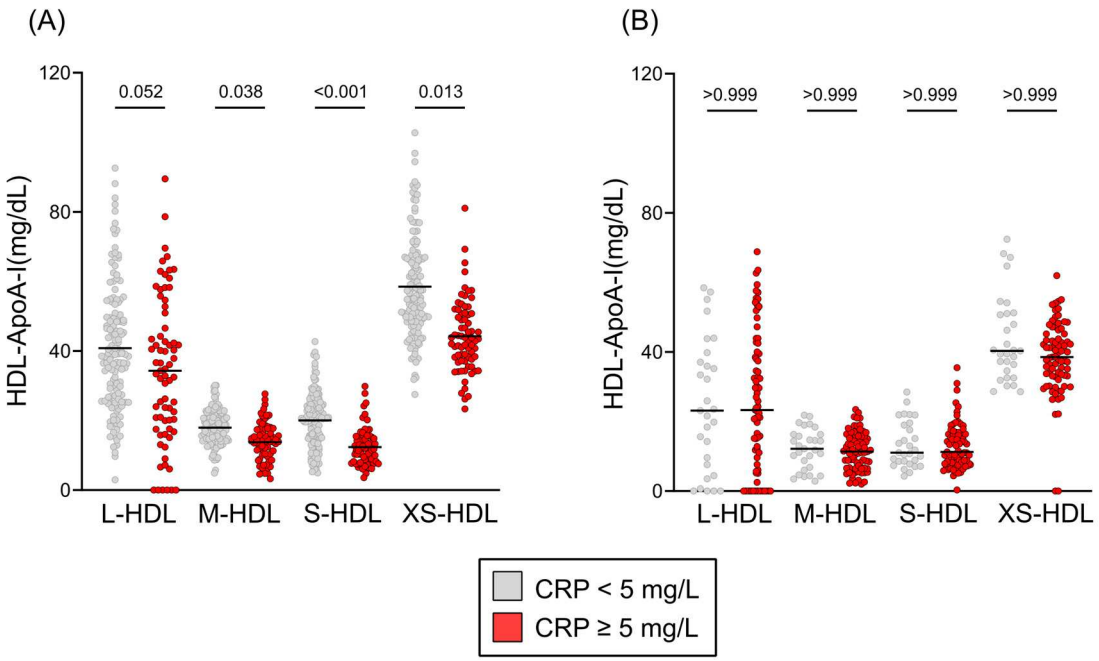

Figure 3. HDL subclass distribution by inflammation status. HDL subclass distributions are presented as Tukey's box plots, comparing patients with C-reactive protein (CRP) levels <5 mg/L (gray) and ≥5 mg/L (red). Distributions are shown for (**A**) compensated cirrhosis and (**B**) decompensated cirrhosis. Statistical differences were determined using the Kruskal–Wallis test with Dunn's multiple comparisons post hoc analysis. Abbreviations: ApoA-I, apolipoprotein A-I; HDL, high-density lipoprotein; L-, large; M-, medium; S-, small; XS-, extra-small.

3.5. Associations of HDL Subclasses with Markers of Oxidative Stress, Inflammation and Liver Dysfunction

A robust inverse correlation was observed between HDL subclasses and C-reactive protein (CRP) levels. The strongest association was noted for XS-HDL particles (see Figure 4). This suggests that XS-HDL particles may be highly responsive to systemic inflammation. Additionally, bilirubin exhibited a significant negative correlation with M- and S-HDL particles, indicating a potential link between HDL subclass distribution and liver dysfunction. Several amino acids exhibited inverse correlations with all HDL subclasses. Tyrosine and phenylalanine, which are aromatic amino acids that are often elevated during metabolic stress, showed particularly strong negative associations with smaller HDL particles. XS-HDL particles demonstrated the most significant negative correlations with glutamine and glycine, which are key glutathione synthesis precursors, as well as with pyruvic acid, a metabolite closely involved in redox homeostasis and antioxidant defense. Together, these results underscore the strong relationships between HDL subclasses and indicators of inflammation, liver dysfunction, and oxidative stress.

3.6. HDL Subclasses and Mortality in Compensated and Decompensated Cirrhosis

Analysis of HDL subclasses demonstrated a strong association with mortality. In patients with compensated cirrhosis, a significant reduction across all HDL subclasses was closely linked to an elevated 12-month mortality risk (all $p < 0.001$) (Figure 5A). Likewise, in patients with decompensated cirrhosis, lower HDL subclass concentrations were associated with increased 90-day mortality (L-HDL: $p = 0.012$, M-HDL: $p < 0.001$, S-HDL: $p = 0.134$, XS-HDL: $p = 0.002$) (Figure 5B). However, this association weakened at 12 months in the decompensated group (L-HDL: $p = 0.024$, M-HDL: $p = 0.011$, S-HDL: $p = 0.277$, XS-HDL: $p = 0.383$) (Figure S2).

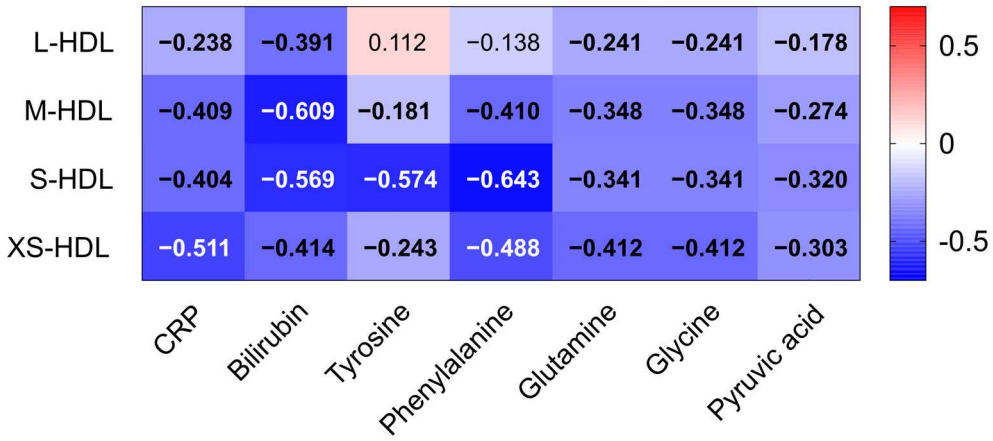

Figure 4. Correlations of HDL subclasses with inflammatory and oxidative markers. Clinical parameters such as CRP and bilirubin as well as NMR-measured amino acid levels of all patients with liver cirrhosis (n = 363) were correlated with HDL subclasses (L-HDL–XS-HDL). Each cell of the heatmap represents a pairwise Spearman's correlation between the two parameters indicated in the respective row and column. Significant values, after Bonferroni correction, are depicted in bold. Abbreviations: CRP, C-reactive protein; HDL, high-density lipoprotein; L-, large; M-, medium; S-, small; XS-, extra-small.

