Dissertation

"Functionality of fetal high-density lipoprotein particles: Heterogeneity and relationship to Gestation Diabetes Mellitus"

submitted by

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DECLARATION

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

Graz, Ivana Sreckovic

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text:

Sreckovic I, Birner-Gruenberger R, Obrist B, Stojakovic T, Scharnagl H, Holzer M, Scholler M, Philipose S, Marsche G, Lang U, Desoye G, Wadsack C – "**Distinct composition of human fetal HDL attenuates its anti-oxidative capacity"** *Biochim Biophys Acta.* 2013; 1831(4):737-746

Sreckovic I, Birner-Gruenberger R, Miljkovic M, Stojakovic T, Scharnagl H, Marsche G, Lang U, Kotur-Stevuljevic J, Jelic-Ivanovic Z, Desoye G, Wadsack C **- "Maternal gestational diabetes mellitus remodels fetal high-density lipoprotein and impacts its functional diversity"** *J Clin Endocrinol Metab,* under review

Sreckovic I, Bernhart E, Sommer N, Sattler W, Marsche G, Lang U, Desoye G, Wadsack C **- "Gestational diabetes mellitus impairs fetal HDL-apoM-S1P vasoprotective action on the feto-placental endothelium"** in preparation

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ABBREVIATIONS

ABC	ATP-Binding Cassette Protein
ACAT	Acyl-coenzyme A:Cholesterol Acyltransferase
apo	apolipoproteins
BSA	Bovine Serum Albumin
CAD	Coronary Artery Disease
CE	Cholesterol Ester
CETP	Cholesterol Ester Transfer Protein
CRP	C-Reactive Protein
DOCK9	Dedicator Of Cytokinesis 9
DOHAD	Developmental Origins of Adult Health and Disease
ECIS	Electrical Cell-substrate Impedance Sensing
EL	Endothelial Lipase
eNOS	endothelial Nitric Oxide Synthase
FFA	Free Fatty Acid
GDM	Gestational Diabetes Mellitus
Gfi-1	Growth factor independent protein 1
GGE	Gradient Gel Electrophoresis
HbA1c	Hemoglobin A1c
HDL	High Density Lipoprotein
HL	Hepatic Lipase
HPAEC	Human Placental Arterial Endothelial Cells
HPEC	Human Placental Endothelial Cells
IGHG1	Ig Gamma-1 Chain C region
IGKC	Ig Kappa Chain C region
IGLC2	Ig Lambda Chain C regions
IDL	Intermediate Density Lipoprotein

Lp	Lipoprotein
LCAT	Lecithin Cholesterol Acyl Transferase
LC-MS/MS	Liquid Chromatography–electrospray ionization tandem Mass Spectrometry
LDL	Low Density Lipoprotein
LDLR	Low Density Lipoprotein Receptor
LPL	Lipoprotein Lipase
MORC3	Microrchidia3
MTP	Microsomal Triglyceride Transfer Protein
NO	Nitric Oxide
PAF-AH	Platelet Activating Factor-AcetylHydrolase
PGI2	Prostacyclin
PI3K	PhosphatidylInositol-3-Kinase
PLBP	PrePlatelet Basic protein
PLTP	Phospholipid Transfer Protein
PON1	Paraoxonase-1
RCT	Reverse Cholesterol Transport
RT	Room Temperature
S1P	Sphingosine-1-Phosphate
S1PR	S1P receptor
SAA	Serum Amyloid A
SPL	S1P Lyase
SR-BI	Scavenger Receptor class BI
TAC	Total Anti-oxidative Capacity
TG	Triglycerides
Tlag	Lag Time
Tmax	Maximum Time
TT	Trophoblasts
VDBP	Vitamin D Binding Protein
VLDL	Very Low Density Lipoprotein

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ZUSAMMENFASSUNG

HDL ist die dominierende Lipoproteinfraktion in humanem Nabelschnurblut, wohingegen im mütterlichen Serum LDL der Transporter für Cholesterin ist. Veränderungen im mütterlichen Lipid- und Cholesterinmetabolismus können Einfluss auf den Fetus und Konsequenzen im späteren Leben des Kindes haben. GDM, eine der häufigsten Komplikationen, steht in Zusammenhang mit postnataler fetaler Fettleibigkeit und einem erhöhten Risiko für vaskuläre Erkrankungen. In dieser Arbeit sollte die Hypothese, dass fetales HDL (fHDL) Proteine trägt, die sich qualitativ wie quantitativ von mütterlichem HDL (mHDL) unterscheiden und dass GDM das HDL-Proteom und auch dessen biologische Funktion verändert, bewiesen werden.

Proteomics und komplementäre Analysemethoden wurden verwendet, um Zusammensetzung und Funktion von fHDL und mHDL zu untersuchen. Isoliertes HDL dafür stammte aus unkomplizierten Schwangerschaften oder solchen, die von GDM betroffen waren. Analysierte Proteine, die in fHDL erhöht waren (apoE und Koagulationsproteine), sind charakteristisch für die HDL_2 Subfraktion. Im Gegensatz dazu sind jene Proteine, die in mHDL erhöht waren (apoL, apoF, PON1, apoD), in der dichteren HDL_3 Fraktion beschrieben, welche eine Rolle bei anti-oxidativen Prozessen und der Immunabwehr spielt. Beachtenswert ist, dass sowohl Menge als auch Aktivität von PON1 im Fetus 5-mal geringer waren *($p<0.01$)* als in der Mutter und gleichzeitig eine Verringerung des anti-oxidativen Potentials von fHDL beobachtet wurde. Obwohl CETP in annährend gleicher Menge in fetalem und mütterlichem Plasma vorliegt, ist seine enzymatische Aktivität in der fetalen Zirkulation um rund 55% geringer *($p<0.001$)*.

Beim Vergleich von HDL aus normalen und GDM Schwangerschaften wurden acht Proteine identifiziert, die in Lipidmetabolismus, Entzündungsprozessen und Immunabwehr eine Rolle spielen und differenziell exprimiert sind. Dazu zählen apoM, PON1, Prothrombin (verringert, $p<0.05$) und SAA (erhöht, $p<0.05$), die sowohl auf mütterlichem als auch fGDM-HDL dieselben Unterschiede zeigten. Die verringerte Expression von

PON1 ging mit geringerer enzymatischer Aktivität ($p<0.05$) und geringerer anti-oxidativer Kapazität der GDM-HDL Partikel einher. GDM hat eine Anreicherung der HDL Partikel mit Triglyzeriden zur Folge, die im Weiteren zu einem verringerten HDL-Cholesterin Level führt. Störungen, die im Lipidteil von GDM-HDL beobachtet wurden, könnten von Veränderungen in Menge und Aktivität von CETP herrühren. Die Kapazität von HDL freies Cholesterin aus TT sowie von HPEC aufzunehmen war in der GDM Gruppe verringert ($p<0.05$).

Aufgrund dass apoM ein wichtiger Träger von S1P ist, und durch die Tatsache, dass apoM Levels in fHDL und mHDL ähnlich sind, haben wir zeigen können, dass auch fHDL S1P trägt. Der Komplex fHDL-apoM-S1P induziert die Migration von HPAEC. Zudem interagiert S1P-HDLvorwiegend mit dem S1PR auf HPAEC und löst damit eine Neu-Organisation von Actinfasern und die Formierung eines korticalen Actomyosin-Ringes aus, was die Barrierefunktion des Endotheliums verstärkt. Blockiert man S1PR1 und S1PR3 mit selektiven Antagonisten, wird diese Wirkung des HDL-S1P aufgehoben ($p<0.001$). Bemerkenswerterweise ist in GDM-Schwangerschaften 46% weniger S1P mit fHDL assoziiert ($p<0.01$) was dazu führt, dass fGDM-HDL nur beschränkt in der Lage ist, die Migration von HPAEC anzuregen und zur Barrierefunktion beizutragen ($p<0.001$).

All diese Beobachtungen deuten darauf hin, dass mHDL sich in Hinsicht auf sein Proteom, Größe und Funktion von fHDL unterscheidet. Die verringerten anti-oxidativen Eigenschaften aufgrund des Fehlens von apoA-1, apoL und PON1 sowie eine mangelhafte angeborene Immunabwehr sind gemeinsam Unterscheidungsmerkmale für fHDL. Veränderungen in der Zusammensetzung und dem Metabolismus von HDL, die durch GDM bedingt sind, stehen in engem Zusammenhang mit der beeinträchtigten anti-oxidativen und vasoprotektiven Funktion des HDL-Partikels.

ABSTRACT

The human high-density lipoprotein (HDL) represents the major cholesterol carrying lipoprotein class in cord blood, while cholesterol is mainly carried by low-density lipoprotein in maternal serum. Changes in maternal lipid metabolism and cholesterol supply might also affect fetal outcome with consequences later in life. Gestational Diabetes Mellitus (GDM), most common complication of pregnancy, is related to postnatal fetal obesity and to higher risk for vascular events. We tested the hypothesis that fetal HDL (fHDL) carries proteins qualitatively and quantitatively different from maternal HDL (mHDL) and that GDM could cause alterations of fHDL proteome and probably its biological function.

Shotgun proteomics and biochemical analyses were used to assess composition/function of fHDL and mHDL isolated from uncomplicated and GDM human pregnancies at term of gestation. The pattern of analyzed proteins that were statistically elevated in fHDL (apoE, proteins involved in coagulation, transport processes) suggests a particle characteristic for the light HDL_2 sub-fraction. In contrast, proteins that were enriched in mHDL (apoL, apoF, PON1, apoD) have been described almost exclusively in the dense HDL_3 fraction and relevant to its anti-oxidative function and role in innate immunity. Strikingly, PON1 mass and activity were 5-fold lower ($p<0.01$) in the fetus, which was accompanied by attenuation of anti-oxidant capacity of fHDL. Despite almost equal quantity of CETP in maternal and fetal plasma, its enzymatic activity was 55% lower ($p<0.001$) in the fetal circulation, whereas LCAT activity was not altered.

When comparing control and GDM HDL eight proteins involved in lipid metabolism, inflammation and innate immunity were differentially expressed. Among them apoM, PON1, prothrombin (decreased, $p<0.05$) and SAA1 (increased, $p<0.05$) showed the same differences on both, mGDM HDL and fGDM HDL. The lower PON1 protein expression was corroborated by lower activity ($p<0.05$) which was accompanied by attenuation of anti-oxidant capacity of GDM HDL. GDM caused enrichment of HDL with triglycerides followed by decreased HDL-cholesterol levels ($p<0.05$). Disturbances observed in GDM HDL lipid moiety might be caused by CETP mass and activity

alterations. Rate of free cholesterol efflux from trophoblasts to maternal and from placental endothelial cells to fGDM HDL was diminished ($p<0.05$).

With the discovery of apoM as an important carrier of S1P in HDL particles and due to the similar apoM levels in mHDL and fHDL, it is comprehensible that fHDL is also the carrier of sphingosine-1-phosphate (S1P). FHDL-apoM-S1P complex is powerful chemoattractant and induced migration of human placental arterial endothelial cells (HPAEC). Secondly, S1P associated to fHDL interaction with S1P receptor (S1PR) on HPAEC and triggered rearrangement of actin fibers and formation of prominent cortical actomyosin ring which further leads to increment of barrier integrity. By blocking S1PR1 and S1PR3 with selective antagonist effect of HDL-S1P were abolished ($p<0.001$). Notably, in GDM 46% less S1P is associated to fHDL ($p<0.01$) which caused that fGDM HDL functions to induce barrier integrity and migration of HPAEC were diminished ($p<0.001$).

These findings indicate that maternally-derived HDL differs from fHDL with respect to its proteome, size and function. Absence of apoA-1, apoL and PON1 on fHDL is associated with decreased anti-oxidative properties together with deficiency in innate immunity collectively indicating distinguish HDLs in fetuses. Alterations occurring in HDL composition and metabolism to GDM are intimately associated with impaired anti-oxidative and vasoprotective properties of this particle.

1. INTRODUCTION

1.1. Cholesterol metabolism

1.1.1. Lipoproteins

Lipoproteins are macromolecular vehicles for transport of hydrophobic lipids throughout the aqueous environment of the circulatory system (Figure 1) (1). They are composed of various lipid species aggregated with specific proteins (apolipoproteins), which act as receptor ligands, stabilize the emulsion and confer structural properties to the lipoprotein particle.

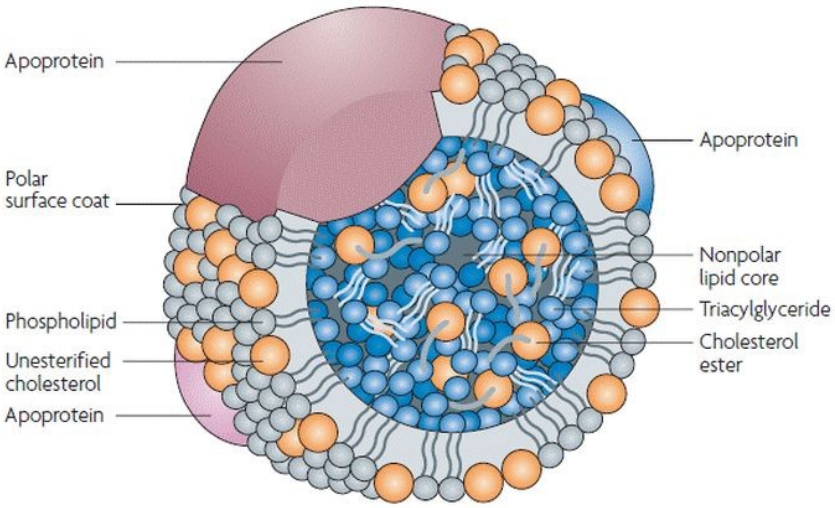


Figure 1: General structure of a lipoprotein.
The core is primarily composed of TG and CE. They are encased by a phospholipid monolayer. Apolipoproteins embedded in the phospholipid layer confer structural and functional properties to the particle. Figure modified from (2)

Lipoproteins are assigned into four main classes according to their particle density (Table 1), with the largest diameter and lowest density particles referred to as chylomicrons (1). Chylomicrons are formed by packing of nascent triglycerides (TG)

together with other lipids and apolipoprotein B-48 (apoB-48). Chylomicrons carry dietary TG to the peripheral tissue, where it serves as an energy source, or is stored in adipose tissue. Another class of lipoprotein particles, termed very low density lipoproteins (VLDLs) is similar to chylomicrons, hence primarily composed of TG, but also contain free cholesterol and cholesterol esters (CE). The removal of TG by lipoprotein lipase (LPL) converts VLDL particles into intermediate density lipoproteins (IDLs) and further to smaller, more stable, low density lipoproteins (LDLs). LDL particles account for 70–80% of the circulating cholesterol in human plasma. In contrast to chylomicrons and VLDL particles, which are loaded with substantial TG, LDL particles are composed primarily of free cholesterol and CE. Whereas VLDL carries TG synthesized in the liver also to the periphery, LDL are delivering cholesterol to peripheral tissues, or back to the liver, through LDL receptors. IDL normally undergo rapid conversion to LDL or are removed by the liver. High density lipoproteins (HDLs) are the smallest of the lipoprotein particles and have the highest protein content (1). HDL transports cholesterol from peripheral tissues mostly to the liver or steroidogenic organs such as adrenals, ovary, and testes.