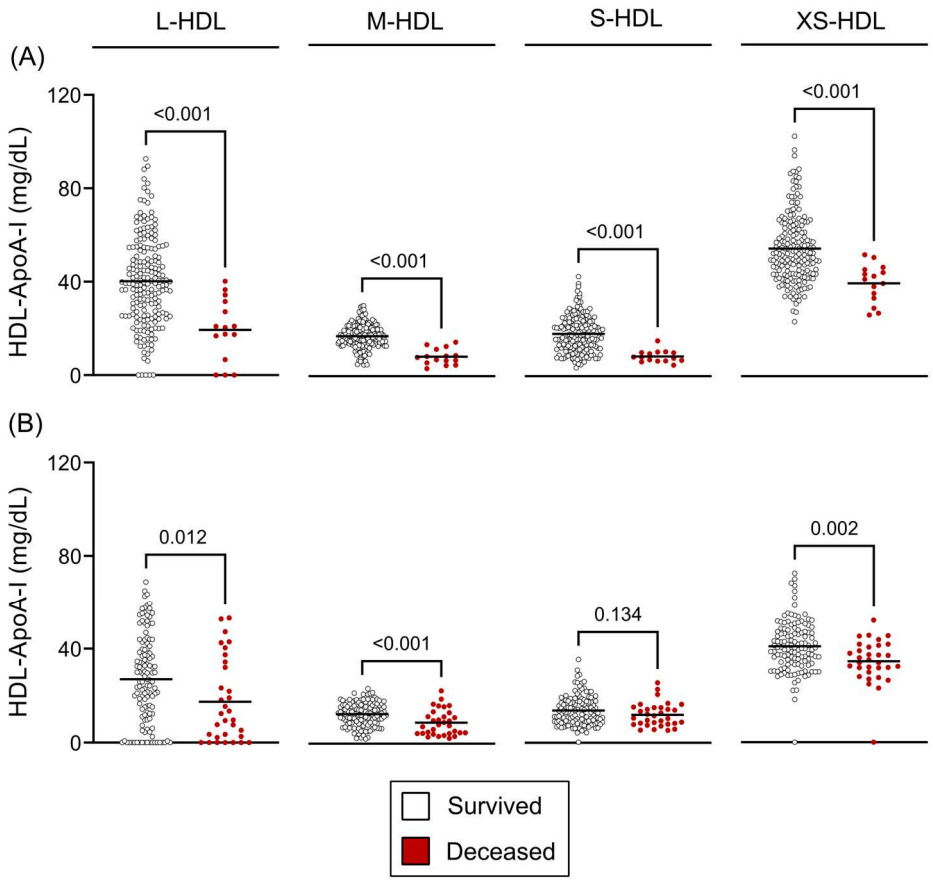

Figure 5. HDL subclass concentrations and survival outcomes. (**A**) Twelve-month survivors versus non-survivors in compensated cirrhosis and (**B**) ninety-day survivors versus non-survivors in decompensated cirrhosis. Statistical significance was assessed using unpaired *t*-tests or Mann–Whitney U tests, as appropriate based on data distribution. Abbreviations: ApoA-I, apolipoprotein A-I; HDL, high-density lipoprotein; L-, large; M-, medium; S-, small; XS-, extra-small.

3.7. HDL Subclasses as Predictors of Mortality in Liver Cirrhosis

To assess the independent prognostic value of HDL subclass parameters for mortality risk in liver cirrhosis, we performed multivariable Cox regression analyses (Table 2). In patients with compensated cirrhosis, after adjusting for age, sex, and Model for End-Stage Liver Disease (MELD) score, M-HDL ($p < 0.001$), S-HDL ($p < 0.001$) and XS-HDL ($p = 0.001$) showed a significant inverse association with 12-month mortality. In contrast, among patients with decompensated cirrhosis, only XS-HDL retained a significant inverse association with 90-day mortality ($p = 0.004$). Following additional adjustment for C-reactive protein (CRP) levels, the inverse association between HDL subclasses and three-month mortality was attenuated in compensated patients, with XS-HDL particles no longer reaching statistical significance ($p = 0.246$). Conversely, in cases of decompensated patients, the correlation with XS-HDL particles exhibited a strengthening *p*-value (Table S1, $p = 0.001$).

Table 2. Multivariable Cox-regression analyses of HDL-related parameters with risk of death in liver cirrhosis.

	Compensated		**Decompensated**	
Parameter	**HR (95% CI) Per 1 SD**	***p*-Value**	**HR (95% CI) Per 1 SD**	***p*-Value**
Total HDL-ApoA-I	0.43 (0.21–0.87)	**0.019**	0.75 (0.49–1.15)	0.189
L-HDL-ApoA-I	0.49 (0.23–1.04)	0.063	0.83 (0.54–1.28)	0.399
M-HDL-ApoA-I	0.09 (0.03–0.33)	**<0.001**	0.73 (0.46–1.16)	0.178
S-HDL-ApoA-I	0.10 (0.03–0.39)	**<0.001**	0.91 (0.54–1.55)	0.730
XS-HDL-ApoA-I	0.24 (0.10–0.58)	**0.001**	0.46 (0.28–0.78)	**0.004**

The independent association between standardized HDL subclasses and mortality was determined using multivariable Cox regression. Analyses were conducted for 12-month mortality in compensated patients and 90-day mortality in decompensated patients. Hazard ratios (HRs) with 95% confidence intervals (CIs) were computed per 1 standard deviation increase in HDL subclass levels, adjusting for age, sex, and MELD score. Significant findings ($p < 0.05$) are indicated in **bold**. Abbreviations: ApoA-I, apolipoprotein A-I; HDL, high-density lipoprotein; HR, hazard ratio, L-, large; M-, medium; S-, small; XS-, extra small.

3.8. Receiver Operating Characteristic Analyses of HDL Subclasses as Mortality Predictors

To assess clinical utility, receiver operating characteristic (ROC) analyses were performed on significant Cox regression predictors. In compensated cirrhosis, M-HDL had a numerically higher area under the curve (AUC) than MELD for predicting 12-month mortality (0.92 vs. 0.89; Figure 6A). The combination of M-HDL-ApoA-I and MELD further improved prognostic accuracy (AUC: 0.93). In decompensated cirrhosis, although XS-HDL-ApoA-I was a significant predictor in Cox regression, it did not significantly improve the 90-day mortality prediction of MELD (Figure 6B). Although the ROC curves of combined MELD and M- HDL or combined MELD and XS-HDL showed comparable or slightly higher AUC than the MELD score alone, statistical comparison using the DeLong test revealed no significant differences between the AUCs of the MELD score and those combined parameters (compensated: $p = 0.612$, decompensated: $p = 0.818$).

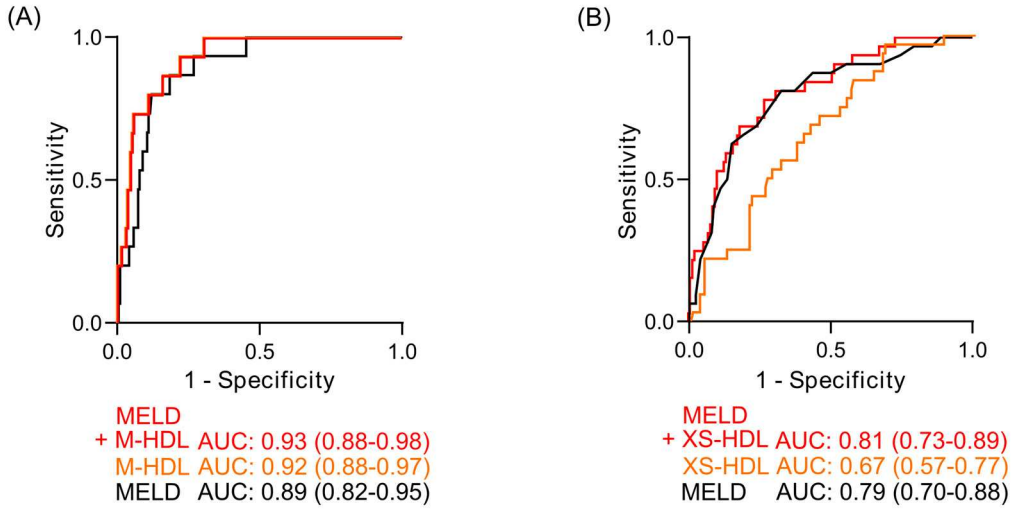

Figure 6. Receiver operating characteristic (ROC) curves for mortality prediction. (**A**) 12-month mortality prediction in compensated cirrhosis (DeLong Test: $p = 0.612$) and (**B**) 90-day mortality prediction in decompensated cirrhosis (DeLong Test: $p = 0.818$). Curves depict the MELD score (black), the HDL subclass significantly associated with mortality (orange), and the combined MELD-HDL subclass model (red). The area under the curve (AUC) is indicated below the graphs, including the 95% confidence interval, which is displayed in brackets. Abbreviations: ApoA-I, apolipoprotein A-I; HDL, high-density lipoprotein; MELD, Model for End-Stage Liver Disease.

4. Discussion

Cirrhosis markedly alters lipoprotein metabolism, leading to substantially reduced HDL and total cholesterol levels. This study offers the first comprehensive analysis of HDL subclass composition in cirrhosis and its clinical implications for mortality. We observed a significant decline in M-HDL to XS-HDL particle concentrations in patients with cirrhosis, particularly those with decompensated disease, whereas L-HDL levels remained relatively unchanged. This decrease was associated with lower cholesterol content within the M-to-XS-HDL, whereas triglyceride levels were increased in all subclasses. Notably, reduced M-to-XS-HDL concentrations were independently linked to higher mortality risk, even after adjusting for established predictors such as age, sex, and MELD score, underscoring their potential as a prognostic marker.

Further adjustment for CRP levels (an indicator of systemic inflammation), revealed nuanced insights. For compensated patients, the protective association between HDL subclasses and three-month mortality weakened, and the effect of XS-HDL particles lost statistical significance. Conversely, for decompensated patients, the association with XS-HDL particles persisted and strengthened. These findings suggest that, for compensated patients, the relationship between HDL subclasses and mortality may be partially influenced by inflammation. For decompensated patients, XS-HDL particles may serve as more robust and independent prognostic markers.

These findings are consistent with the recognized biological roles of HDL subclasses, wherein critical functions—such as cholesterol efflux capacity, anti-oxidative capacity, anti-inflammatory effects, and antiapoptotic activity—are predominantly mediated by small, dense, protein-rich HDL particles [11]. Specifically, these smaller HDL particles may confer significant protection against oxidative stress induced by free radicals [11].

Our study shows a significant inverse correlation between circulating levels of small HDL subclasses and key markers of oxidative stress and liver dysfunction, namely phenylalanine, tyrosine, bilirubin, and C-reactive protein. This observed negative association strongly suggests that the antioxidant and anti-inflammatory functions of small HDL subclasses may play a protective role in liver failure. The observed elevation of phenylalanine

and tyrosine in liver disease has been linked to oxidative stress-induced inhibition of phenylalanine hydroxylase [18]. The observed strong negative correlation suggests a possible direct interaction between the depletion of small HDL subclasses and the development of metabolic dysfunction in this patient population. Similarly, elevated bilirubin levels, indicative of impaired hepatic detoxification, and elevated CRP, a marker of increased systemic inflammation, both showed negative correlations with all HDL subclasses.

Our previous research showed a significant reduction in the activities of key enzymes involved in HDL maturation and metabolism in patients with cirrhosis, including phospholipid transfer protein, lecithin–cholesterol acyltransferase (LCAT), cholesteryl ester transfer protein (CETP), and lipoprotein lipase (LPL) [3]. LCAT facilitates HDL maturation by esterifying free cholesterol, which is then incorporated into the HDL core, while CETP regulates HDL composition by transferring cholesteryl esters between HDL and very-low-density lipoprotein. The marked reduction in the S-HDL and XS-HDL subclasses observed in cirrhosis is probably due to impaired hepatic ApoA-I synthesis combined with these enzymatic deficiencies. Specifically, reduced LCAT activity impairs the conversion of pre-β-1 HDL to α-migrating HDL, limiting cholesterol esterification and resulting in lower cholesterol content within these smaller HDL subclasses. At the same time, reduced CETP activity limits the transfer of cholesteryl esters from HDL, potentially preserving L-HDL levels. During LPL-mediated lipolysis of triglyceride-rich lipoproteins, surface remnants such as phospholipids and apolipoproteins are transferred to HDL [19], supporting HDL remodeling and maturation. However, reduced LDL activity in cirrhosis may interfere with this process and further increase triglyceride levels in HDL by inhibiting the degradation of triglycerides in HDL. These complex changes in HDL subclass distribution and composition warrant further investigation, given the different contributions of each subclass to lipid homeostasis and innate immunity.

The immunomodulatory role of HDL may be particularly important in chronic liver failure. Patients with cirrhosis are highly susceptible to Gram-negative bacterial infections, which cause excessive release of the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-alpha), exacerbating liver damage [20]. As a result, mortality from septic shock in cirrhotic patients reaches approximately 80% [21], far exceeding the 30% rate observed in individuals without cirrhosis [22]. The diminished quantity and impaired functionality of HDL may substantially contribute to the pathophysiology of systemic inflammation, a key driver in the progression to acute-on-chronic liver failure (ACLF) [23]. HDL exerts a critical protective role against sepsis by neutralizing deleterious bacterial cell wall components, such as lipopolysaccharide (LPS) [10]. Notably, restoring HDL levels and functions with reconstituted HDL significantly attenuated LPS-induced inflammatory pathways in an ex vivo study of patients with advanced chronic liver failure [24]. While our study did not directly assess the risk of decompensation, the observed associations support the hypothesis that HDL subclass profiling may offer early predictive insight into decompensating events and serve as a potential biomarker for risk stratification in chronic liver disease. Future prospective studies are warranted to explore these links.

Lipoprotein receptors play a dual role in viral infection. While they are crucial for lipid metabolism, viruses can exploit these receptors to enter host cells, evade immune responses, and disseminate throughout the body. Specifically, the HDL scavenger receptor B1 (SR-BI) has been shown to facilitate hepatitis B virus (HBV) entry into hepatocytes by interacting with the viral preS1 envelope protein [10]. Notably, HBV infection upregulates SR-BI expression in hepatocytes, potentially enhancing viral replication. However, HDL can competitively bind to SR-BI, blocking viral access and enabling the receptor's protective function in innate immunity [25]. Understanding the complex relationship between HDL and the immune system may reveal innovative targets for developing new treat-

ments to combat infectious diseases and improve patient outcomes. Implementing NMR spectroscopy in routine clinical laboratories can facilitate high-throughput, standardized measurement of lipoprotein subfractions [26], thereby offering enhanced risk stratification. Furthermore, NMR facilitates comprehensive profiling of HDL subclasses, which have demonstrated potential as biomarkers in conditions such as Alzheimer's disease [27], acute heart failure [13], and myocardial infarction [12]. Interpreting HDL-ApoA-I in mg/dL offers a protein-centric view of HDL composition rather than a direct count of particles. Reductions in ApoA-I mass within small HDL subclasses may indicate structural and functional impairment, such as reduced antioxidant and anti-inflammatory potential. These changes are consistent with dysfunctional HDL profiles observed in chronic disease states and may have prognostic significance. Although the Bruker IVDr NMR platform used in this study provides standardized and reproducible lipoprotein subclass data, its current application is largely limited to research environments. Broader clinical implementation may be constrained by the need for specialized equipment, technical expertise, and cost considerations. Nevertheless, as NMR technology becomes more automated and cost-effective, its integration into clinical laboratories may become increasingly feasible, particularly for high-throughput risk stratification or biomarker panels, potentially expanding their use from research to clinical diagnostics [28].

Certain limitations of the current study warrant consideration. This study's observational nature limits our ability to establish causality between HDL subclass changes and mortality. We acknowledge that relying solely on plasma measurements represents a limitation regarding spatial specificity. These systemic markers cannot definitively distinguish the precise contribution of hepatic oxidative stress from oxidative processes originating in other organs or from broader systemic inflammation. Ideally, future studies incorporating direct assessments of intrahepatic oxidative stress through liver tissue biopsies or hepatic-specific imaging/biomarkers would provide a more direct and spatially resolved measure.

The observed depletion of S-HDL and XS-HDL particles in cirrhosis, especially in the compensated stage, points to a potentially modifiable factor in disease progression. Since these HDL subclasses are known for their antioxidant, anti-inflammatory, and cholesterol efflux capabilities, their loss may fuel systemic inflammation and oxidative stress in cirrhotic patients.

Our findings suggest that HDL subclasses may offer superior clinical utility as biomarkers for disease monitoring and risk stratification compared to total HDL cholesterol. This highlights the importance of investigating interventions aimed at preserving or restoring small HDL particles, including ApoA-I mimetic peptides, lifestyle modifications, and novel pharmacological agents, for their potential to ameliorate cirrhosis-related complications. Furthermore, incorporating HDL subclass analysis into clinical practice could significantly improve prognostic assessment beyond established tools like the MELD score. While acknowledging the observational nature of this study precludes causal inference, these insights are pivotal for designing future trials on HDL-targeted therapies in liver disease.

5. Conclusions

Taken together, these findings suggest that reductions in specific HDL subclasses may amplify oxidative stress, potentially creating a detrimental feedback loop that exacerbates liver dysfunction and systemic complications in cirrhosis. The robust inverse association between small HDL and these markers of oxidative stress highlights therapeutic potential of strategies targeting restoration of small HDL subclasses. HDL subclass analysis provides robust mortality prediction in compensated cirrhosis, comparable to the MELD score. This indicates the potential for incorporating HDL subclass profiling into clinical practice to enable personalized therapeutic strategies. Such strategies could

enhance HDL-mediated functions, including cholesterol efflux, anti-inflammatory, and antioxidant activity, ultimately leading to improved patient outcomes and more targeted anti-inflammatory interventions.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/antiox14060664/s1, Figure S1: HDL Subclass Concentrations by Cirrhosis Etiology; Figure S2: HDL-ApoA-I Subclass Concentrations and 12-Month Survival; Table S1: To assess the impact of inflammation, we incorporated logarithmic C-reactive protein (CRP) levels into the multivariable Cox Regression Model.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Medical University of Graz (23-056 ex 10/11, 23-096 ex 10/11, 23-285 ex 10/11).