Characteristics	Chylomicrons	Very low-density lipoproteins	Low density lipoproteins	High density lipoproteins
Density (g/mL)	< 0.95	0.95–1.006	1.019–1.063	1.063–1.210
Particle diameter (nm)	> 75	30–80	18–25	5–12
Protein composition (% dry weight)	1–2	8–10	20–25	52–60
Triacylglycerol composition (% dry weight)	80–88	45–53	5–9	2–3
Cholesterol composition (% dry weight)	2–4	17–27	43–50	12–25
Phospholipid composition (% dry weight)	7–9	17 – 19	19–21	17–24

Table 1: Density, size and physical composition of human plasma lipoproteins (1)

1.1.2. Lipoprotein metabolism

The handling of lipoprotein particles in systemic circulations is referred to as *lipoprotein metabolism* (3). It is divided into two separate pathways, *exogenous* and *endogenous* (Figure 2), depending on whether the lipoprotein particles are composed of dietary (exogenous) lipids or whether they originated in the liver (endogenous), through *de novo* synthesis of TG.

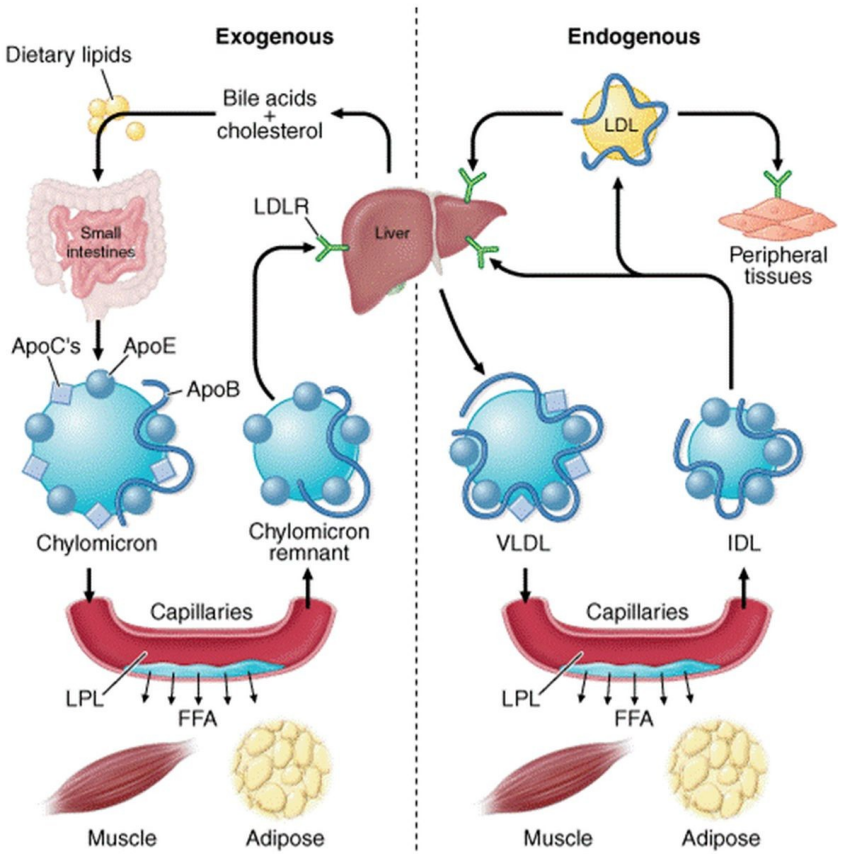


Figure 2: The exogenous and endogenous lipoprotein metabolic pathways
The exogenous pathway transports dietary lipids to the periphery and the liver. The endogenous pathway transports hepatic lipids to the periphery. Picture taken from Internal medicine, 17th edition.

The exogenous pathway (Figure 2, left part) starts with the intestinal absorption of dietary cholesterol and free fatty acids (FFAs). Within the intestinal cell, FFA reacts with glycerol to form TG, and cholesterol is esterified by acyl-coenzyme A:cholesterol acyltransferase (ACAT) to form CE. TGs assemble with phospholipids, cholesterol, CE and apoB-48 to form chylomicrons. These particles are then secreted from the enterocyte into the mesenteric lymph. As they circulate through the lymphatic vessels, nascent chylomicrons bypass the liver circulation and are drained via the thoracic duct into the bloodstream. In the blood stream, chylomicrons can interact with HDL particles which donate apoC-2 and apoE to the chylomicron. ApoE and apoC-2 rich chylomicrons are considered mature. Beside, apoC-2 is a cofactor for LPL which makes the chylomicrons progressively smaller primarily by hydrolysing the core TGs and releasing FFAs. The FFAs are then used either as an energy source, converted to TG, or stored in adipose tissue. The end-products of chylomicrons are chylomicron remnants that are cleared from the circulation by hepatic low-density lipoprotein receptor (LDLR) for which apoB is a high-affinity ligand (4).

The endogenous pathway (Figure 2, right part) of lipoprotein metabolism starts with the synthesis of VLDL by the liver. Microsomal triglyceride transfer protein is an intracellular lipid-transfer protein found in the endoplasmic reticulum of hepatocytes. It is essential for the transfer of the lipid molecules (principally TGs) onto apoB-100 in the liver (5,6). The main surface apolipoproteins for VLDL include apoB-100, apoC-2, apoC-3 and apoE. In the absence of functional MTP, VLDL is not secreted into the circulation. The TG core of nascent VLDL particles is hydrolyzed by LPL. During lipolysis, the core of the VLDL particle is reduced, generating VLDL remnant particles (also called IDL) that are depleted of TG via a process similar to the generation of chylomicron remnants. Some of the excess surface components in the remnant particle, including phospholipid, unesterified cholesterol, and apoA, C and E, are transferred to HDL. VLDL remnants can either be cleared from the circulation by the LDLR or remodelled by hepatic lipase (HL) to form LDL particles. LDL particles are the primary transport molecules for the delivery of cholesterol to peripheral tissues, and account for 70–80% of the circulating cholesterol in humans (7). LDL can be up-taken by the target tissue through endocytosis which begins with an interaction between LDLR and apoB-100 on the LDL particle. The internalized LDL particles are hydrolysed within lysosomes, which enables lipids release, chiefly cholesterol.

1.1.3. Composition of high density lipoprotein (HDL)

The HDL paticle is a macromolecule made up of different lipids and proteins and is highly heterogeneous in its physiochemical properties, function, and biological activity (8). Such heterogeneity is the result of differences in the relative distribution of apoliporoteins and lipids on and within the HDL-particle. Multiple subfractions of HDL can be identified in the plasma based on density, size, charge, and composition (Figure 3) (9).

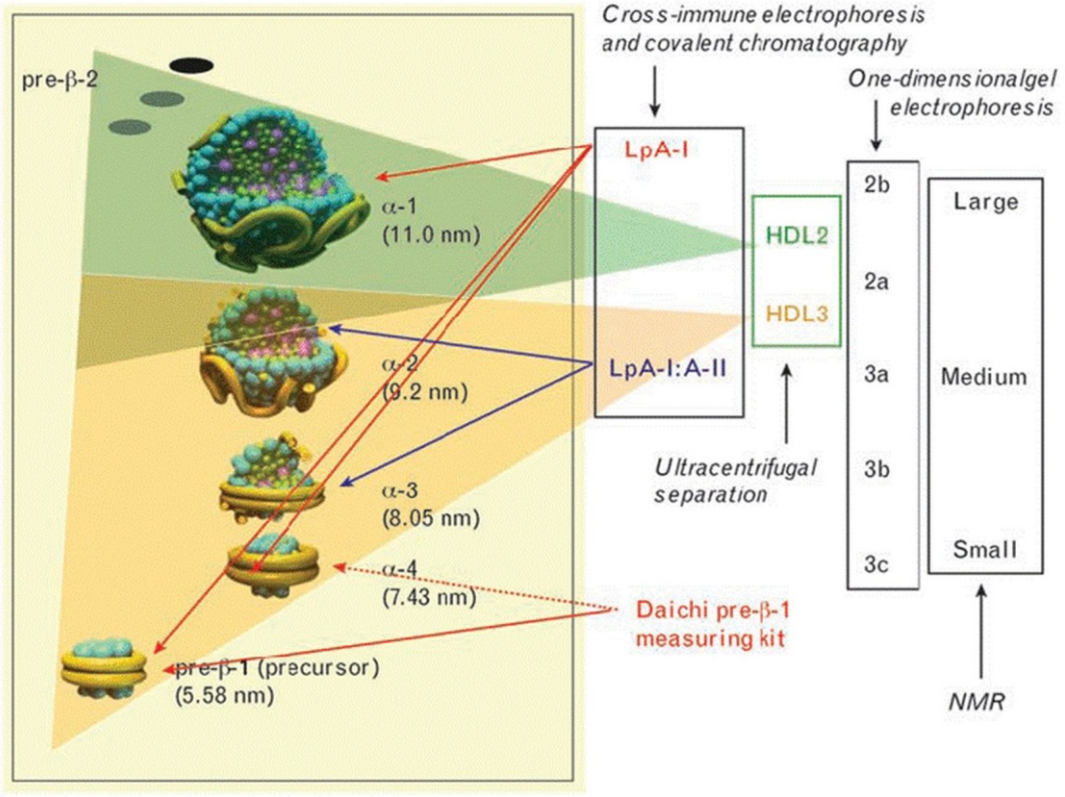


Figure 3: Comparing the nomenclature of HDL subclasses determined by different separation methods (9)

Based on density, plasma HDL are divided into HDL_2 (larger and less dense, 1.063–1.125 g/ml) and HDL_3 (smaller and denser, 1.125–1.21 g/ml due to high protein/lipid ratio), while agarose gel electrophoresis further discriminates the basic HDL fraction (α-lipoproteins) and a small fraction, pre-β-HDL. Another example of separation is by sequential immunoaffinity chromatography to Lp-A-I (all lipoprotein particles containing apoA-1), Lp-AI:A-II (particles containing apoA-1 and apoA-2) and Lp-A-II HDL subfractions (particles with apoA-2 without apoA-1). Newer methods, such as fast liquid chromatography, nondenaturing PAGE, NMR and high-resolution ion mobility technique, measure various HDL subclasses by different properties.

HDL is highly loaded with proteins compared to other plasma lipoproteins, with a protein to lipid ratio ranging from 1:2 in large light HDL_2 to 10:1 in small dense preβ-HDL particles. Proteomics studies have identified between 28 and 67 HDL-associated proteins using liquid chromatography–electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) (10). ApoA-1, quantitatively represents the major protein constituent of HDL (10) and accounts for approximately 70% of protein mass, followed by apoA-2 proportionate 15 to 20% of total proteins (11). The remaining 10 to 15% of HDL protein mass is composed

of minor amphipathic exchangeable proteins, including apoC, apoE, apoD, apoM, and apoA-4, completed with enzymes and lipid transfer proteins such as lecithin:cholesterol acyl transferase (LCAT), CE transfer protein (CETP) and paraoxonase-1 (PON1). Interestingly, proteomic studies of HDL have found not only proteins with well-characterized roles in lipid metabolism and anti-oxidant properties of HDL, but also a number of proteins involved in the acute phase response, complement regulation and proteinase inhibition.

In addition to proteins, HDL cholesterol also contains lipids including free cholesterol and CE; phospholipids (phosphatidyl-choline, phosphatidyl-ethanolamine, lysophosphatidyl-choline, and plasmalogen); FFA; mono-, di-, and triacylglycerols and sphingolipids (ceramide, sphingomyelins and sphingosine-1-phosphate) (12). Interestingly, it has been demonstrated that phospholipids on HDL particles determine cholesterol efflux capacity (13), that phosphatidyl-choline species influences HDL´s anti-inflammatory activity (14) and recently, huge attention is given to HDL associated S1P and its anti-atherogenic role. These data is suggesting that, besides proteins, consistence of HDL lipid composition might be the crucial variable of HDL functionality.

1.1.4. Metabolic pathways of HDL

Nascent HDL particles are synthesized by the intestine and the liver (15). Newly secreted apoA-1, by the liver and intestine, rapidly acquires phospholipids and unesterified cholesterol from its site of synthesis via efflux promoted by the membrane protein ATP-binding cassette protein A1 (ABCA1) (16). This process results in the formation of discoidal HDL particles, which then recruit additional unesterified cholesterol from the periphery. Within the HDL particle, the cholesterol is esterified by LCAT and the more hydrophobic CE moves to the core of the HDL. As HDL acquires more CE it becomes spherical, bigger, and additional apolipoproteins and lipids are transferred to the particles from the surfaces of chylomicrons and VLDLs during lipolysis.

HDL cholesterol is transported to hepatocytes by both an indirect and a direct pathway. Indirect pathway is mediated by CETP, which is facilitating transfer of HDL-CEs to apoB-containing lipoproteins in exchange for TG. The CE rich lipoproteins are then removed from the circulation by LDLR mediated endocytosis. HDL-cholesterol can also

be taken up directly by hepatocytes mainly by the scavenger receptor class BI (SR-BI) (17), a cell surface receptor that mediates the selective transfer of lipids into the cells.

HDL particles can be remodeled within the plasma compartment by a variety of lipid transfer proteins and lipases (18). The phospholipid transfer protein (PLTP) has the net effect of transferring phospholipids from other lipoproteins to HDL. After CETP-mediated lipid exchange, the TG-enriched HDL becomes a much better substrate for HL, which hydrolyzes the TG and phospholipids to generate smaller HDL particles. A related enzyme called endothelial lipase (EL) hydrolyzes HDL phospholipids, generating smaller HDL particles that are catabolized faster. Remodeling of HDL influences the metabolism, function, and plasma concentrations of HDL and is driven by physiological conditions of the body.

1.1.5. Biological and functional diversity of HDL

HDL possesses several properties which involve cholesterol efflux from cells, anti-oxidative, anti-inflammatory, endothelium protective, vasodilatory, anti-thrombotic and anti-infectious activities. HDL functions are specified in detail below (Figure 4).

Cholesterol efflux capacity – One of the most recognized roles of HDL is the transfer of excess cholesterol from vascular back to the liver and steroidogenic tissues (reverse cholesterol transport – RCT). The concept that RCT has a significant role in cardiovascular diseases got a substantial boost when it was published that HDL-mediated cholesterol efflux from macrophages had a strong inverse association with both carotid intima-media thickness and the likelihood of coronary artery disease (CAD) (19). These effects were shown to be independent of HDL-cholesterol level (20). Cholesterol efflux can occur by several mechanisms, including the following: a unidirectional ATP-dependent pathway mediated by the ATP binding cassette A1 transporter (ABCA1); a unidirectional ATP-dependent pathway mediated by the ABCG1; an ATP-independent, bidirectional pathway involving scavenger receptor class B type I (SR-BI); and receptor-independent passive diffusion according to cholesterol concentration gradient (21,22). The relative efficiency of different HDL subpopulations in promoting cholesterol efflux via the receptor-mediated pathways crucially depends on the composition of acceptors and receptors involved. Thus, lipid-free and/or lipid-poor HDL apolipoproteins, primarily apoA-1, potently and dose-dependently induce cholesterol efflux via interaction with

ABCA1. Despite the major role of ABCA1 and small HDL in cholesterol efflux from lipid-loaded cells, pathways promoted by large HDL via SR-BI and ABCG1 can also contribute significantly to net cholesterol efflux. Moreover, large, lipid-rich HDL particles represent a more efficient ligand for cellular CE uptake mediated by SR-BI compared with small, lipid-poor HDL (23), consistent with the role of these particles in RCT (23). When comparing cholesterol efflux properties of HDL subpopulations, it is essential to keep in mind the concentration basis employed for such comparison. Thus, on the basis of phospholipid content, small, dense HDLs more potently promote cholesterol efflux, whereas on a particle number basis, large HDLs are more effective (24,25).