Informed Consent Statement: Informed written consent was obtained from all subjects involved in the study.

Data Availability Statement: Data are contained within the article and the Supplementary Materials.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ApoA-I	apolipoprotein-A-I
AUC	area under the curve
CETP	cholesteryl ester transfer protein
CRP	c-reactive protein
HR	hazard ratio
LCAT	lecithin-cholesterol acyltransferase
L-HDL	large HDL
LPL	lipoprotein lipase
MELD	model for end-stage liver disease
M-HDL	medium HDL
NMR	nuclear magnetic resonance
S-HDL	small HDL
SR-BI	scavenger receptor B1
XS-HDL	extra small HDL

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Cardiovascular Diabetology

RESEARCH

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Effects of dietary interventions and intermittent fasting on HDL function in obese individuals with T2DM: a randomized controlled trial

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Abstract

Background Cardiovascular disease represents a significant risk factor for mortality in individuals with type 2 diabetes mellitus (T2DM). High-density lipoprotein (HDL) is believed to play a crucial role in maintaining cardiovascular health through its multifaceted atheroprotective effects and its capacity to enhance glycemic control. The impact of dietary interventions and intermittent fasting (IF) on HDL functionality remains uncertain. The objective of this study was to assess the effects of dietary interventions and IF as a strategy to safely improve glycemic control and reduce body weight on functional parameters of HDL in individuals with T2DM.

Methods Before the 12-week intervention, all participants ($n=41$) of the INTERFAST-2 study were standardized to a uniform basal insulin regimen and randomized to an IF or non-IF group. Additionally, all participants were advised to adhere to dietary recommendations that promoted healthy eating patterns. The IF group ($n=19$) followed an alternate-day fasting routine, reducing their calorie intake by 75% on fasting days. The participants' glucose levels were continuously monitored. Other parameters were measured following the intervention: Lipoprotein composition and subclass distribution were measured by nuclear magnetic resonance spectroscopy. HDL cholesterol efflux capacity, paraoxonase 1 (PON1) activity, lecithin cholesterol acyltransferase (LCAT) activity, and cholesterol ester transfer protein (CETP) activity were assessed using cell-based assays and commercially available kits. Apolipoprotein M (apoM) levels were determined by ELISA.

Results Following the 12-week intervention, the IF regimen significantly elevated serum apoM levels ($p=0.0144$), whereas no increase was observed in the non-IF group ($p=0.9801$). ApoM levels correlated with weight loss and fasting glucose levels in the IF group. Both groups exhibited a robust enhancement in HDL cholesterol efflux capacity ($p<0.0001$, $p=0.0006$) after 12 weeks. Notably, only the non-IF group exhibited significantly elevated activity of PON1

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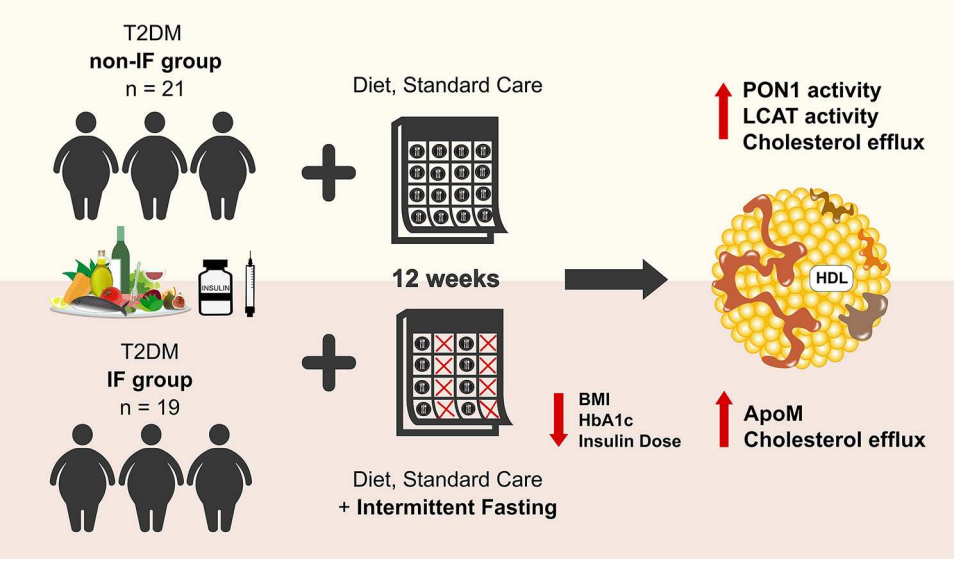
($p = 0.0455$) and LCAT ($p = 0.0117$) following the 12-week intervention. In contrast, the changes observed in the IF group did not reach statistical significance.

Conclusions A balanced diet combined with meticulous insulin management improves multiple metrics of HDL function. While additional IF increases apoM levels, it does not further enhance other aspects of HDL functionality.

Trial registration The study was registered at the German Clinical Trial Register (DRKS) on 3 September 2019 under the number DRKS00018070.

Keywords T2DM, Obesity, Intermittent fasting, Diet, HDL, Cardiovascular health, Cholesterol efflux, ApoM, PON-1, LCAT

Graphical Abstract



Background

Diet and fasting are the cornerstones of lifestyle modifications and essential factors in promoting cardiovascular health. The Mediterranean diet is associated with lower cardiovascular risk and diabetes incidence [1]. Various fasting strategies, from intermittent fasting to fasting-mimicking diets, may also offer advantages for preventing and treating chronic metabolic diseases like type 2 diabetes (T2DM) [2–4]. Significant weight loss from short-term calorie restriction can lower blood sugar and HbA1c levels, potentially leading to remission of T2DM [5]. Previous findings of the INTERFAST-2 study demonstrated that alternate day fasting (IF) over 12 weeks in insulin-treated people with T2DM is safe, and reduces HbA1c, body weight, and total daily insulin dose, while the resting metabolic rate and the physical activity levels remained unaltered [6].

T2DM and the cluster of pathologies including glucose intolerance/insulin resistance, obesity, and high plasma triglycerides that comprise the metabolic syndrome are associated with low and dysfunctional HDL. In addition to its established positive effects on cardiovascular health, HDL is emerging as a significant factor in enhancing glycemic control. HDL lowers plasma glucose by increasing plasma insulin and activating the adenosine monophosphate-activated protein kinase (AMPK) pathway in skeletal muscle [7]. HDL binds to cell surface receptors on skeletal muscle, including ATP-binding cassette transporter A1, leading to the mobilization of intracellular calcium ions and activation of calcium/calmodulin-dependent protein kinase kinase. This cascade promotes the phosphorylation and activation of AMPK, resulting in downstream effects such as glucose uptake. The most extensively studied and significant function of HDL is its cholesterol efflux capacity, which quantifies the capacity to remove cholesterol from cells. Recent large-scale clinical studies have demonstrated a correlation between in vitro HDL cholesterol efflux capacity and the prevalence and incidence of cardiovascular disease, which appears to be independent of HDL-C concentration [8]. Paraoxonase 1 (PON1), an HDL-associated enzyme, enhances insulin sensitivity by promoting GLUT4 translocation in myocytes [9]. ApoM is a lipocalin, primarily associated with HDL particles, that has a distinct hydrophobic binding pocket that allows it to bind functional lipids such as sphingosine-1-phosphate (S1P). This interaction plays a

critical role in preventing the degradation of S1P, enhancing the formation of atheroprotective preβ-HDL, and promoting insulin release [10, 11]. Lecithin–cholesterol acyltransferase (LCAT) as well as cholesterol ester transfer protein (CETP) play a crucial role in HDL maturation [12, 13]. These mechanisms suggest a potential link between low circulating HDL levels and metabolic dysfunction [7].

Understanding the factors influencing HDL function in T2DM could pave the way for novel biomarkers to track disease progression and develop personalized treatment plans. While specific dietary components have demonstrated improvements in HDL function [14], the influence of fasting on HDL structure, function, and metabolism remains unclear. In this study, we examined the effects of IF on various functional parameters of HDL in patients with T2DM, including (i) HDL subclass distribution, (ii) serum apoM levels, (iii) HDL cholesterol efflux capacity, (iv) PON1 activity and (v) serum LCAT and CETP activities.