Anti-oxidative activity – HDL particles are also heterogeneous in their capacity to protect LDL from oxidative damage induced by one-electron oxidants such as free radicals. Small, dense HDL_3 particles may be superior to large, light HDL_2 in terms of their capacity to remove oxidised lipids from other lipoproteins and cellular membranes (26). Moreover, a distinct conformation of apoA-1 in HDL_3 might facilitate the redox reaction between methionine residues of apoA-1 and lipid hydroperoxides. Second, hydrolysis of short-chain oxidised phospholipids by HDL-associated hydrolytic enzymes also appears to be enhanced in small, dense HDL_3. Finally, the unique proteome of HDL_3 might have implications for its anti-oxidative activity, which is highly correlated with the presence of apoJ, apoM, serum amyloid A4 (SAA4), apoD, apoL-1, PON1 and PLTP (10).

Anti-inflammatory activity – HDLs display multiple anti-inflammatory effects including inhibition of monocyte activation. This is further resulting in an inhibition of pro-inflammatory cytokine and chemokine production, cytokine-induced adhesion molecule expression in endothelial cells and inhibition of monocyte adhesion to the endothelium. Reduction in cytokine and chemokine production also diminishes neutrophil activation and neutrophil infiltration in the arterial wall (27,28). Anti-inflammatory actions of HDL may also involve hydrolysis of pro-inflammatory oxidized lipids by HDL-associated platelet activating factor-acetylhydrolase (PAF-AH) and PON1, which is mechanistically similar to the role of these enzymes in HDL anti-oxidative activity (11,29). The anti-inflammatory activities of HDL appear to be primarily mediated by apoA-1, with a contribution by phospholipids, including S1P and sphingosylphosphorylcholine (30).

Endothelium protective activity – Recent studies indicated HDL protective functions on human endothelium. HDL stimulates endothelial nitric oxide production (31),

inhibits formation of reactive oxygen species (32,33), LDL oxidation (34) and oxidized LDL induced apoptosis (35), improves endothelium-dependent vasodilation and early endothelial progenitor cell mediated endothelial repair (36,37). It has been hypothesized that apoA-1, PON1, LCAT and S1P play a significant role in these HDL functions (38-41).

Vasodilatory activity – HDLs can contribute to the maintenance of vascular endothelium function by stimulating nitric oxide (NO) release and production of prostacyclin (PGI2) by human endothelial cells (42). Activation of NO production involves HDL binding to SR-BI, which activates downstream the phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway and the phosphorylation of endothelial nitric oxide synthase (eNOS). This activation also depends on S1P receptors (43,44).

Anti-thrombotic activity – The anti-thrombotic activity of HDL is observed as inhibitory actions on platelet activation as well as on factors that promote blood coagulation, including tissue factor, and factors X, Va, and VIIIa. Mechanistically, whereas HDL may reduce platelet activation directly (45,46) it also may act indirectly on platelet activation by one or more of its effects on both, human and animal endothelial cells (47,48).

Anti-infectious activity – HDL is also a part of the innate immunity, as it carries several complement proteins (9,49,50) and is able to bind lipopolysaccharide through apoA-1 (50). Another innate immune-defense function of HDL is its ability to bind and/or neutralize certain invading pathogens (51).

Modulation of glucose metabolism – It has been shown by cell culture, animal and human studies that apoA-1 gene expression is decreased by elevated glucose levels and increased by insulin (52), but there is emerging evidence that HDL, and apoA-1 in particular, might also modulate glucose metabolism directly. Recombinant apoA-1 improves glucose uptake in mouse skeletal muscle and decreases hepatic gluconeogenesis. Infusion of reconstituted HDL particles reduces plasma glucose, increases insulin secretion and promotes glucose uptake in skeletal muscle of patients with type 2 diabetes. In addition, HDL is able to increase pancreatic β-cell insulin secretion (53,54) and decrease β-cell apoptosis (55). In adipocytes HDL increases glucose uptake and mediates release of adiponectin (56), a hormone that increases insulin sensitivity (57,58).

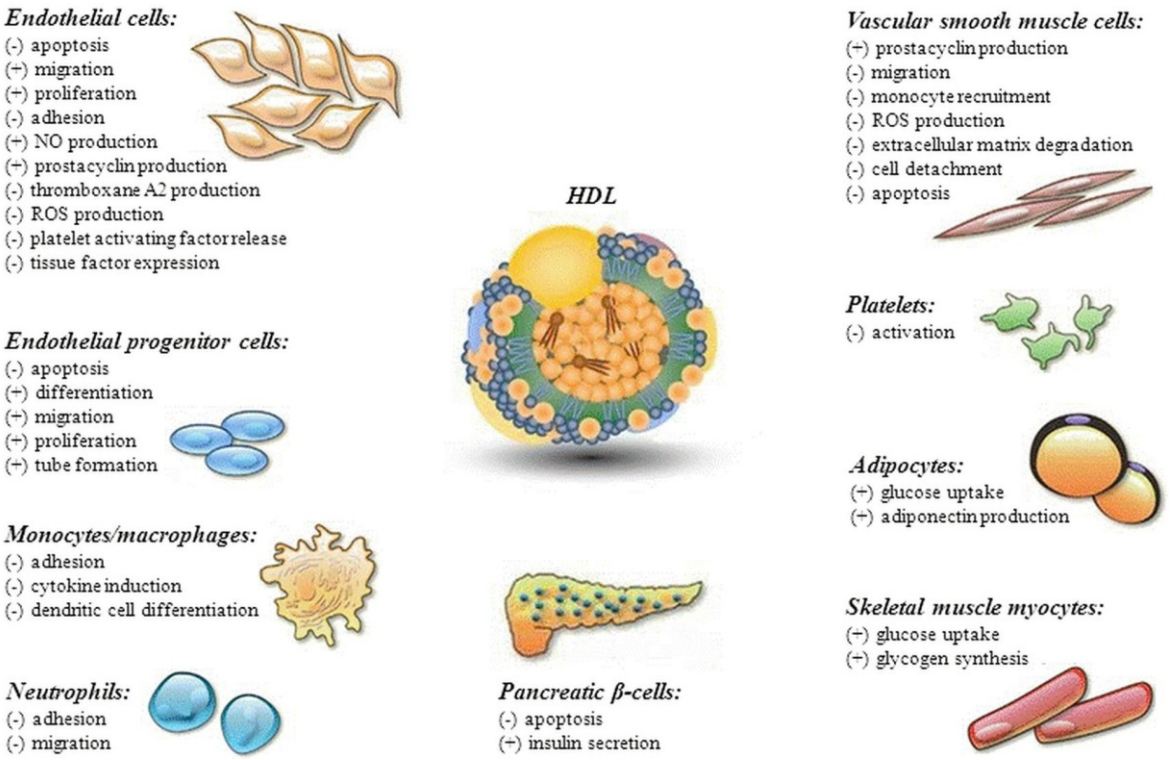


Figure 4: Cell targets of HDL action
Figured modified from (59)

1.1.6. HDL as a carrier of sphingosine-1-phosphate (S1P)

S1P is blood born bioactive phospholipid. The sources of S1P in blood are mostly platelets and erythrocytes but S1P could also be secreted from vascular endothelial cells by ABC-transporters (60). Once released S1P mainly binds to HDL through interaction with apoM (Figure 5).

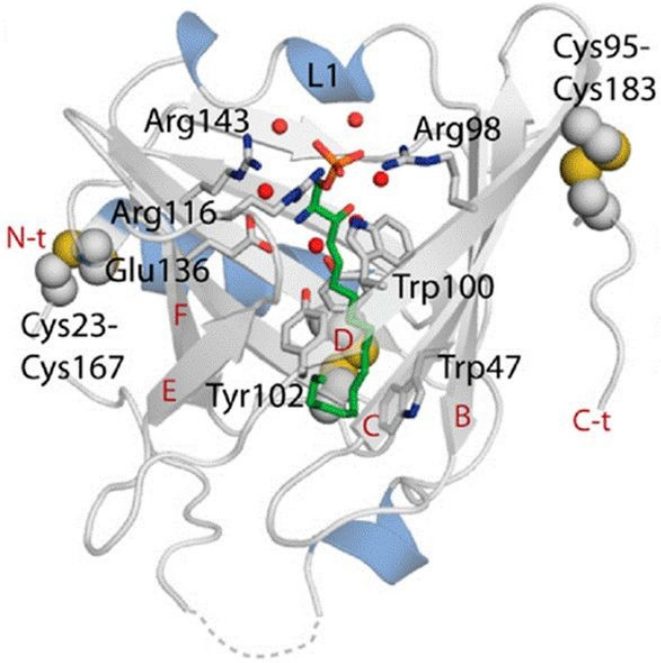


Figure 5: Stereo view of the crystal structure of apoM with S1P at 1.7-Å resolution
S1P is shown as green sticks together with interacting residues. Strands B–F, the N terminus (N-t), and the C terminus (C-t) are labeled in red, and the interacting residues are labeled in black (61).

Approximately 70% of circulating S1P is bound to HDL while remaining 30% associates to albumin (62). The percentage of S1P transported in plasma lipoproteins may be positively correlated with HDL-cholesterol concentration suggesting that individuals with a high HDL-cholesterol level may have a high HDL-S1P level, which further supports the role of S1P as a mediator of HDL-induced antiatherogenic action (62). Recent evidence suggest S1P role in HDL mediated macrophage-cholesterol efflux, maintenance of endothelial function, anti-inflammatory, anti-oxidant, and profibrinolytic activities (59,63). HDL associated S1P may be responsible for the beneficial effects of this lipoprotein on vasodilatation, protection against postischemic inflammation, inhibition of oxidation, and synthesis of nitric oxide and prostacyclin (PGI2) (44,59,64). S1P may also mediate HDL-induced endothelial cell survival through S1PR1 pathways and migration through the S1PR1 and S1PR3 (38,65). These observations may support the hypothesis that raising plasma level of HDL-bound S1P may be associated with beneficial effects in cardiovascular diseases patients.

1.2. Cholesterol metabolism in pregnancy

1.2.1. Lipoproteins in maternal and fetal systemic circulation

Pregnancy is characterized by complex changes in glucose, protein, and lipid metabolism. Interestingly, lipid metabolism is especially affected during pregnancy (66). Maternally derived lipoproteins have been studied extensively in human pregnancies (67-69), but little is known about the differences between maternal and fetal lipoproteins in late pregnancy and their possible interdependency. By the third trimester most women have a lipid profile, which would be considered highly atherogenic in the non-pregnant state (70). It is characterized by an increase of 300% in TG levels, a 25–50% increase in total cholesterol, accompanied by higher levels of HDL cholesterol and mass of small dense LDL (71,72). Increased insulin resistance and estrogen stimulation during pregnancy are responsible for this state of maternal hyperlipidemia (73). Hyperphagia and increased lipid synthesis (74) contribute to maternal fat accumulation typically associated with the first two-thirds of gestation. In the last trimester fat storage declines as a consequence of enhanced lipolytic activity and decreased LPL activity in adipose tissue. Although for most women this undoubtedly represents a transient change, which reverts to normal after delivery, the long-term consequences of variations on lipid profile are still unknown. Moreover, changes in maternal lipid metabolism and cholesterol supply might also affect fetal outcome with consequences later in life (75,76). In general, fetal cord and neonatal plasma lipid levels are reported to be very much lower than those in adults (77,78), with a relatively larger proportion of cholesterol carried in HDL particles (77), resulting on a lower total cholesterol/HDL-cholesterol ratio (78). Concentration and composition of fetal high density lipoprotein (HDL) are different from those in adults. While low-density lipoprotein (LDL) represents the major class of lipoprotein in maternal serum, more than 50% of fetal total cholesterol is carried by HDL. LDL and very low-density lipoprotein (VLDL) are present in the fetal circulation, but only at a low concentration (79-83). Previous studies on lipoproteins in human cord blood showed that only apoE was present at a level similar to that in adult plasma, whereas other apolipoproteins were all lower than those in adults (Table 2) (79-81,84).

	Cord serum	Adult serum
Total cholesterol (mg/dl)	65 ± 17[a]	187 ± 25
Free cholesterol (mg/dl)	20 ± 6[a]	44 ± 6
Triglyceride (mg/dl)	23 ± 10[a]	71 ± 38
Phospholipid (mg/dl)	107 ± 19[a]	195 ± 22
ApoA-1 (mg/dl)	84 ± 16[a]	130 ± 17
ApoA-2 (mg/dl)	18 ± 3[a]	31 ± 5
ApoB (mg/dl)	19 ± 5[a]	81 ± 14
ApoC-2 (mg/dl)	1.7 ± 0.6[a]	3.1 ± 1.1
ApoC-3 (mg/dl)	3.9 ± 1.6[a]	7.4 ± 2.2
ApoE (mg/dl)	4.6 ± 1.4	4.6 ± 1.2

Table 2: Lipids and apolipoproteins in cord and adult serum
Table taken form (85). n(cord serum)= 75; n(adult serum)= 38; $X^a = p<0.001$

1.2.2. Lipid transport/transfer across the human placenta

Cholesterol has key roles in fetal development. In addition to serving as a structural component of all membranes it is a precursor for oxysterols, it has been implicated in the activation and propagation of the Sonic hedgehog signals (86). In the fetus it mostly originates from *de novo* synthesis in many compartments, foremost adrenals, gonads and liver. Interestingly, fetuses with a genetic disorder of cholesterol biosynthesis (Smith-Lemli-Opitz syndrome), where *de novo* synthesis of cholesterol is not possible, still have measurable cholesterol concentrations (87). Also offspring with other defects in endogenous sterol synthesis (homozygous familiar hypobetalipoproteinemia) have no birth defects (88). These observations demonstrate the existence of external cholesterol sources for the fetus. It was estimated that up to 40% of the serum cholesterol in the term fetus originates from maternal sources by transfer across the placenta (89,90). The existence of maternal-fetal cholesterol transfer is also supported by significantly higher levels of HDL-, LDL- and total cholesterol in umbilical venous than umbilical arterial plasma (91).

Substance, to enter fetal circulation, has to be transported across the human placenta. The placenta is a peculiar organ of limited life span (Figure 6). It connects the mother and the fetus and at the same time represents a barrier between the two circulations. Many diverse functions are attributed to the placenta which is vital for normal fetal development and thus initially the placenta substitutes for fetal organs. Key roles of the placenta are supply of the fetus with maternal nutrients, exchange of gases between the

maternal and fetal circulation, and returning waste products from the fetus to the mother for excretion. Consequently, the placenta is responsible for facilitating adequate fetal development (92).

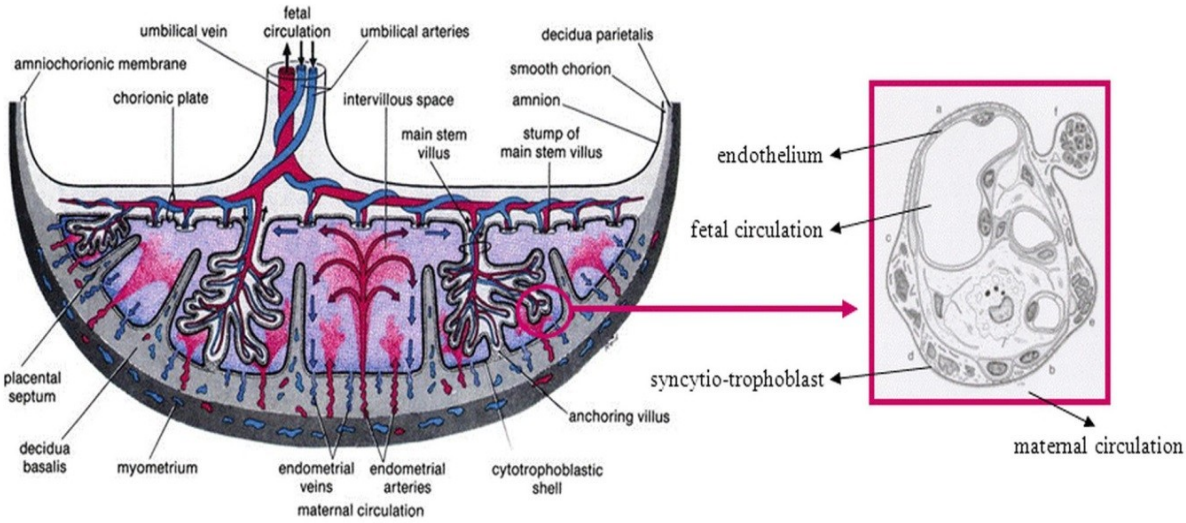


Figure 6: Structure of the human placenta
The inset image shows a cross-section through a chorionic villus.

Many studies have been conducted to provide better understanding of fetal external cholesterol supply. The lipoprotein receptors described to be expressed in the placenta include the LDL preceptor, the VLDL receptor, the class A scavenger receptor, the LDL receptor related protein, the apoE receptor 2, megalin, cubilin, and SR-BI (93), while cholesterol transporters found in the placenta are ABCA1 and ABCG1. In animal models with yolk sac, the lipoprotein cholesterol can be metabolized trough the lysosomal pathway (by LDL receptors family) or by cytosolic esterases (by SR-BI). In BeWo cells (cell line highly similar to trophoblasts) maternally derived LDL-cholesterol can be effluxed on the basolateral side to HDL and phospholipids but not to apoA-1, indicating ABCG1 as the main cholesterol transporter in these cells (94,95). Once cholesterol is effluxed into the extracellular matrix it can be taken up by the placental endothelial cells and further released into the fetal circulation by ABCA1 and ABCG1 to apoA-1, apoE, and HDL (96). In addition, cholesterol might be also transported in receptor independent manner (97). Finally, it is important to mention that exogenous maternal cholesterol does not have to originate from maternal circulation. It is possible that that cholesterol be synthesized by the human placenta could be released to fetal circulation just like cholesterol taken from the maternal circulation. The scheme below demonstrates sequential steps in transplacental

transfer of lipoprotein-derived cholesterol at the end of gestation in humans with proposed pathway for cholesterol efflux from placental ECs into the fetal circulation.

In the case of lipoprotein/cholesterol this multistep process will encompass 1) an 'influx/uptake' component allowing maternal HDL and/or their cholesterol backbone to get into the syncytiotrophoblast, the placental tissue which is in contact with maternal blood, 2) then a transport of HDL-derived cholesterol or the HDL particle to the basal side of the syncytiotrophoblast, 3) a component of intra-placental cholesterol transport from outside the syncytio-trophoblast across the basal lamina to the endothelium, 4) transport into the placental endothelium and 5) finally an efflux component by which cholesterol is released from the placental endothelium into the fetal circulation (Figure 7) (96).

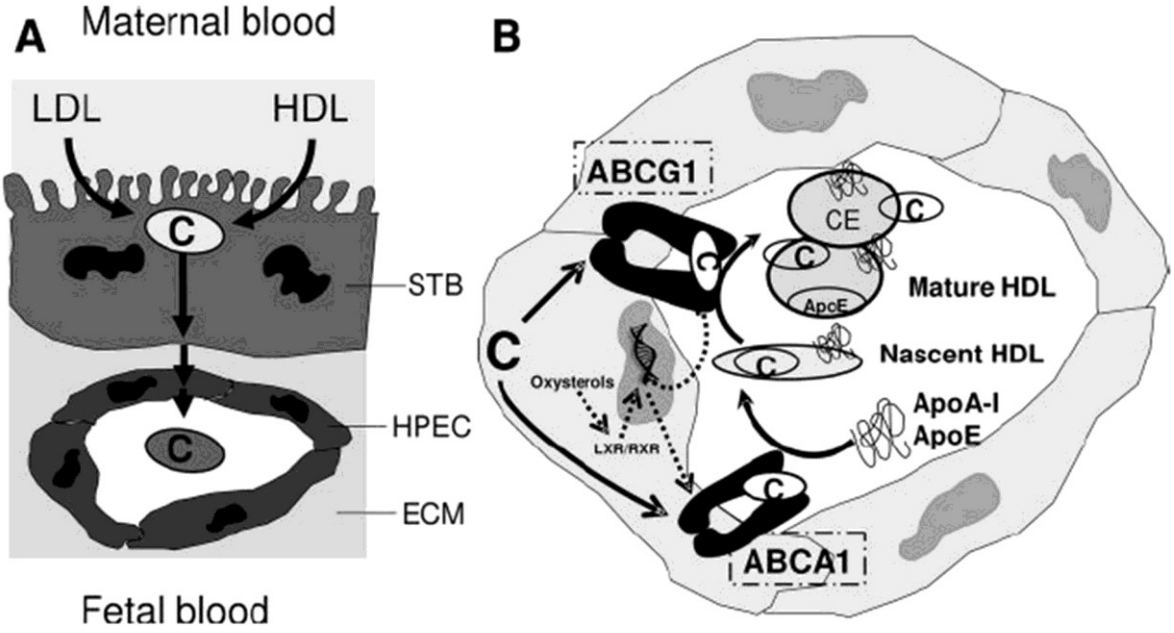


Figure 7: Sequential steps in transplacental transfer of lipoprotein-derived cholesterol at the end of gestation

A) Overall maternal–fetal transfer likely encompasses an "uptake/influx" component for maternal lipoproteins and/or their cholesterol into the syncytiotrophoblast (STB), transport of lipoproteinderived cholesterol (C) to the basal side of the STB, subsequent release into the villous core for passage through the extracellular matrix (ECM), uptake into HPECs, and, finally, an efflux component by which cholesterol is released from HPECs into the fetal circulation

B) The efflux of cholesterol (C) from HPECs into the fetal circulation involves an interactive role of ABCA1, ABCG1, and fetal apolipoproteins. ABCA1 promotes cholesterol efflux to lipid-free/poor apoA-1 or apoE in the fetal circulation, thus initiating the formation of nascent, discoidal HDL, which will be further enriched with cholesterol by the cooperation of ABCA1 and ABCG1. ApoE-enriched HDL particles may facilitate cholesterol efflux via the ABCG1 pathway. Esterification of cholesterol by lecithin cholesterol acyl transferase will eventually result in the formation of mature spherical HDL. Oxysterols activate LXRs, which induce expression of ABCA1 and ABCG1, and, hence, will represent an effective mechanism regulating cholesterol efflux (dotted lines). (96)

1.3. Lipid metabolism in Gestation Diabetes Mellitus

1.3.1. Definition and consequences of Gestational Diabetes Mellitus (GDM)

Glucose metabolism is altered during pregnancy due to an increase in insulin resistance as a result of an anti-insulin action of hormones produced by the placenta (98). Thus, pregnant women need to produce elevated amounts of insulin to meet the requirements of the tissues. In gestational diabetes mellitus (GDM) either the production of insulin is insufficient (e.g. pancreatic ß-cell dysfunction) or the insulin resistance becomes pathological (98,99). Therefore GDM is defined as glucose intolerance with onset or first recognition during pregnancy (98). Its prevalence ranges between 1.3 and 19.9% (100) depending on diagnostic criteria and ethnicity (101).

Despite its short duration, GDM affects fetal growth and has long term consequences for both mother and offspring. The main adverse outcome on progenies from pregnancy complicated with maternal diabetes appears to be macrosomia, but the complications also include metabolic abnormalities, degraded antioxidant status, disrupted immune system and potential metabolic syndrome in adult offspring (102). Several indicators point towards GDM to promote a vicious trans-generational cycle of increasing obesity and diabetes in the offspring leading to mounting rates of GDM, type 2 diabetes and obesity in subsequent generations (102). In recent years alterations in the intrauterine environment associated with pregnancy conditions have received considerable attention because of mounting evidence that maternal conditions during pregnancy affect postnatal and adult morbidity including disorders such as hypertension, coronary heart disease, type 2 diabetes mellitus or metabolic syndrome (103). The basis of this Developmental Origins of Adult Health and Disease (DOHAD) concept is an altered intrauterine milieu that induces fetal adaptations with the long-term consequences for the offspring. As an example maternal hypercholesterolemia is associated with fatty streak formation in fetal arteries and with accelerated progression of atherosclerosis in childhood and probably later in life (104,105). Considering that progression of atherosclerosis in adults takes ages, these striking results support the assumption of a strong maternal impact on the comparatively short period of fetal development.

1.3.2. Lipoproteins in GDM and implications for the newborns

As far as lipid metabolism is concerned, experimental diabetes has been shown to impair maternal and fetal lipid metabolism (106,107). In animal maternal models, hyperinsulinemia and hepatic hyperlipogenesis, common features of experimental obesity and diabetes, are causing significant increase in serum TG and total cholesterol. Macrosomic and obese offspring of diabetic animals' exhibit high adipose tissue weight, together with high adipose tissue lipid contents (108), followed by high serum and liver lipid levels (107,109,110).

In human pregnancies complicated with GDM changes in lipoprotein concentrations in the maternal and fetal circulation are controversial and may relate to the quality of glycemic control achieved in the diabetic mothers, to the type of diabetes i.e., pre-gestational type 1 vs gestational diabetes, and to treatment modalities (111). In GDM mothers higher TG levels have been found in the first trimester of gestation compared to normal pregnancy (112,113), although there are also studies where no change in plasma TG levels was found (71). Plasma cholesterol levels in the third trimester were lower in GDM than in normal pregnancies in some studies (112), unchanged (71) or even higher (113) in others.

Whereas maternal diabetes-associated dyslipidemia has been well characterized (104), little is known about GDM effects on lipoprotein metabolism in the placenta and fetus. Disturbances in maternal lipid metabolism caused by GDM have been associated with increased fat accumulation in the offspring (114). Infants of diabetic mothers have higher plasma cholesterol and phospholipid levels (111) and altered lipoprotein composition (115). Additionally, early changes in the lipid and protein compositions of specific HDL subspecies in youth males with type 2 diabetes are shown to be early markers of arterial disease (116). Higher HDL-cholesterol, as well as higher HDL-cholesterol/apoA-1 and HDL-cholesterol/apoA-2 ratios are strongly and independently related to lower risk of future type 2 diabetes (117). Collectively, these findings suggest that analyzing the composition of HDL, rather than HDL-cholesterol, may be useful in assessing postnatal risk for future cardiovascular diseases. Identifying molecular targets might offer options for therapeutic intervention.

1.4. Hypothesis and objectives

While maternal lipoprotein profile was extensively described (67-69,112,113), almost nothing is known about the full HDL proteome in the fetal circulations and the associated functions. Under the pathophysiological conditions (e.g. dyslipidemia, insulin resistance, inflammation, infection and cardiovascular disease) HDL particles can progressively lose normal biological activities and acquire altered properties as a result of alterations in HDL composition, structure and metabolism.

Therefore we are aiming to show:

(i) that maternal and fetal HDL are composed differently and translated into a different functional particles. As an initial step towards verifying this hypothesis, maternal and corresponding fetal HDLs obtained from pregnancies without complications were analyzed by proteomics.

(ii) that GDM is associated with alterations of the maternal and fetal HDL proteome and that these alterations have an impact on HDL protective properties.

(iii) that fetal HDL is also a carrier of S1P, due to the finding that apoM is associated with fetal HDL, and to describe its regulatory effect on feto-placental endothelium in pregnancies without complications and in pregnancies complicated with GDM.

Accordingly, results will be explained and discussed in a three subsections to enable a better understanding.

2. RESEARCH DESIGN, MATERIALS AND METHODS

2.1. Blood sampling

The study protocol was approved by the local ethical committee of the Medical University of Graz. Women without pregnancy complications (n=11) or with GDM (n=9) were recruited at the time of delivery and gave informed written consent. All women in the study underwent a 75g oral glucose tolerance test. GDM was defined as abnormal glucose tolerance according to the IADPSG statement (118). Only patients who were diagnosed with GDM for the first time and treated with diet only were included in the study. Healthy participants were selected based on negative oral glucose tolerance test, absence of medical complications during pregnancy, and were matched to the lean GDMs in their pre-pregnancy BMI (<25kg/m^2), total serum cholesterol, TG and PL levels. Venous blood from pregnant women at term was collected before delivery, while their corresponding umbilical cord blood was taken maximally 10min after delivery. EDTA-plasma and serum were isolated by centrifuging at 3500rpm for 10 minutes in a refrigerated centrifuge and stored at -80°C until further analyses. Since fetal TG concentrations are higher after vaginal delivery when compared to caesarean-section (119), we decided to include in the study only pregnancies which mode of delivery was caesarean-section.

2.2. Subjects characteristics and their lipoprotein profile

Subject characteristics are summarized in Table 3. Total cholesterol, TG, free cholesterol and phospholipid levels were measured enzymatically (DiasSys GmbH, Holzheim, Germany). HbA1c% and C-reactive protein (CRP) was determined by immunoassays (Greiner BioChemica GmbH, Flacht, Germany). All measurements were performed on an Olympus AU640 analyzer (Olympus Diagnostic, Hamburg, Germany).