Materials and methods

This is an analysis of the INTERFAST-2 study, a single-center, randomized, controlled trial, investigating the effect of intermittent fasting in people with T2DM already injecting insulin (INTERFAST-2). This study was conducted at the University Hospital Graz, Austria, and approved by the ethics committee of the Medical University of Graz, Austria (EK 30–350 ex 17/18). This research adhered to the tenets of the Declaration of Helsinki, and GCP-ICH guidelines, and complied with the protocol and requirements of the relevant regulatory authorities. The study population consisted of individuals with T2DM, aged 18 to 75 years, with glycated hemoglobin A1c ≥ 7.0% (≥ 53 mmol/mol). The primary inclusion criteria were as follows: a total daily insulin dose of ≥ 0.3 units per kilogram of body weight and stable body weight over the previous three months (weight change < ± 3 kg). Participants had to be willing to comply with the study procedures, attend the study site, participate in the required protocols, and adhere to the fasting protocols.

Major exclusion criteria included active known malignancy within the past year (excluding prostate, gastrointestinal, and basal cell carcinoma intraepithelial neoplasia), pregnancy or intent to become pregnant, lactation, and any chronic disease that might interfere with the interpretation of study results. In addition, participants were excluded if they had started a new hormonal supplement or changed their hormonal contraceptive in the previous two months. Participants with type 1 diabetes mellitus or other forms of diabetes mellitus, acute or chronic inflammatory diseases, or who consumed more than 15 standard alcoholic drinks per week were also excluded. In addition, individuals who worked night shifts or used illicit substances were not included.

Four weeks before the start of the dietary intervention, participants were switched to the same basal insulin regimen. A trained physician made dose adjustments for participants in both groups during the intervention period. Further information can be found in the published study protocol [15].

A registered dietitian provided an educational intervention focused on individualized dietary strategies to promote optimal health outcomes. The session emphasized on creating a balanced and varied plate with a rainbow of vegetables. Participants were encouraged to reduce their added sugars and salt intake while adding more whole grains and legumes to their meals. All patients had the same number of interactions with the dietitian during both on-site and telephone visits. Adherence to the diet was continuously monitored voluntarily.

Blood samples were collected from the participants at the outset of the study, which occurred after the insulin switch phase but before the commencement of the intervention. The second blood draw was conducted after 12 weeks of intervention. Blood samples were drawn after a minimum of 8 h of overnight fasting.

ApoB-Depletion of serum

Apolipoprotein B (apoB) was depleted from serum using a polyethylene glycol (PEG) precipitation method. A 20% (w/v) stock solution of PEG (P1458, Sigma-Aldrich, Darmstadt, Germany) was prepared by dissolving it in 200 mmol/L glycine buffer. Forty microliters (µL) of this PEG solution were then added to 100 µL of serum. The mixture was gently mixed and incubated at room temperature for 20 min. Following incubation, the samples were centrifuged at 10,100 × g for 30 min at 4 °C. The resulting HDL-containing apoB-depleted serum was collected and stored at −70 °C for further analysis.

Lecithin–cholesteryl acyltransferase (LCAT) activity

LCAT activity was assessed using a commercially available kit (MAK107, Merck, Darmstadt, Germany) following the manufacturer's guidelines. The serum samples were incubated with the LCAT substrate for four hours at 37 °C. The fluorescent substrate emits at 470 nm, and upon LCAT-mediated hydrolysis, a monomer with fluorescence at 390 nm is released. LCAT activity was quantified by monitoring the change in the ratio of emission intensities at λ = 470 nm and λ = 390 nm over time.

Arylesterase activity of Paraoxonase

The Ca2+-dependent arylesterase activity of PON1 was determined using a photometric assay involving phenylacetate substrate, following a previously described protocol [16]. Briefly, 1.5 µL of 1:10 phosphate-buffered saline

diluted apoB-depleted serum was added to a 200 µL buffer solution containing 100 mM Tris, 2 mM CaCl2 (pH 8.0), and 1 mM phenylacetate. The hydrolysis of phenylacetate was monitored at a wavelength of 270 nm. The enzymatic activity was determined using the Beer-Lambert law, with a molar extinction coefficient of 1,310 L mol-1 cm-1.

Cholesterol Ester Transfer Protein (CETP) activity

Serum CETP activity was determined using a commercially available kit (MAK106, Merck, Darmstadt, Germany) following the manufacturer's instructions. The CETP Activity Assay Kit uses a proprietary substrate to measure CETP-mediated neutral lipid transfer. 3 µl of serum samples diluted 1:10 in phosphate-buffered saline are incubated with the donor and acceptor molecules at 37 °C for three hours. The reaction produces a fluorescent signal (λEx=465 nm/λEm=535 nm) proportional to CETP activity.

Serum levels of apolipoprotein M

Serum levels of apoM were quantified using a sandwich enzyme-linked immunosorbent assay method described in a prior study [17]. For apoM measurement, capture antibody (clone 1G9) (Abnova, Taipei City, Taiwan) detection antibody (clone EPR2904) (Abcam, Cambridge, UK), and HRP-conjugated anti-rabbit IgG antibody (cat. No. PO448) (DAKO, Glostrup, Denmark) were used. Briefly, a high-binding ELISA plate (Corning, Arizona, US) was coated with a capture antibody overnight and blocked with 2% bovine serum albumin. Serum samples (10 µl) were treated with 1,4-dithiothreitol (Sigma-Aldrich) and iodoacetamide (Sigma-Aldrich) to cleave disulfide bonds in apoM. The 1:50 diluted samples (in tris-buffered saline+1% bovine serum albumin) were incubated overnight in the ELISA plate. After washing and the addition of detection and secondary antibodies, the absorbance of the colorimetric reaction was measured at 492 nm to determine the apoM concentration.

Cholesterol efflux capacity

The cholesterol efflux capacity of apoB-depleted serum was determined following established protocols [18, 19]. In brief, J774.2 cells (Sigma Aldrich, Darmstadt, Germany) were labeled with 0.5 µCi/mL radiolabeled [3 H]-cholesterol (Hartmann Analytic, Braunschweig, Germany) in DMEM media containing 2% FBS, 1% penicillin/streptomycin, and 8(4-chlorophenylthio)-cyclic adenosine monophosphate (0.3 mM) (Sigma-Aldrich, Darmstadt, Germany) overnight. After two washes, the cells were equilibrated for 2 h in serum-free DMEM supplemented with 2% bovine serum albumin from Sigma-Aldrich (Darmstadt, Germany). The cells were then rinsed and incubated with 2.8% apoB-depleted serum samples for 3 h. Cholesterol efflux capacity was expressed as the ratio of radioactivity in the media to the total radioactivity in the media and lysed cells.

NMR analysis

HDL subclasses and composition were assessed using a Bruker 600 MHz Avance Neo NMR spectrometer (Bruker, Rheinstetten, Germany) according to the Bruker IVDr Lipoprotein Subclass Analysis Protocol. Lipoprotein quantification was performed by analyzing the data using the Bruker IVDr Lipoprotein Subclass Analysis (B.I.LISATM) method as described previously [20]. The Bruker IVDr Lipoprotein Subclass Analysis identifies four HDL subclasses, labeled HDL-1 through HDL-4, based on increasing density and decreasing size. The defined density ranges for these subclasses are HDL-1 (1.063 to 1.100 kg/L), HDL-2 (1.100 to 1.112 kg/L), HDL-3 (1.112 to 1.125 kg/L), and HDL-4 (1.125 to 1.210 kg/L). For simplicity, these subclasses are designated as L-HDL (HDL-1), M-HDL (HDL-2), S-HDL (HDL-3), and XS-HDL (HDL-4).

Statistical analysis

All statistical analyses were performed with SPSS (version 29.0.0.0) (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 8.0. A p-value of <0.05 was used to determine statistical significance. Participant characteristics are reported as means±standard deviation, median with interquartile range or counts, and proportions. Statistical differences between the groups were calculated using paired t-test or Wilcoxon signed rank test, depending on the normality of the data. Fisher's exact test was used to identify differences in comorbidities and used medication. The Spearman correlation coefficient was used to assess correlations between HDL functions and clinical parameters. The results are presented as a scatterplot. Differences before and after intervention within each group were calculated using the paired t-test or Wilcoxon test, depending on the normality of the data.