Maternal characteristics	Control (n=11)	GDM (n=9)	Fetal characteristics	Control (n=11)	GDM (n=9)
Height (cm)	165.1 ± 7.3	165.6 ± 7.1			
Weight before (kg)	58.7 ± 10.5	68.3 ± 8.7	**Length (cm)**	49.2 ± 2.5	50.4 ± 2.0
Pre-pregnancy BMI (kg/m^2)	21.2 ± 3.0	23.1 ± 2.1	**Weight (g)**	3244.2 ± 553.2	3427.3 ± 429.0
Weight at childbirth (kg)	74.5 ± 16.4	80.3 ± 10.3	**Placental weight (g)**	616.2 ± 93.9	658.5 ± 146.2
BMI at childbirth (kg/m^2)	27.5 ± 5.0	29.2 ± 3.2	**PI (kg/m^3)**	26.1 ± 2.6	26.8 ± 3.3
Cholesterol (mg/dl)	269.8 ± 80.1 ###	261.1 ± 23.7 $$$	**Cholesterol (mM)**	68.4 ± 13.7	68.8 ± 23.0
Triglyceride (mg/dl)	220.8 ± 86.3###	220.9 ± 95.7 $$$	**Triglyceride (mM)**	26.2 ± 14.3	33.8 ± 10.8
Phospholipids (mg/dl)	301.1 ± 71.7 ###	287.8 ± 26.6 $$$	**Phospholipids (mM)**	110.9 ± 20.5	113.9 ± 25.5
CRP (mg/l)	5.8 ± 4.5	45.8 ± 21.2 ***	**CRP (mg/l)**	0.6 ± 0.0	4.0 ± 7.0 **
HbA1c (%) (mmol/mol)	n.a.	6.0 ± 0.6 (42.0 ±0.4)	**C-peptide (ng/ml)**	n.a.	0.8 ± 0.9
			Insulin (µIE/ml)	n.a.	19.7 ± 10.5

Table 3: Subjects characteristics
***$p<0.001$, **$p<0.01$ differences control vs GDM subjects; ###$p<0.001$, differences maternal vs fetal, control subjects; $$$$p<0.001$ differences maternal vs fetal, GDM subjects; n.a.: not analyzed

2.3. Isolation and characteristics of HDL

Due to the small volume of plasma obtained from the umbilical cord, fetal and maternal HDLs were isolated by an adapted ultracentrifugation method (120). HDL was centrifuged in the TL-100 Tabletop centrifuge using the TLA-100.4 rotor (Beckman Coulter, CA, USA). After adjusting plasma density to 1.24g/ml with potassium bromide, 1.7ml plasma was transferred to a Quick-Seal Bell-top tube (Beckmann Coulter, CA, USA) eligible for the TLA-100.4 rotor. Plasma was centrifuged at 100,000rpm 2 for 2.5hrs after refilling the centrifugation tube with a potassium bromide solution (density 1.006g/ml). To visualize the HDL fraction within the tubes, DiI-dye (1-1´dioctadecyl-3-3-3´-tetramethyl indocarbocyanine perchlorate) was added in one reference tube as an indicator. After re-isolation, HDL was mixed with EDTA, covered with argon and placed at 4°C for longer storage. Before further analyses HDL was desalted with PD10 columns with sefadex (GE Healthcare, Vienna, Austria) (120). To exclude contaminations with other lipoprotein classes and non HDL associated proteins (e.g. albumin, apoB), maternal and fetal HDL were also isolated by Dynabeads Protein G (Invitrogen, New York, USA). Dynabeads (65µl) were completely resuspended and incubated for 15min at room temperature (RT) with 10µg anti-apoA-1 antibody (Novus biologicals, Littleton, CO, USA). The tube with beads was then placed on a Dynal magnet where beads migrated to the side of the tube enabling easier removal of the supernatant and washing buffers (first with 200µl PBS w/Tween20 followed by 200µl conjugation buffer (20mM sodium phosphate, NaCl, pH=7-9)). After the last washing step, Dynabeads plus protein complex were immediately incubated for 30min at RT with 250µl 5mM BS3 (Bissuberate, Thermo Fisher Scientific,

Waltham, USA) diluted in conjugation buffer. To avoid coeluation of the antibody, 12.5µl quenching buffer was added for 15min at RT and beads were again placed on magnet for washing. Plasma samples (200µl) were gently resuspended in Dynabeads-antibody complex for 60min at RT. After series of washing steps with PBS HDL was eluated with 200µl elution buffer (50mM Glycine, pH=2,8) for 2min and the supernatant containing isolated HDL was collected.

By comparing two different lipoprotein isolation methods and subsequent proteomic analysis of the obtained HDL´s revealed that 23% more HDL associated proteins (n=35) were detected on maternal and fetal HDL isolated by ultracentrifugation method than with Dynabeads method (n=27). Among proteins additionally detected in HDL isolated by ultracentrifugation method were apoH, SAA-1 and vitamin D binding protein, which are known to be functionally relevant HDL proteins (49). Albumin was detected to similar quantities in HDLs isolated by both methods with slightly higher (+20%) apoB levels in HDL isolated by ultracentrifugation. Since contamination with non HDL associated proteins (e.g. albumin, apoB) was identified in both HDL isolation methods and 12 more proteins were detected as part of HDL proteome when lipoprotein was isolated by ultracentrifugation method, we decided to proceed with isolating HDL by ultracentrifugation.

Characteristics and purity of isolated HDLs was confirmed by lipid electrophoresis by matching its migration distance with distance of healthy, nonpregnant, female, adult HDL isolated with multistep ultracentrifugation method as described elsewhere (121). Protein concentration was determined by the Bradford (Sigma Aldrich, St. Luis, USA) method. Esterified cholesterol was calculated as the difference between total and free cholesterol. ApoA-1 levels were determined by immunoturbidimetry (Greiner BioChemica GmbH, Flacht, Germany). Isolated maternal and corresponding fetal HDL was stored in salt, covered with argon gas to avoid oxidation at 4°C. Proteomic studies and functional assays were done within three months after lipoprotein isolation.

2.4. Shotgun proteomics by LC-MS/MS

For tryptic digest, 20µg of HDL protein (n=11 for control group, n=9 for GDM group) were precipitated with 3 volumes of acetone at -20°C overnight, solubilized in 10µL 8M ammonium guanidinium hydrochloride and 25 µl 100mM ammonium

bicarbonate, reduced with 35µl of 10mM DTT for 20min by shaking at 550rpm at 56°C and alkylated with 8µl of 55mM iodoacetamide by shaking at 550 rpm at RT for 15 min. Protein was digested by adding 1µg of Promega modified trypsin and shaking over night at 550rpm at 37°C. The resulting peptide solution was acidified by adding 1.6µl of 5% formic acid and diluted in 0.3% formic acid to a theoretical final concentration of 50ng/µl. 40µl were separated by nano-HPLC on an Agilent 1200 system equipped with a Zorbax 300SB-C18, 5µm, 5 x 0.3mm enrichment column and a Zorbax 300SB-C18, 3.5µm, 150 x 0.075mm nanocolumn. Samples were injected and concentrated on the enrichment column for 6min using 0.1% formic acid as isocratic solvent at a flow rate of 20µl/min. The column was then switched into the nanoflow circuit, and the sample was loaded on the nanocolumn at a flow rate of 300nL/min and separated using the following gradient: solvent A: water, 0.3% formic acid; solvent B: acetonitril/water 80/20, 0.3% formic acid; 0-10min: 10% B; 10-120min 10-60% B, 120-122min 60-95% B, 122-130min 95% B, 130-132min 95-10% B, 132-140min re-equilibration at 10% B. The sample was ionized in the nanospray source equipped with nanospray tips (PicoTipTM Stock# FS360-75-15-D-20, Coating: 1P-4P, 15±1µm Emitter, New Objective) and analyzed in a Thermo LTQ-FT mass spectrometer in positive ion mode by alternating full scan MS (m/z 400 to 2000) in the ICR cell and MS/MS by CID of the 5 most intense peaks in the ion trap with dynamic exclusion enabled (for a duration of 10s).

2.5. LC-MS/MS data analysis

The LC-MS/MS data were analyzed by searching the human NCBI nonredundant public database (downloaded on March 11th, 2011) with Spectrum mill Rev. A.03.03.078 (Agilent) and Mascot 2.2 (MatrixScience). Detailed settings: Enzyme: trypsin, max. missed cleavage sites: 2, N-terminus: hydrogen, C-terminus: free acid, carbamidomethylation on cysteine as fixed modification, oxidised methionine as variable modification, maximum precursor charge 3; precursor mass tolerance ± 0.05Da, product mass tolerance ± 0.7Da; acceptance parameters were 2 or more identified peptides after automatic validation (Mascot: decoy search, FDR<5%; Spectrum Mill: for precursor charge of 2: score threshold was 6.0, %SPI threshold was 60.0, Fwd-Rev score threshold was 2.0 and rank 1-2 score threshold was 2.0, for precursor charge of 1: score threshold was 6.0, %SPI threshold was 70.0, Fwd-Rev score threshold was 2.0 and rank 1-2 score threshold was 2.0, for precursor charge of 3: score threshold was 8.0, %SPI threshold was 70.0, Fwd-Rev

score threshold was 2.0 and rank 1-2 score threshold was 2.0). Spectral counting of the total peptides identified was used to compare relative protein abundances of the same protein between samples (122). A threshold of a minimum of 4 spectral counts was used for relative quantitation (123).

2.6. Western blotting

Lysed proteins (15µg/µl) from different control (n=5) and GDM (n=3), maternal and their corresponding fetal HDL were separated by 4-20% precise protein gels (Thermo Scientific, Rockford, USA) for 45min at 120V, 400mA with 1x Tris-HEPES-SDS running buffer. Separated proteins were transferred to PVDF membrane (GE Healthcare, Vienna, Austria) with blotting buffer (Tris-Glycin-Methanol, pH=8.3) for 1h at 45V, 40mA. Membranes were blocked at RT in blocking buffer (5% non-fat dry milk in 1x TBE buffer) for 1h, and then incubated with different primary antibodies overnight at 4ºC as shown in Table 4.

protein	antibody	dilution
albumin	abcam – ab83465 Cambridge, UK	1:2000
apoA-1	novus biologicals nb100-65491 Littleton, USA	1:3000
apoA-2	abcam – ab54796 Cambridge, UK	1:1000
apoA-4	abcam - ab72395 Cambridge, UK	1:1000
apoE	abcam - ab1906 Cambridge, UK	1:1000
apoF	abcam - ab92301 Cambridge, UK	1:1000
apoM	abcam - ab57471 Cambridge, UK	1:1000
PON1	abcam – ab24261 Cambridge, UK	1:1000
SAA1	abcam - ab50232 Cambridge, UK	1:1000

Table 4: Primary antibodies used for proteomics results validation

ApoA-2 was chosen as a loading control, since its average number of peptides was similar on maternal and fetal, control and GDM HDL. After washing with 1x TBE (0.1% Tween, 20, 0.01M TBS) the membrane was incubated with secondary antibodies (goat anti mouse or anti rabbit/ HRP conjugate, 1:2000, Bio Rad Laboratories, Hercules, CA) for 1h at RT. After a subsequent washing immuncomplexes were visualized using the ECL development (GE Healthcare, Vienna, Austria) and quantified by densitometry using Quantity One 1-D (Bio-Rad Laboratories) and DigiDoc software.

2.7. Native gradient gel electrophoresis (Native GGE)

In order to investigate protein intactnes of maternal and fetal, control and GDM HDL *"in vivo"*, intact HDL particles were analyzed by native GGE. Isolated HDL (10µg protein) was electrophoresed on 4 – 15% TrisHCl gels (Bio-Rad) upon dilution with native sample buffer (Invitrogen, New York, USA) as described (124) with minor modifications (125). Gels were pre-run (PowerPac Basic, Bio-Rad, Vienna, Austria) for 20min at 125V, in running buffer (0.09 mol/L Tris, 0.08 mol/L boric acid, 3 mmol/L EDTA, pH 8.3), then samples were applied and gels were electrophoresed for 30min at 70V before increasing to 125V for another 6h, all at 4°C. Gels were fixed in 10% sulfosalicylic acid for 30min and then stained with Coomassie Brillant Blue G250. The high molecular weight marker NativeMark (Invitrogen, New York, USA) was used as standard.

2.8. CETP and LCAT ELISA

CETP and LCAT were quantified using commercially available assays (Alpco, Salem, USA).

2.9. CETP activity assay

The commercial CETP activity assay (Kamiya Biomedical Company, Seattle, USA) uses a fluorescent self-quenched neutral lipid that is transferred to an acceptor molecule in the presence of the enzyme. CETP-mediated transfer results in an increase in fluorescence (excitation: 465nm, emission: 535nm). Concentrations of enzymes from samples were extrapolated from a standard curve.

2.10. LCAT activity assay

The LCAT activity assay (Calbiochem, Darmstadt, Germany) used 1µl of each plasma sample, which was incubated for 4h at 37ºC with LCAT fluorescent substrate (kit component No.KP23001) that emits fluorescence at 470nm. Upon hydrolysis of the substrate by LCAT, a monomer with a fluorescence maximum at 390nm is released. The LCAT activity was assessed as change in 470/390 emission intensity and compared with the known activity of the referent sample.

2.11. Paraoxonase 1 activity assay

The rate of hydrolysis of paraoxon (Sigma-Aldrich, St. Louis, USA) into 4-nitrophenol was measured by monitoring the increase in absorbance at 405nm. Serum (10µl) was added to 200µl buffer (100mmol/L Tris, 2mmol/L $CaCl_2$, 1mmol/L paraoxon, pH 8.0). The assay was performed in 96-well plate (Greiner Diagnostics GmbH, Bahlingen, Germany) at room temperature and measurements were taken after 30min at 405nm on a plate reader (Spectra Max 250, Molecular Devices, USA). Enzymatic activity was calculated by multiplying the molar extinction coefficient of ε_{405}=17100 l/mol*cm with the absorbance measured after 30min. Paraoxonase activity was expressed as a rate of 4-nitrophenol formation (nmol/l per minute).

2.12. Determination of the oxidative susceptibility of HDL

The intrinsic susceptibility of maternal and fetal HDL to "*in vitro*" copper-mediated oxidation was assessed by the technique described by Esterbauer et al (126). Briefly, 50µg/ml maternal and fetal HDLs (n=6) were incubated in duplicates with 1µM CuSO4 at 37ºC. The conjugated dienes formed during HDL protein oxidation produce a distinct absorbance peak at a wavelength of 234nm. The absorbance was recorded every 5min (over 200min) for each individual HDL sample after subtracting basal absorbance of each HDL at 234 nm (control, without Cu^{2+}). From the absorbance profile obtained for each HDL three characteristic parameters were calculated. First, the lag time (Tlag), was defined as the intersection of the baseline with the tangent to the slope of the absorbance curve during the propagation phase. Second, the maximum time (Tmax) was defined as the time at the end of the propagation phase, when diene production reached its maximum.

Third, the maximal amount of dienes produced, was calculated by using the molar extinction coefficient for conjugated dienes (ε_{234}=29500 l/mol*cm).

2.13. Determination of total anti-oxidative capacity of HDL

Total anti-oxidant capacity (TAC) was determined using an automated method (127). This method is based on the de-coloration of 2,2′ -azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS) by anti-oxidants present on HDL. The color change was measured using an ILab 300 Plus autoanalyzer. The assay was calibrated with Trolox (a water-soluble analog of vitamin E, 6-hydroxy-2.5.7.8-tetramethylchroman-2-carboxylic acid). Due to the limitation of the volume of collected cord blood samples TAC was estimated in 6 control and 6 GDM maternal/fetal subjects and is expressed as mmol Trolox equivalent/L. The intra- and inter-assay coefficients of variance were 4.3 % and 8.8 %, respectively.