Results

Baseline characteristics of the study cohort

Baseline characteristics of the study cohort are presented in Table 1. The INTERFAST-2 trial included 41 participants, with 19 (45%) assigned to the IF group and 22 (55%) to the non-IF group. The mean age of the study cohort was 63±7 years, and body mass index (BMI) was 34±5 kg/m². While lipid profiles were generally within normal limits, glucose metabolism was impaired, indicated by elevated fasting glucose (184±42), insulin (18.3±9.4), and glycated hemoglobin (HbA1c) levels (68±12 mmol/mol). Hypertension was common, with elevated systolic blood pressure (141±22 mmHg). Additionally, elevated levels of inflammatory markers, CRP

Table 1 Baseline characteristics of the study cohort

	Non-IF group	IF group	All	*p*-value
	(*n* = 22)	(*n* = 19)	(*n* = 41)	
Demographic parameters				
Age (years)	61 ± 7	66 ± 6	63 ± 7	0.025
Sex, Female	9 (40.9)	9 (47.4)	18 (43.9)	0.758
Physical examination and laboratory parameters				
BMI (kg/m^2)	34 ± 5	35 ± 4	34 ± 5	0.418
Systolic BP (mmHg)	145 ± 24	137 ± 20	141 ± 22	0.281
Diastolic BP (mmHg)	85 ± 11	79 ± 10	82 ± 11	0.093
Total Cholesterol (mg/dL)	169 (107, 271)	157 (114, 277)	166 (107, 277)	0.353
Fasting Insulin (mU/L)	18 ± 7	19 ± 11	18.3 ± 9.4	0.910
Fasting Glucose (mg/dL)	180 ± 45	187 ± 39	184 ± 42	0.638
HbA1c (mmol/mol)	69 ± 13	67 ± 11	68 ± 12	0.591
HDL-C (mg/dL)	53 ± 11	45 ± 10	49 ± 11	0.023
Triglycerides (mg/dL)	212 (93, 528)	161 (52, 321)	181 (52, 528)	0.009
Albumin (g/dL)	4.4 ± 0.2	4.3 ± 0.2	4.3 ± 0.2	0.266
AST (U/L)	25 (13, 9)	24 (14, 6)	24.5 (13, 9)	0.957
ALT (U/L)	23 (14, 6)	25 (18,0)	24 (15,0)	0.288
GGT (U/L)	24.5 (4.0, 101.0)	28.00 (14.0, 158.0)	25.0 (4.0, 158.0)	0.313
CRP (mg/L)	3.0 (0.6, 16.8)	2.1 (0.6, 18.8)	2.6 (0.6, 18.8)	0.704
IL-6 (pg/mL)	3.3 (1.6–15.1))	3.60 (1.5, 16.3)	3.4 (1.5, 16.3)	0.541
Total Protein (g/dL)	7.32 ± 0.41	7.12 ± 0.31	7.2 ± 0.37	0.093
Uric acid (mg/dL)	4.95 (2.90, 9.00)	5.40 (2.60, 9.10)	5.10 (2.60, 9.90)	0.374
Comorbidities				
Hypertension	18 (81.8)	18 (94.7)	36 (87.8)	0.350
Heart failure	1 (4.5)	4 (21.1)	5 (12.2)	0.164
Coronary heart disease	6 (27.3)	6 (31.6)	12 (29.3)	1.000
Myocardial infarction	3 (13.6)	7 (36.8)	10 (24.4)	0.144
Stroke	2 (9.1)	0 (0.0)	2 (4.9)	0.490
Retinopathy	5 (22.7)	5 (26.3)	10 (24.4)	1.000
Polyneuropathy	10 (45.5)	7 (36.8)	17 (41.5)	0.752
Amputation	1 (4.5)	1 (5.3)	2 (4.9)	1.000
Medication				
Metformin	17 (77.3)	14 (73.7)	31 (75.6)	1.000
Sulfonylurea	0 (0.00)	0(0.00)	0(0.00)	1.000
DPP4 inhibitors	5 (22.7)	8 (42.1)	13 (31.7)	0.313
SGLT2 inhibitors	9 (40.9)	9 (47.4)	18 (43.9)	0.758
GLP-1 receptor agonist	11 (50.0)	8 (42.1)	19 (46.3)	0.756
Pioglitazon	0 (0.0)	0(0.0)	0(0.0)	1.000
Premixed insulin	1 (4.5)	0 (0.0)	1 (2.4)	1.000
Basal insulin	20 (90.9)	19 (100.0)	39 (95.1)	0.490
Bolus insulin	18 (81.8)	18 (94.7)	36 (87.8)	0.350

Data are reported as means ± standard deviation, median with interquartile range or counts, and proportions. Differences between non-IF and IF individuals were tested with a t-test, Mann–Whitney U test, or Fisher's exact test. P values less than 0.05 were considered statistically significant. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; BP, blood pressure; CRP, C-reactive protein; DPP4, dipeptidyl peptidase 4; GGT, gamma-glutamyl transferase; GLP1, glucagon-like peptide-1; HbA1c, hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; IL-6, interleukin-6, SGLT2, sodium-glucose linked transporter type 2

and IL-6, suggested low-grade inflammation, highlighting the metabolic and inflammatory burden associated with the study population. A significant proportion of the study cohort also had other comorbidities such as heart failure, coronary artery disease, and myocardial infarction. Participants were taking a variety of medications for diabetes management, including metformin, DPP-4 inhibitors, SGLT2 inhibitors, GLP-1 receptor agonists, and basal insulin. While the groups were generally well-matched, some differences have to be noted in age (61 ± 7; 66 ± 6), HDL-C (53 ± 11; 45 ± 10), and triglyceride levels (212 [93, 528]; 161 [52, 321]). No significant differences between other baseline characteristics were observed.

Effects of IF on HDL composition

Lipoprotein profile analysis by NMR revealed changes in HDL composition after 12 weeks. When comparing the IF group to the non-IF group, we observed a non-significant upward trend in several components of large HDL (L-HDL) after the intervention in the IF group. This includes apoA-I, apoA-II, cholesterol, and phospholipids ($p = 0.083$, 0.126, 0.198, and 0.061, respectively) (Fig. 1A). Plasma apoM levels increased significantly after 12 weeks of IF (Fig. 1B). In agreement with our results, previous studies have shown an association between apoM and BMI [21, 22]. Consistent with this observation, our data showed a negative correlation between changes in BMI due to weight loss and changes in apoM levels (Fig. 1C). In addition, we observed a negative association between the change in apoM (delta apoM) and the change in fasting glucose (delta fasting glucose) (Fig. 1D).

Effects of IF on metrics of HDL function and metabolism

Next, we determined whether IF affects the functional metrics of HDL. Interestingly, the ability of HDL to remove cholesterol from macrophages was elevated in both the IF and non-IF groups after 12 weeks of intervention (Fig. 2A). To gain insight into the changes in serum antioxidant and anti-inflammatory activities upon fasting, we examined the activity of the HDL-associated enzyme PON1. After 12 weeks, there was a significant increase in PON1 activity among the non-IF group, while the increase in the IF group did not reach significance (Fig. 2B). LCAT is an enzyme that plays a crucial role in the maturation of HDL particles [23]. Interestingly, we observed a significant increase in LCAT activity in the non-IF group, whereas again the increase in the IF group did not reach significance (Fig. 2C). CETP facilitates the redistribution of cholesteryl esters, triglycerides, and phospholipids between plasma lipoproteins. Notably, no change in CETP activity was observed in either of the groups following the intervention period (Fig. 2D).

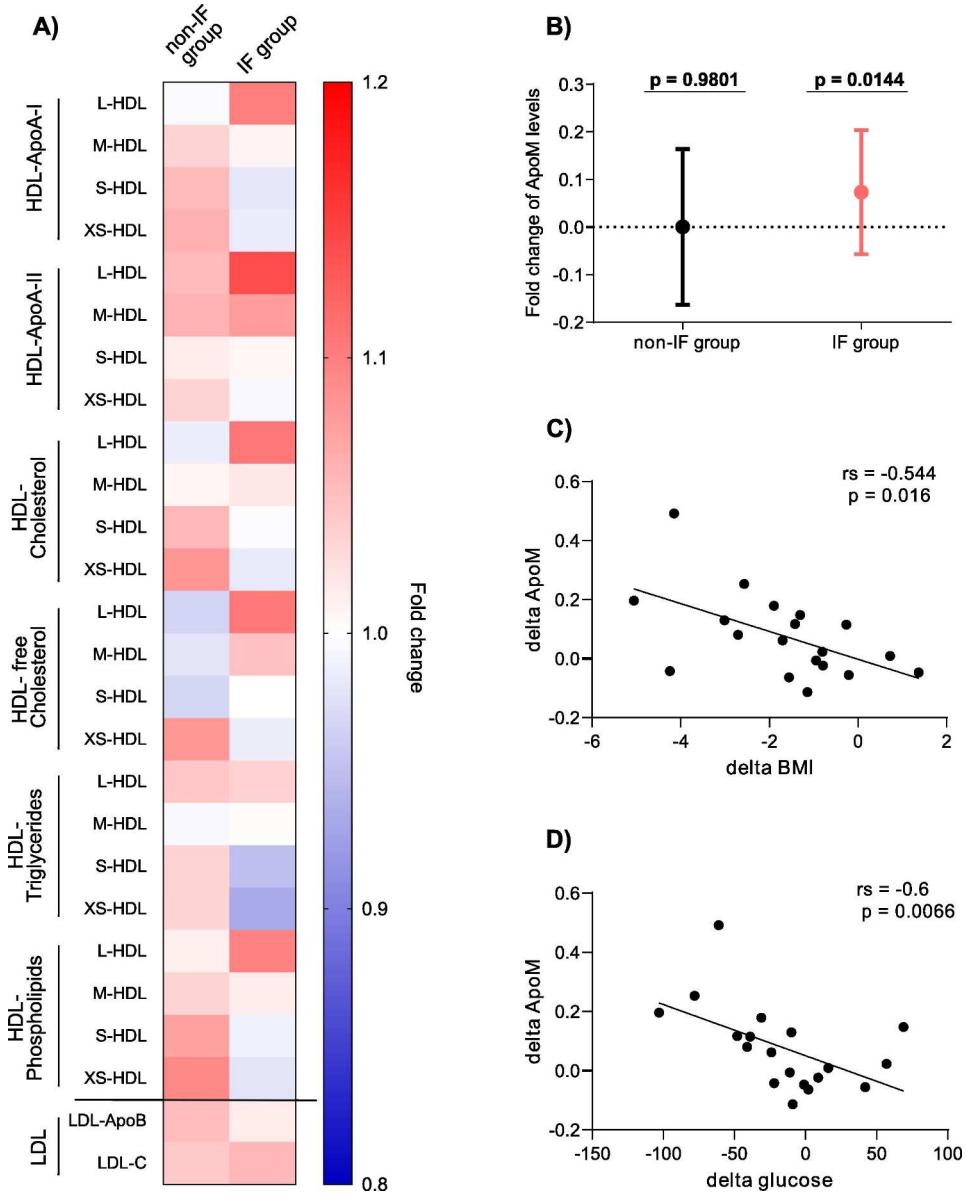


Fig. 1 Effects of IF on HDL composition. (**A**) HDL compositional parameters measured by NMR spectroscopy. (**B**) Fold change of apoM levels after 12 weeks of intervention. (**C**) Correlation analysis of delta apoM levels with delta BMI of the IF group; difference from before to after 12 weeks intervention measurements. (**D**) Correlation analysis of delta apoM with delta fasting glucose parameters of the IF group. HDL, high-density lipoprotein; HDL-A1, HDL-associated apolipoprotein A1; HDL-A2, HDL-associated apolipoprotein A2; HDL-C, HDL cholesterol; HDL-FC, HDL free cholesterol; HDL-TG, HDL triglycerides; HDL-PL, HDL phospholipids; LDL-ApoB, LDL associated apolipoprotein B; LDL-C, LDL cholesterol

Discussion

While fasting is practiced by billions worldwide for health or religious reasons, the larger picture of human adaptation to prolonged food deprivation remains elusive. Furthermore, the long-term health effects, both positive and negative, are areas of ongoing research. In patients with T2DM, previous studies suggested that IF may offer several benefits such as weight loss, which may lead to a lower daily insulin requirement [6, 24]. In addition, integrating a balanced diet and proper insulin management with IF may positively impact HDL metabolism and function. As previously shown, a 12-week IF regimen in insulin-treated people with T2DM is safe, and reduces HbA1c, body weight, and total daily insulin dose. IF may be a promising approach for some T2DM patients, potentially improving blood sugar control [6].

This study explored the effects of dietary intervention and fasting on HDL metabolism and function in individuals with type 2 diabetes. Our findings underscore the potential of nutritional modifications to enhance various HDL-related functional parameters after a 12-week intervention. These results are particularly noteworthy

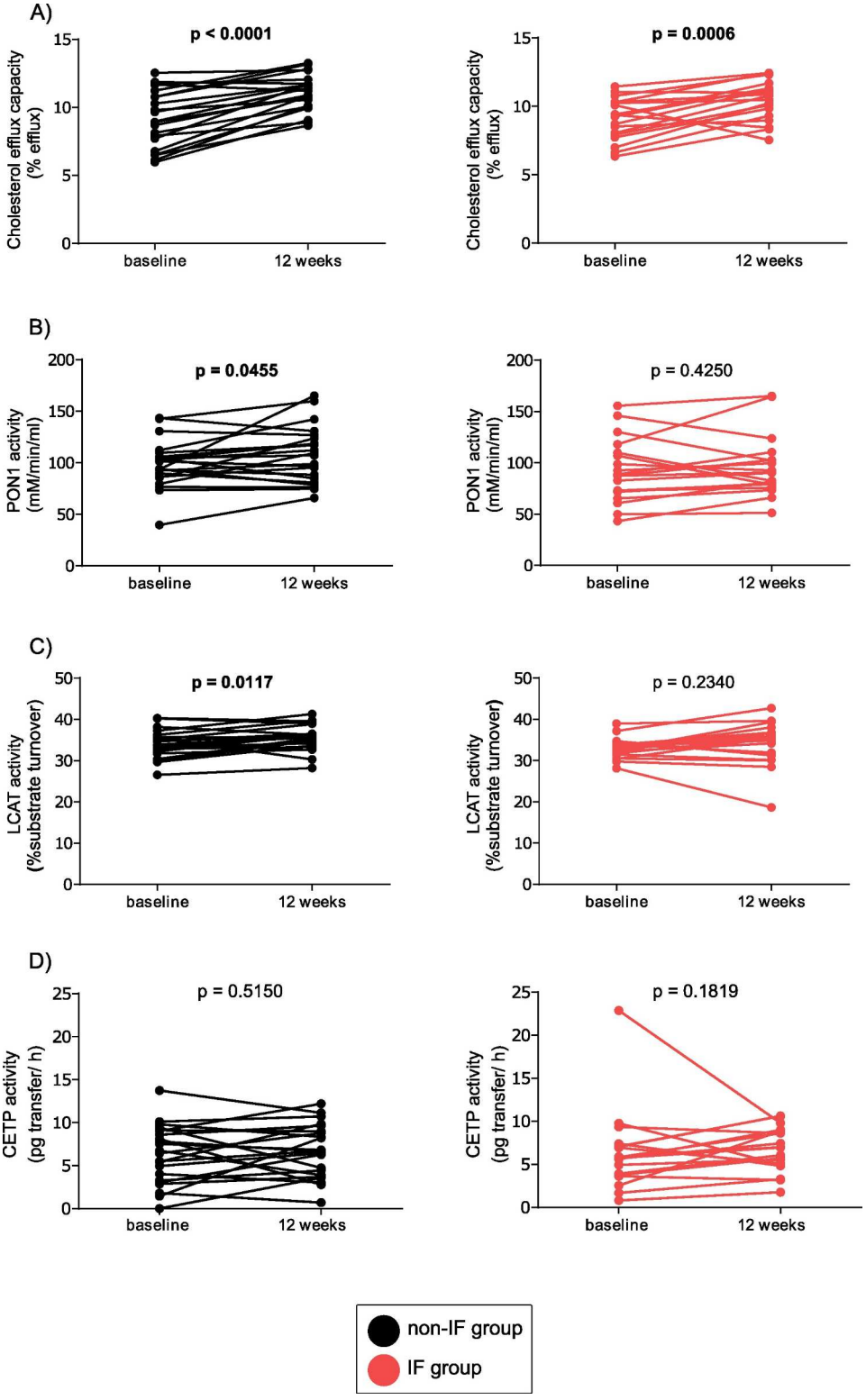


Fig. 2 Effects of IF on HDL functionality and metabolism. Line plots of cholesterol efflux capacity, enzymes involved in HDL functionality, and metabolism before and after the intervention period. (**A**) Cholesterol efflux capacity (% efflux); (**B**) PON1 activity (mM/min/ml); (**C**) LCAT activity (% substrate turnover); (**D**) CETP activity (pg transfer/h). Differences between baseline and after intervention (12 weeks) were analyzed by paired t-test or Wilcoxon test depending on the normality of the data. CETP, cholesterol ester transfer protein; LCAT, lecithin cholesterol acyltransferase; PON1, paraoxonase 1