2.14. Primary placental cells

Primary human term trophoblasts (TT) and placental endothelial cells (HPEC) from third trimester placentas were isolated after caesarean section as described (128,129). The quality and purity of isolated cell populations was assessed by indirect immuno-fluorescence staining (129). Purified trophoblasts were plated at a density of 500 cells/mm^2 and cultured for 24 h in Dulbecco's minimum essential medium (DMEM, Gibco, Life Technologies, Paisly, UK) containing 10% fetal calf serum (FCS, HyClone, Utah, USA), 1% glutamine and 1% penicillin/streptomycin. Functionality of HPEC was assessed by internalization of acetylated low-density-lipoprotein (Biomedical Technologies, Stoughton MA) after they were cultured on 1% (v/v) gelatin-coated plates using Endothelial Basal Medium (EBM, Cambrex, CloneticsTM, USA) supplemented with the EGMTM-MV BulletKit (CloneticsTM) containing gentamicin/amphotericin, hydrocortisone, recombinant-human epidermal growth factor, bovine brain extract and 5% FCS.

2.15. Cholesterol efflux assay

Efflux of cellular cholesterol to several acceptors was analyzed as described previously (96) with minor modifications. Cell were seeded on 12-well culture plates

(70,000 endothelial cells/well and 1.000,000 trophoblasts/well) and metabolically labeled with 0.5µCi/ml [3H]-cholesterol in medium containing supplements and 5% FBS for 24h. Subsequently, cells were washed with PBS and incubated with serum-free medium at 37°C for 2h in order to equilibrate cellular radiolabeled cholesterol pools. Cells were washed again (PBS) and serum-free medium containing cholesterol acceptor particles (maternal (n=3) and fetal HDL (n=3) from control and GDM subjects, 14µg/ml HDL protein) were added to the wells. After 6h cell supernatants were collected centrifuged and radioactivity was determined in aliquots from each well by liquid scintillation counting. Monolayers were washed twice in cold PBS and lysed in 0.3mol/L NaOH (4h, 4°C). Aliquots of the lysate were used for counting the cell-associated radioactivity and for determining cellular protein contents using the BCA protein kit (Invitrogen, New York, USA). [3H]-cholesterol efflux was expressed as percentage of radioactivity measured in supernatants relative to the total radioactivity (medium plus cell lysate) relative to total cell protein per well.

2.16. Quantitative real-time PCR

Isolation of total cellular RNA was performed using RNeasy Mini (QIAGEN, Hilden, Germany) from 22 different human placental arterial endothelial cells (HPAEC). Quality and concentration of obtained RNA were determined by Bio-Photometer using 260/280 ratio (Eppendorf-AG, Hamburg, Germany). cDNA was generated using iScript cDNA Synthesis Kit (Bio-Rad) and the C1000 Thermal Cycler (Bio-Rad). Quantitative real-time PCR (RTQ-PCR) was carried out at the Core-Facility Molecular Biology center of the Medical University of Graz by using the AB7900-2 Syllabus thermal cycler (Life technologiesTM, Vienna, Austria). The cycling conditions were: 2 minutes at 50°C, initial denaturation at 95°C for 10 minutes and followed by 40 cycles of 95°C for 15 seconds and as annealing temperature 60°C was chosen. Gene specific primers for S1PR1 (HS00173499_m1, Applied Biosystems, USA) and S1PR3 (HS00245464_s1, Applied Biosystems, USA) were synthesized by Taqman. The results were normalized to the house-keeping gene RPL-30.

2.17. S1P measurements

S1P plasma and HDL concentration was estimated with commercially available ELISA (Echelon Biosciences Inc., Salt Lake City, USA).

2.18. S1P depletion by S1P lyase

To deplete S1P from fetal HDL 5µg of active, human SPL lyase (SPL) (Sigma-Aldrih, Saint Louis, USA) was incubated with 1nmol S1P-HDL for 60min at 37ºC. S1P is an enzyme which irreversibly hydrolases S1P to hexadecenal and ethanolamine-phosphate. Amount of S1P depletion was analysed by S1P ELISA.

2.19. Migration Assay

Migration assays were performed using a 96-well chemotaxis chamber system (Neuroprobe) (61). After serum starvation for 3 h in EBM medium, HPAEC were placed in the upper well of the chemotaxis chamber at a density of 10 000 cells per well and were allowed to migrate toward chemoattractants in the lower well, which was separated from the upper well by a fibronectin-coated polycarbonate filter with 8-µm pores. After incubation for 4 h at 37°C, the upper surface of the filter was wiped clean of nonmigrating cells, and the cells were fixed with 4% formaldehyde and stained with DAPI. Pictures were taken from each well of the filter, and single, migrated cells were quantified by Dot count software.

2.20. Electrical cell-substrate impedance sensing (ECIS)

To determine the effects of control and GDM fetal HDL on barrier function, impedance measurement were performed using an ECIS System (Applied Biophysics, Troy, NY, USA). HPAEC were plated on gelatine-coated gold electrodes of 8W10E+ arrays and impedance was recorded in real time at 1-min intervals at 4 and 64 kHz. After approximately 24h, when cells reached stable basal resistance, HPAEC were treated with different control and GDM fetal HDLs, commercially available selective inhibitor of S1PR1 – 10µM W146 (Avanti Polar Lipids, Alabaster, USA) and 2ng/ml VPC23019 (Torcis bioscience, Bristol, UK). As control in all experiments 5% FCS in EBM was used. When cell were treated with S1P vehicle control was albumin, with W146 inhibitor - 20% (2-hydroxypropyl)-beta-cyclodextrin and with VPC 23019 - DMSO.

2.21. F-actin immunofluorescence staining

HPAEC (75,000 cells/well) were seeded in 1% gelatine coated chamber slides. After 24h, the monolayers were washed with HBSS and treated with 1μM S1P associated to bovine serum albumin (BSA) and 100μg/ml fetal control and GDM HDL protein in 5% serum EBM, for 30min. After washing, cells were fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. After washing three times with PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for 25 min at room temperature. The slides were again three times washed with PBS and then blocked with 1% BSA in PBS for 25 min at room temperature. After blocking, slides were washed once with PBS and then stained overnight with 1 U/200 ll methanolic phalloidin-Texas Red (Molecular Probes, Invitrogen) for 1 h in the dark at room temperature. Stained cells were washed three times with PBS and slides mounted with Dako fluorescent mounting medium (Dako, Denmark) with DAPI (1:2.000, Invitrogen, New York, USA). After overnight drying, actin organization and focal adhesions were observed in a Zeiss LSM 510 Meta microscope, objective Plan-Apochromat 639/1.4 Oil DIC, lasers 405, 488 and 543 nm and LSM Image Browser software.

2.22. Statistical analysis

Differences in plasma and HDL parameters between maternal and fetal samples were analyzed using tests for related samples - paired samples T-test and the Wilcoxon test and between control and GDM group 2-tailed Mann-Whitney U-test was used. To assess for data normality, Kolmogorov-Smirnov and Shapiro-Wilk tests were used. Changes in the HDL proteome were evaluated from spectral counts of automatically validated proteins (i.e., the number of MS/MS spectra assigned to a protein). Pearson´s correlation was applied to measure association between different maternal and fetal variables. Significance was accepted at $*p<0.05$, $**p<0.01$ and $***p<0.001$. Statistical analyses were performed with PASW Statistics Version 18 and SigmaPlot 12.

3. RESULTS

3.1. Distinct composition of human fetal HDL attenuates its anti-oxidative capacity

3.1.1. Fetal HDL carries a unique protein cargo

To determine the differences between maternal and fetal HDL we used shotgun proteomics liquid chromatography-electrospray ionization-tandem MS (LC-ESI-MS/MS) of tryptic digests. HDL isolated by ultracentrifugation fractioned on a density gradient and isolated from 11 individual plasmas of normolipidemic, healthy, pregnant women at term and fetal HDL from corresponding umbilical cord blood samples (from 55% male and 45% female fetuses) were analyzed. When the maternal and fetal plasma lipid profiles were compared, total cholesterol, TG and phospholipid concentrations were considerably lower in fetal samples (Table 3), which is consistent with previous results (85). Fetal HDL-cholesterol levels were similar to maternal levels, but fetal HDL contained significantly less TG and apoA-1 (Table 5), suggesting that, like LDL, its function is primarily to deliver cholesterol to the developing tissue.

Characteristics of HDL	Maternal	Fetal
Cholesterol (mg/mg protein)	0.81 ± 0.23	0.78 ± 0.24
Triglycerides (mg/mg protein)	0.27 ± 0.17 ***	0.08 ± 0.01
Non-esterified cholesterol (mg/mg protein)	0.13 ± 0.06 *	0.27 ± 0.09
Esterified cholesterol (mg/mg protein)	0.68 ± 0.18	0.51 ± 0.15
Phospholipids (mg/mg protein)	1.41 ± 0.42	1.42 ± 0.39
ApoA-1 (mg/mg protein)	2.21 ± 0.58 **	1.19 ± 0.31

Table 5: Characteristics of maternal and fetal HDL.
****p<0.001, **p<0.01, *p<0.05*

To exclude that labor and delivery have an impact on maternal or fetal lipid profiles, individual levels of CRP, which rise in response to inflammation was determined.

The mean CRP values ranged from <0.6 to 4.6±2.2 mg/L (Table 1A) in fetuses and mothers, respectively, similar to published values (130) and are considered low.

Shotgun proteomic analyses detected 35 different proteins associated with HDL (Figure 8) in accordance with recent literature (10,49,131). Seventeen of the identified HDL-associated proteins are linked to cholesterol- and lipoprotein metabolism, four are acute-phase-response proteins, while six are involved in immune response, which implicates a role of HDL also in inflammation and immunity. Other HDL associated proteins are involved in coagulation processes (n=4) or act as proteinase inhibitors (n=2) and transport proteins (n=6).

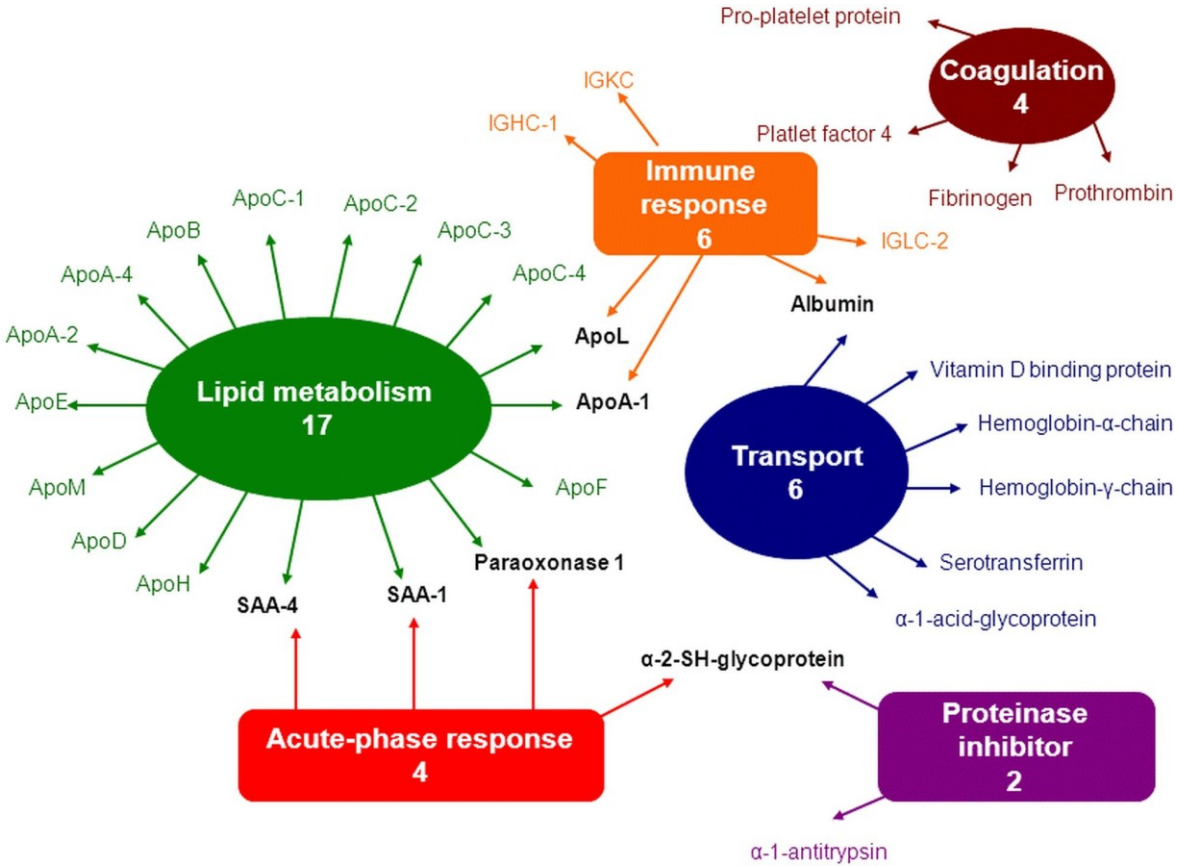


Figure 8: General illustration of HDL-associated proteins categorized with their established biological functions in the maternal and fetal circulation
HDL-associated proteins were identified by LC-ESI-MS/MS. The global scheme categorizes the proteins according to their assigned functions into those involved in lipid metabolism, acute-phase response, transport, proteinase inhibition, immune response, coagulation and complement regulation. Proteins with more than one function are in bold.

Proteins not shown on the Figure 1, dedicator of cytokinesis 9 (DOCK9), which function as activators of small G proteins, and two zinc finger proteins - growth factor independent protein 1 (Gfi-1) and microrchidia3 (MORC3) - were also found in the HDL

fraction. These proteins are part of intracellular signalling networks and hematopoietic process, therefore probably originating from cellular debris (132-134).

Figure 9 indicates the total peptide counts for each protein, while Figure 10 emphasizes differences in average number of proteins identified on maternal and fetal HDL. Peptides derived from apoA-1 prevailed, as expected. ApoF ($p=0.007$) and apoL ($p=0.005$) were only part of the maternal HDL protein cargo, which was also enriched in apoA-1 ($p=0.006$), apoC-1 ($p=0.016$), apoC-2 ($p=0.045$), apoC-3 ($p=0.005$), apoC-4 ($p=0.027$), apoD ($p=0.046$) and PON1 ($p=0.003$) (Figure 10B). HDL isolated from umbilical cord plasma contained statistically higher levels of albumin ($p=0.041$), apoA-4 ($p=0.027$), apoE ($p=0.021$), α-2-HS-glykoprotein ($p=0.024$), platelet basic protein ($p=0.017$), while haemoglobin α ($p=0.005$) and γ chain ($p=0.005$), Ig gamma-1 chain C region (IGHG1) ($p=0.043$), Ig lambda chain C regions (IGLC2) ($p=0.042$), platelet factor 4 ($p=0.026$) and vitamin D binding protein ($p=0.046$) were almost exclusively present on fetal HDL (Figure 10A).