because improved HDL functionality is closely linked to reduced cardiovascular risk and better glycemic control. Metrics of HDL function, such as cholesterol efflux capacity, have been demonstrated to strongly correlate with a decreased risk of cardiovascular disease [8]. It is interesting to note that HDL cholesterol efflux capacity is inversely related to T2DM in the EPIC Norfolk study [25]. HDL-associated PON1 plays a crucial role in preventing LDL oxidation and atherosclerosis by hydrolyzing lipid peroxides [26]. Oxidized LDL is associated with insulin resistance. The prevention of LDL oxidation by PON1 helps to maintain insulin sensitivity [9]. Moreover, PON1 reduces insulin resistance in mice fed a high-fat diet, and promotes GLUT4 overexpression in myocytes, via the IRS-1/Akt pathway [27]. Beyond its primary role in cholesterol esterification, LCAT exhibits additional antioxidant and anti-inflammatory properties. LCAT can neutralize the platelet-activating factor and oxidized phospholipids [12], and its activity has been independently associated with all-cause mortality in patients with chronic kidney disease [28]. Moreover, HDL-associated apoM inhibits the degradation of S1P, stimulates the formation of atheroprotective small preβ-HDL particles, and facilitates insulin release [10, 11]. These findings suggest a potential correlation between low or dysfunctional circulating HDL levels and metabolic dysfunction. Notably, the administration of reconstituted HDL to patients with T2DM has been shown to decrease plasma glucose by increasing plasma insulin and activating skeletal muscle AMP-activated protein kinase [29], which is in line with the aforementioned assumption. These results indicate that therapies aimed at raising functional HDL levels may have broader clinical applications beyond atherosclerosis in the management of T2DM.

Notably, whereas HDL cholesterol efflux capacity increased in both the IF and non-IF groups, the activities of PON1 and LCAT were significantly enhanced only in the non-IF group. IF increased apoM levels after 12 weeks. The activity of CETP, a protein that facilitates the redistribution of cholesterol esters and triglycerides between lipoproteins [30], was not altered in both groups.

All participants were given recommendations for a diet similar to the Mediterranean diet. This included eating at least three servings of vegetables and two servings of fruit per day, which was not possible for the IF group on fasting days. This could explain why some parameters such as PON1 and LCAT activity only increased significantly in the non-IF group. Additionally, participants were encouraged to reduce sugar and salt intake, choose whole grains and legumes over refined options, and limit red meat, dairy products, and saturated fats. Simmering was recommended as a healthier cooking method compared to frying [31]. Previous research demonstrated, that the Mediterranean diet markedly improves HDL functional parameters [14]. In the PREDIMED trial, the cholesterol efflux capacity of HDL was increased after 1 year of intervention compared to baseline levels [32]. The Mediterranean diet, especially when supplemented with extra virgin olive oil rich in phenolic compounds, has been shown to markedly improve metrics of HDL functionality. Particularly, the phenolic compounds of extra virgin olive oil seem to exert significant positive effects on HDL function [14]. Moreover, supplementation of anthocyanins as well as antioxidants such as lycopene or the omega-3 fatty acid eicosapentaenoic acid improve parameters of HDL function. Especially the consumption of nuts, legumes, and fish was previously reported to be associated with elevated PON1 antioxidant activity [33]. It is documented that the consumption of tomatoes is associated with an increase in LCAT activity, which is believed to be linked to the high lycopene content of tomatoes [34].

While the recommended Mediterranean-style diet rich in fruits, vegetables, and whole grains might be the primary driver of the improved cholesterol efflux observed in both groups, the lack of a significant PON1 and LCAT activity increase in the IF group remains unclear and further research is needed to draw firm conclusions.

It is important to note that alternate-day fasting appears to have the opposite effect in mice lacking LDL receptors. Two studies reported that alternate-day fasting unexpectedly increased the development of atherosclerosis in mice lacking LDL receptors [35, 36].

The results of our study suggest that a balanced Mediterranean-style diet enhances HDL function. However, the addition of fasting might negate some of these advantages, with the notable exception of increasing apoM levels. Our findings align with previous research showing that apoM levels are lower in individuals with obesity and metabolic syndrome, conditions often characterized by insulin resistance, and that caloric restriction increases the production of apoM [37]. ApoM expression is controlled by transcription factors associated with hepatic glucose and lipid metabolism [38]. ApoM, a carrier for S1P has been shown to enhance insulin secretion through S1P signaling, potentially impacting glycemic control [39]. The observed association between apoM levels and blood glucose in the IF group is consistent with this concept. Furthermore, the observed rise in apoM levels in the IF group could potentially lead to improved blood sugar regulation, given that apoM has been shown to enhance insulin secretion through S1P [10].

In addition to diet and fasting, regular aerobic exercise training is tightly linked with improved glycemic control and can significantly improve lipid profiles in healthy and obese individuals [40–43]. In individuals with T2DM, supervised and structured aerobic exercise training was

associated with increased HDL cholesterol levels and reductions in plasma triglycerides and LDL cholesterol [44]. Combining a Mediterranean diet, intermittent fasting, and regular physical activity could further improve the health of T2DM patients.

Some limitations of our study have to be noted. Primarily, the relatively small sample size limits the generalizability of our findings. Secondly, the open-label design may have introduced bias. Participant's awareness of their fasting status may have altered their behavior, impacting the results. Those fasting may have indulged in unhealthy food choices post-fasting, potentially negating some benefits. Conversely, participants in the non-IF group may have consciously adhered to a healthier diet. Moreover, the non-IF group had higher baseline HDL cholesterol and triglyceride levels, which might have influenced the results. If baseline HDL cholesterol levels are already high, there might be a limited capacity for further improvement through dietary and fasting interventions. However, both groups showed significant improvements in HDL cholesterol efflux capacity, suggesting that baseline differences likely did not substantially affect the results. The slight age difference of approximately five years between the groups is unlikely to have impacted the findings, as previous research suggests that cholesterol efflux capacity and LCAT activity are generally independent of age [45]. While PON1 activity declines in individuals over 65 in comparison to individuals of about 26 years of age [45], the relatively small age difference in our study is unlikely to have had a significant impact.

Conclusions

Our findings underscore the continued importance of a healthy lifestyle, particularly the Mediterranean diet, in addition to well-managed insulin therapy for individuals with T2DM. While we observed improvements in HDL function, as indicated by increased cholesterol efflux capacity, in both the non-IF and IF groups, the exclusive increase in apoM levels in the IF group suggests an additional mechanism that may contribute to the metabolic benefits. The established role of apoM in promoting insulin secretion is consistent with the observed improvements in glycemic control. While the recommended Mediterranean-style diet rich in fruits, vegetables, and whole grains might be the primary driver of the improved cholesterol efflux observed in both groups, the lack of a significant PON1 and LCAT activity increase in the IF group remains unclear and further research is needed to draw firm conclusions.

In conclusion, our study highlights the importance of a comprehensive approach to managing T2DM, which includes a healthy diet, well-regulated insulin therapy, and potentially intermittent fasting, as strategies to reduce cardiovascular risk and enhance overall metabolic health. Additionally, increasing functional HDL levels may offer broader clinical benefits in T2DM management, extending beyond just the prevention of atherosclerosis.

Abbreviations

Apo	Apolipoprotein
CETP	Cholesterol ester transfer protein
HDL	High-density lipoprotein
IF	Intermittent fasting
LCAT	Lecithin-cholesterol acyltransferase
PON1	Paraoxonase 1
S1P	Sphingosine-1-phosphate
T2DM	Type 2 diabetes mellitus

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Author contributions

Authors' contributions: Conceptualization, G.M., H.S.; methodology, A.P., H.H., T.M., formal analysis, A.P.; investigation, A.P., G.M.; writing—original draft preparation: A.P., G.M. writing—review and editing, A.P., G.M., J.T.S., A.O., P.P., N.J.T., H.H., T.M., H.S.; visualization A.P.; supervision G.M; project administration, G.M., A.O.,P.P., H.S.; funding acquisition, G.M., T.M., H.S. All authors have read and agreed to the published version of the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

The protocol was approved by the Ethics Committee of the Medical University of Graz (EK 30–350 ex 17/18).

Consent for publication

All participants gave written consent before any study-related procedure.

Competing interests

The authors declare no competing interests.

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