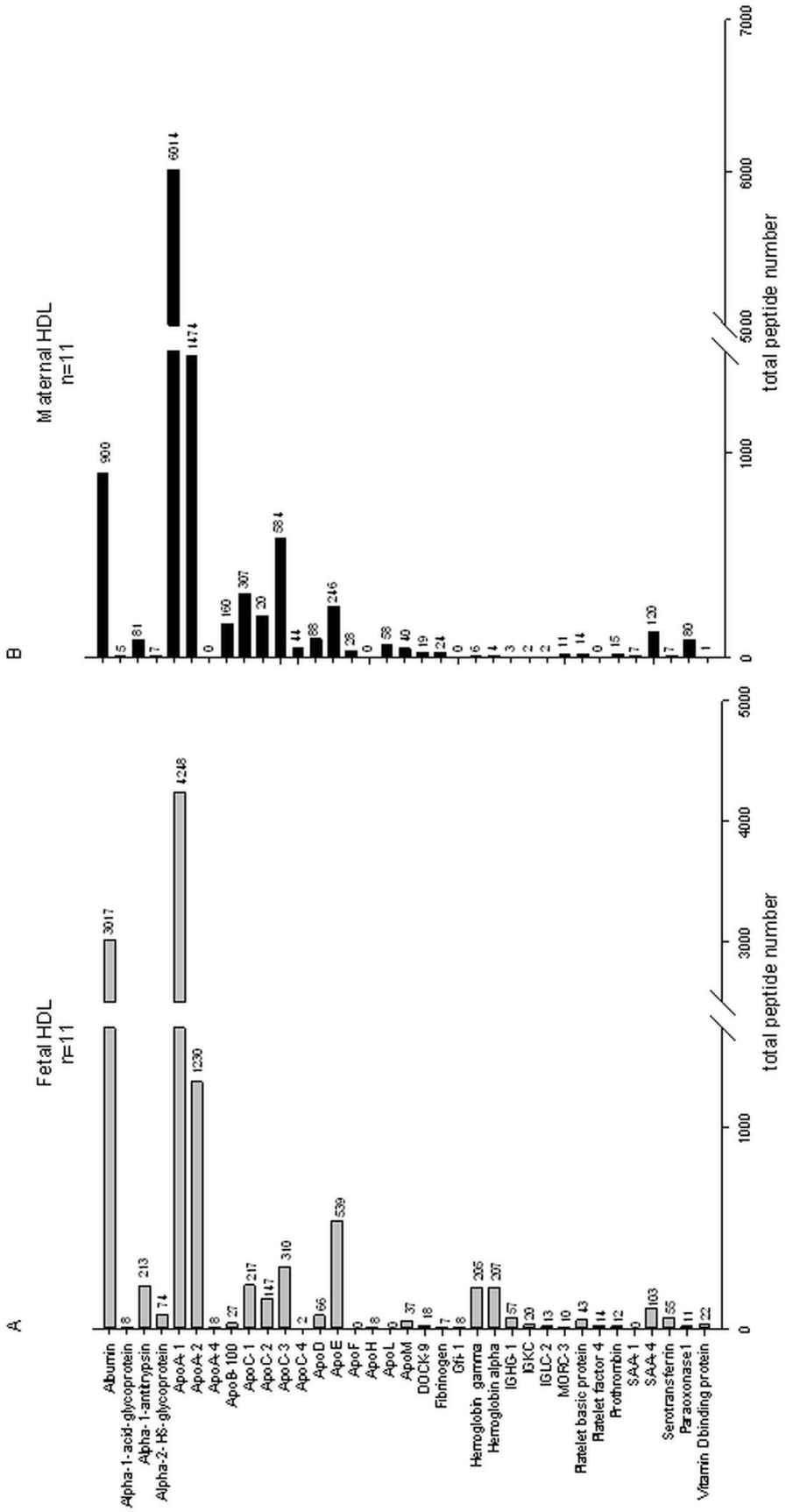


Figure 9: Number of total peptides found for each protein

A) Summed from 11 individual fetal HDL samples; B) Summed from 11 individual maternal HDL samples.

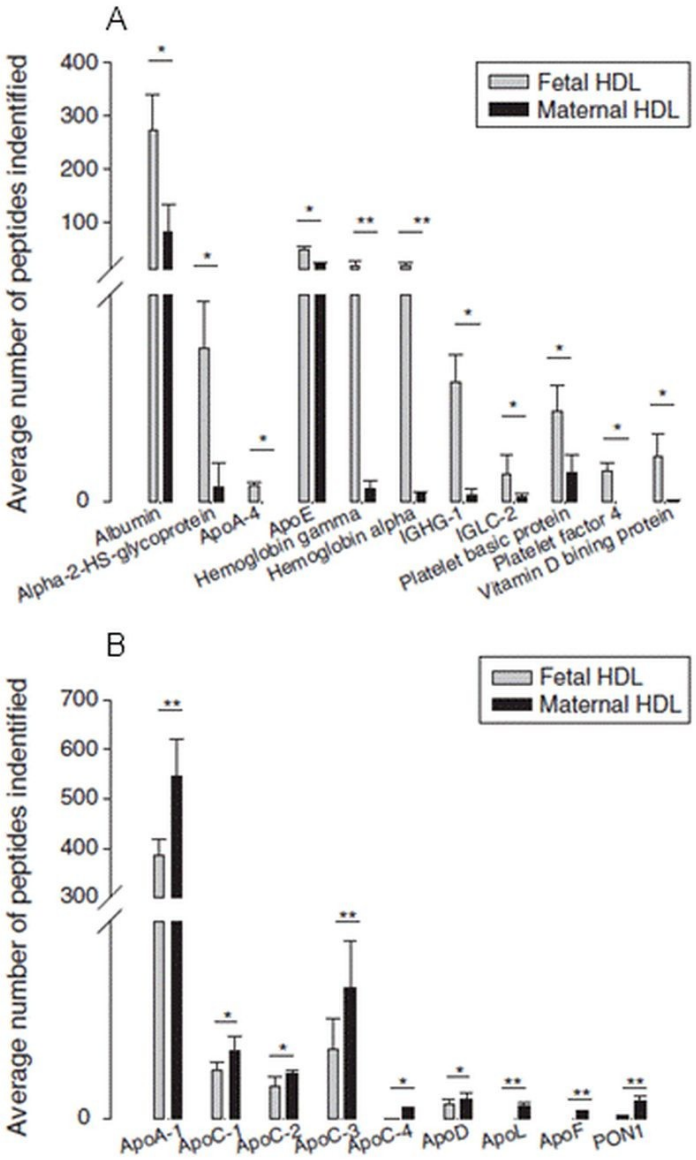


Figure 10: Abundance of proteins on fetal and maternal HDL
Peptides predominately present on either fetal HDL (A) or maternal HDL (B) in comparison of the different circulations. (n=11) *$p<0.05$; **$p<0.01$

To confirm the proteomics results by an independent method, some HDL-associated proteins were selected based on their particular role in maternal and fetal lipid metabolism. Amount of these proteins was measured by Western blotting of five individual maternal and fetal HDLs. The bands were subsequently quantified by densitometric estimation of bands specific for albumin (60kDa), for apoA-1 (29kDa), for apoA-2 (32kDa), for apoA-4 (50kDa), for apoE (38kDa), for apoF (33kDa) and for PON1 (40 kDa) (Figure 11). ApoA-2 could be used as loading control, since its average number of peptides was not statistically different between maternal (134 ± 32) and fetal HDL (111 ± 42). Observations obtained with Western blot analyses indicate that MS peptide counts provide

a valid approach for determination of the relative abundance of proteins on maternal and fetal HDL.

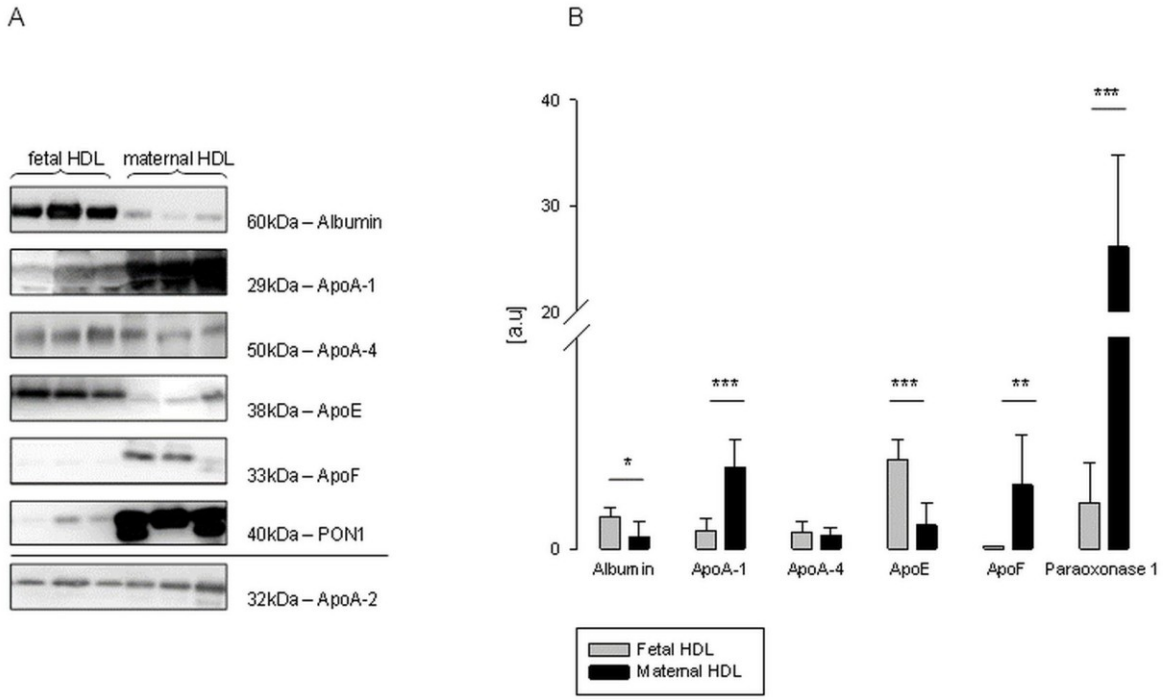


Figure 11: Western blot of HDL-associated proteins
A) To validate the results obtained by LC-MS/MS, five maternal and their corresponding fetal HDLs were subjected to immunoblot analyses. ApoA-2 was used as loading control, since its average number of pepetides was similar on maternal (134 ± 32) and fetal HDL (111 ± 42). B) Densitometric quantification of specific bands expressed relative to ApoA-2. **p<0.05; **p<0.01; ***p<0.001*

3.1.2. Apolipoprotein distribution remodels fetal HDL

Lipids and apoA-1concentrations of maternal and fetal HDLs are shown in Table 5. While HDL-triglycerides were significantly lower in fetal HDL, phospholipid (PL) levels were about similar, whereas non esterified cholesterol was higher in fetal HDL as compared to maternal, respectively. These results are consistent with previous reports (85).

Interestingly, as shown by native gel electrophoresis (Figure 12A), fetal HDL migrated more slowly than maternal HDL and was followed by a more indistinct apolipoprotein distribution. As a consequence of lower apoA-1 and higher apoE expression, fetal HDL particle size was slightly increased compared to maternal HDL (11.4 ± 0.2 nm vs. 9.6 ± 0.3 nm) (Figure 12B). This altered protein composition resulted in an altered negative particle charge which further led to a slower migration of fetal HDL.

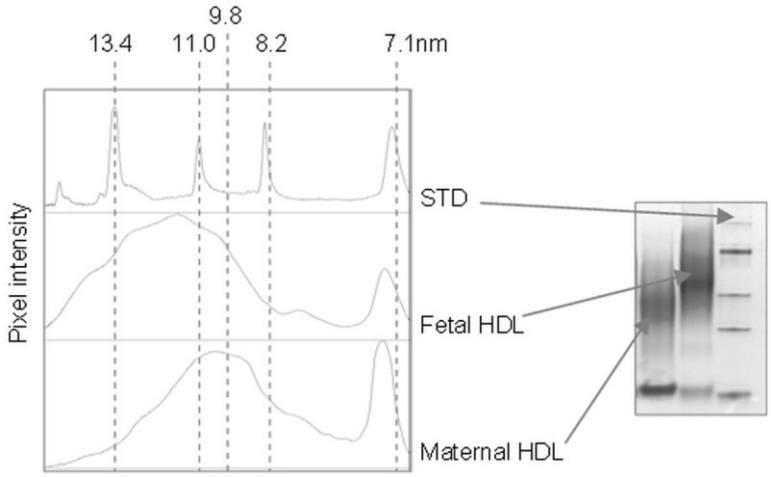


Figure 12: Native GGE analysis of HDL
Maternal and fetal HDL (10µg total protein) were used for native GGE analysis and stained with Coomassie Brillant Blue G250. The gel shown is representative of 3 independent experiments. Standard proteins used wer bovine serum albumin (7.1nm), lactate dehydrogenase (8.2nm), B-phycoerythrin (11.0nm, Ficner et al., 1992), apoferritin (13.4nm, de Haen, 1987).

3.1.3. Fetal LCAT and CETP levels and activity are altered in cord serum

LCAT concentration was higher (18.84 ± 5.80µg/ml, *p<0.001*) in the maternal as compared to the fetal circulation (5.08 ± 1.46µg/ml), while LCAT activity was similar (Table 6). In contrast, CETP enzymatic activity was 55% lower in fetal plasma (*p<0.001*) despite similar concentrations of the enzyme in maternal and fetal serum (Table 6).

	Maternal	Fetal
PON1 activity (nmol/min)	75.12 ± 18.21 ***	13.88 ± 3.34
LCAT mass (µg/ml)	18.80 ± 5.80 ***	5.08 ± 1.46
LCAT activity (470/390nm)	1.07 ± 0.12	1.01 ± 0.13
CETP mass (µg/ml)	3.75 ± 1.13	3.05 ± 1.52
CETP activity (pmol/µl/30 min)	97.95 ± 22.40 ***	51.39 ± 13.40

Table 6: Characteristics of HDL-associated enzymes
*** *p<0.001*

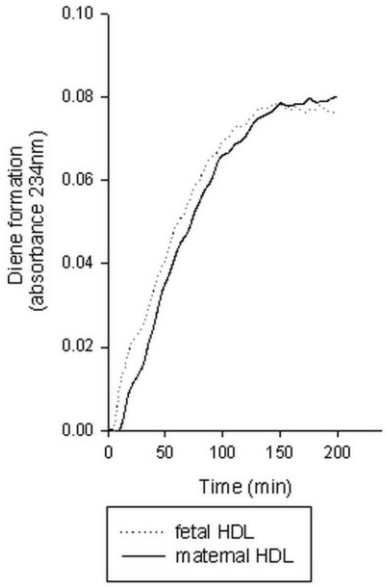
3.1.4. Impaired anti-oxidative capacity of fetal HDL

Besides the important role in lipid metabolism, enzymatic components, including PON1, are potentially contributing to the anti-oxidative properties of HDL. Strikingly, HDL-associated PON1 protein content was 7-fold lower in the fetal than in the maternal circulation (7.27 ± 1.83 vs. 1.00 ± 0.41, *p<0.001*), reflected by a lower PON1 activity (maternal: 75.12 ± 18.21nmol/min; fetal: 13.88 ± 3.34nmol/min, *p<0.001*; Table 4).

Positive correlations were found between PON1 mass and activity in both circulations (fetal: r^2= 0.825, p=0.004; maternal: r^2=0.924, p=0.000).

To examine whether lower PON1 activity affects the anti-oxidative capacity of fetal HDL, we monitored maternal and fetal HDL-oxidation spectrophotometrically after the addition of Cu^{2+}. Strikingly, fetal HDL (dotted line) needed a shorter time period to achieve the same oxidation level than maternal HDL (Figure 13A, solid line) over all time points measured, as reflected by a shorter calculated Tlag time (10±2.6 vs. 17±3.2, *p<0.05*) (Figure 13B). Tmax was achieved faster in the case of fetal HDL (127.2±11.2min vs. 142.8±6.8min, *p<0.05*), while the maximal dien production was about equal (Figure 13B). In addition, we found positive correlations among anti-oxidative parameters of maternal, but not of fetal HDL. Maternal PON1 mass and activity positively correlated with Tlag (r^2=0.688, p=0.019, r^2=0.614, p=0.044). Also both PON1 mass and activity correlate with Tmax (r^2=0.652, p=0.03; r^2=0.603, p=0.05). This correlation results support the strong association of HDL associated PON1 and its anti-oxidative capacity, which is obviously relevant in both maternal and fetal circulations.

A:



B:

	Fetal HDL n=(6)	Maternal HDL n=(6)
Tlag (min)	10 ± 2.6	17 ± 3.2*
Tmax (min)	127.2 ± 11.2	142.8 ± 6.8*
maximal diene production (mmol)	1.4 ± 0.6	1.4 ± 0.2

Figure 13: Cu^{2+} induced oxidation of HDL
Maternal and fetal HDL (50 protein µg/ml) were oxidized in the presence of 1µM Cu2+, 200min at 37°C as described in Methods. Continuous (maternal HDL) and dotted line (fetal HDL) represents the mean of six individual HDL isolations (A). The time course of HDL oxidation was monitored by the change in absorbance at 234nm. Tlag, Tmax and the maximal amount of conjugated dienes formed at Tmax are shown in inserted Table (B). * *p<0.05*

3.2. Maternal gestational diabetes mellitus remodels fetal high-density lipoprotein and impacts its functional diversity

3.2.1. Clinical characteristics and lipid profile of the study cohort

The clinical characteristics of the study subjects are shown in Table 3. Although controversially discussed as a predictor of metabolic characteristics (135) pre-pregnancy BMI and BMI at childbirth of the mothers in both groups showed no significant differences. The women in the GDM group (n=9) were well controlled since HbA1c levels at term were around 6.0 ± 0.6%. For comparison control HDL was obtained from normolipidemic, control pregnant women at term and from corresponding umbilical cord plasmas described in detail above (n=11) (in further text defined as control group). Mean CRP values ranged from <0.6 to 5.8 ± 4.5 mg/L in control fetuses and mothers, respectively, similar to published values (130) and are considered low, while in GDM CRP levels reached 4.03 ± 7.0 mg/l in the fetal and 45.8 ± 21.2 mg/l in the maternal circulation. HbA1c levels strongly correlated with maternal CRP levels in GDM (r^2=0.881, p=0.002). When the maternal and fetal plasma lipid profiles were compared, total cholesterol, TG and PL concentrations were considerably lower in fetal samples consistent with previous results (85). In the GDM group total cholesterol, TG and PL levels in mothers and fetuses were not changed further indicating that glycemic control for GDM subjects was appropriate.

3.2.2. Fetal HDL protein cargo is altered in GDM

To determine the protein differences between maternal and their correspondent fetal HDL in control and GDM subjects, we used shotgun proteomics after tryptic digests of HDLs. In our initial analysis 37 different proteins associated with HDL were identified by shotgun proteomic analyses (Table 7) in accordance with recent literature (10)(85). Thirty-five proteins were already described above, as part of control, maternal and fetal HDL protein cargo (136), while complement C3 and hemopexin were two proteins additionally detected. Complement C3 plays a central role in the activation of the complement system (137) and, therefore, could contribute to an acute phase-response. Hemopexin is a high-affinity transport protein for heme. It scavenges the heme released or

lost by the turnover of heme proteins such as hemoglobin and thus protects the body from the oxidative damage that free heme can cause (138).

	Maternal HDL	Maternal GDM HDL	P	Fetal HDL	Fetal GDM HDL	p
Albumin	81.9 ± 51.8	103.8 ± 55.7	0.305	272.4 ± 65.5 $^{\#}$	162.2 ± 78.4 $^{\$}$	0.214
Alpha-1-acid GP	0.5 ± 0.9	Nd	0.446	0.4 ± 1.1	nd	0.302
Alpha-1-antitrypsin	7.4 ± 5.7	8.9 ± 8.2	0.320	**19.0 ± 5.5**	**7.7 ± 2.5**	**0.021**
Alpha-2-HS-GP	0.6 ± 1.0	0.7 ± 1.1	0.795	6.7 ± 2.0 $^{\#}$	4.7 ± 7.3 $^{\$}$	0.964
ApoA-1	546.7 ±74.9 $^{\#\#}$	594.7 ± 187.6	0.568	404.1 ± 31.4	478.2 ± 245.2	0.164
ApoA-2	134.0 ± 42.0	125.8 ± 32.2	0.137	124.5 ± 47.7	118.3 ± 61.8	0.343
ApoA-4	**Nd**	**1.4 ± 1.5**	**0.044**	0.9 ± 0.2 $^{\#}$	0.5 ± 1.0	0.922
ApoB-100	14.6 ± 14.6	25.1 ± 35.9	0.567	3.7 ± 4.4	3.9 ± 5.8	0.152
ApoC-1	27.9 ± 5.6 $^{\#}$	29.6 ± 15.7	0.819	21.0 ± 2.9	27.1 ± 16.4	0.273
ApoC-2	18.3 ± 1.6 $^{\#}$	19.7 ± 8.7	0.493	15.4 ± 3.8	21.2 ± 9.0	0.178
ApoC-3	52.9 ± 19.3 $^{\#\#}$	38.7 ± 23.9	0.518	29.5 ± 2.6	30.6 ± 18.6	0.594
ApoC-4	4.0 ± 1.1 $^{\#}$	0.9 ± 1.3 $^{\$}$	0.472	0.3 ± 0.1	nd	0.453
ApoD	8.0 ± 2.8 $^{\#}$	7.5 ± 2.7	0.492	6.4 ± 2.2	6.1 ± 3.2	0.824
ApoE	22.4 ± 2.7	27.5 ± 18.2	0.703	**49.6 ± 9.0** $^{\#}$	**78.1 ± 26.4** $^{\$}$	**0.046**
ApoF	2.5 ± 0.9 $^{\#\#}$	1.7 ± 2.0 $^{\$}$	0.333	nd	nd	1.000
ApoL	5.3 ± 1.8 $^{\#\#}$	1.8 ± 1.5 $^{\$}$	0.137	nd	nd	0.241
ApoM	**3.6 ± 1.7**	**2.3 ± 1.1**	**0.033**	**3.7± 1.8**	**2.7 ± 1.1**	**0.035**
ApoH	Nd	Nd	1.000	0.5 ± 0.7	nd	0.084
Complement C3	0.2 ± 0.4	0.9 ± 2.0	0.396	0.1 ± 0.3	nd	0.097
Gfi-1	Nd	Nd	1.000	0.9± 1.2	nd	0.136
DOCK9	**1.7 ± 2.4**	**Nd**	**0.011**	**1.5 ± 1.8**	**nd**	**<0.001**
Fibrinogen alpha	**2.1 ± 2.7**	**Nd**	**0.025**	0.9 ± 1.5	0.5 ± 0.9	0.364
Hemoglobin γ	0.5 ± 0.3	4.0 ± 11.6	0.802	18.3 ± 8.6 $^{\#\#}$	8.9 ± 7.7 $^{\$\$}$	0.540
Hemoglobin α	0.4 ± 0.1	5.0 ± 15.0	0.760	18.1 ± 6.8 $^{\#\#}$	8.6 ± 7.7 $^{\$\$}$	0.941
Hemopexin	0.3 ± 0.6	0.7 ± 0.7	0.304	nd	nd	0.779
IGHG1	0.3 ± 0.3	0.3 ± 0.8	0.942	5.2 ± 1.2 $^{\#}$	1.4 ± 1.2 $^{\$}$	0.162
IGKC	0.2 ± 0.6	Nd	0.826	**1.5 ± 2.0**	**nd**	**0.025**
IGLC2	0.2 ± 0.2	Nd	0.759	**1.2 ± 0.8** $^{\#}$	**nd**	**0.026**
MORC 3	1.0 ± 2.1	1.4 ± 3.1	0.321	1.1 ± 2.1	1.8 ± 3.0	0.198
Platelet basic protein	**1.3 ± 0.7**	**Nd**	**0.018**	4.0 ± 1.1 $^{\#}$	2.2 ± 2.5 $^{\$}$	0.264
Platelet factor 4	Nd	Nd	1.000	1.2 ± 0.4 $^{\#}$	0.2 ± 0.4 $^{\$}$	0.215
Prothrombin	**1.4 ± 1.3**	**0.7 ± 1.8**	**0.037**	**1.0 ± 1.8**	**nd**	**0.019**
SAA1	**0.6 ± 0.1**	**11.1 ± 3.3** $^{\$}$	**0.028**	**nd**	**4.2 ± 2.4**	**0.006**
SAA4	10.9 ± 9.0	12.9 ± 8.8	0.517	9.6 ± 8.6	13.7± 9.9	0.127
Serotransferrin	0.6 ± 1.0	2.3 ± 3.0	0.169	3.6 ± 4.5	2.9 ± 3.6	0.631
Serum paraoxonase 1	**7.3 ± 1.8** $^{\#\#}$	**2.1 ± 1.2** $^{\$\$}$	**0.008**	**1.0 ± 0.4**	**nd**	**0.033**
Vitamin D-BP	0.1 ± 0.0	Nd	0.827	2.0 ± 1.0 $^{\#}$	nd	0.130

Table 7: Average number of peptides identified for each HDL-associated protein
Data are mean number of spectral counts ± sd per subject. ***p<0.01, *p<0.05* for differences control vs GDM HDL; *##p<0.01, #p<0.05* for differences maternal vs fetal, control HDL; *$$p<0.01, $p<0.05* for differences maternal vs fetal, GDM HDL. nd = not detectable; dedicator of cytokinesis 9 (DOCK9), growth factor independent protein 1 (Gfi-1), microrchidia3 (MORC3), Ig gamma-1 chain C region (IGHG1), Ig kappa chain C region (IGKC) , Ig lambda chain C regions (IGLC2)

Figure 14 emphasizes differences in average number of proteins identified on GDM and control HDL. Maternal GDM HDL was carrying less apoM (p=0.033), fibrinogen α (p=0.025), platelet basic protein (p=0.018), prothrombin (p=0.037) and PON1 (p=0.008), while apoA-4 (p=0.044) and SAA1 (p=0.028) have become more abundant (Figure 14A). Similar changes were seen on the fetal side. Fetal GDM HDL was enriched with SAA1 (p=0.006) and apoE (p=0.046), while α-1-antitrypsin (p=0.021), apoM (p=0.035), Ig kappa chain region (IGKC) (p=0.024), Ig lambda chain C regions (IGLC2) (p=0.026), prothrombin (p=0.019) and PON1 (p=0.033) were lacking (Figure 14B). Importantly, neither maternal nor fetal apoA-1 levels were altered in GDM.

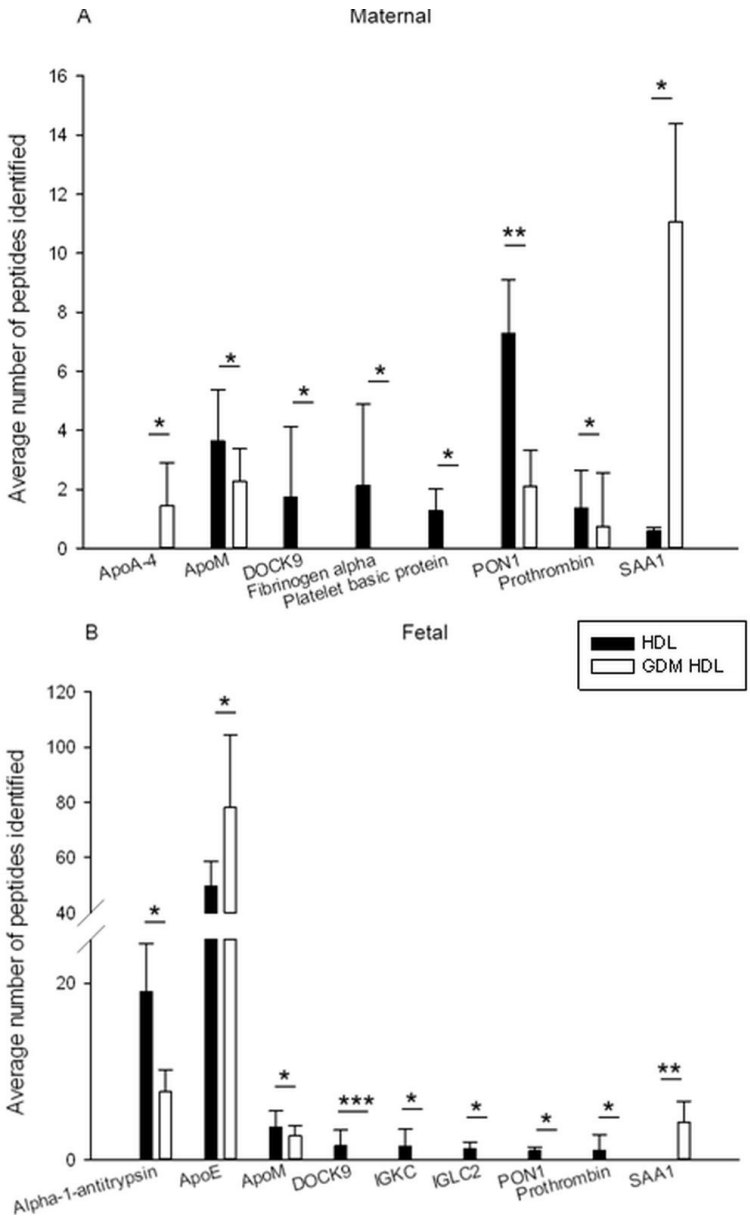


Figure 14: HDL proteome differences between control and GDM pregnancies
Peptides differently associated with HDL in control and GDM pregnancies in the maternal (A) and fetal (B) total HDL fraction. **p<0.05, **p<0.01, ***p<0.001*; dedicator of cytokinesis (DOCK 9)

To confirm the proteomics results by an alternative method, HDL-associated proteins were selected based on their particular role in maternal and fetal lipid metabolism. These proteins were quantified by immunoblotting of three representative, individual maternal and fetal HDLs. The bands were subsequently quantified by densitometric estimation of bands specific for apoA-1 (29kDa), for apoE (38kDa), for PON1 (40 kDa) and for SAA1 (14kDa) (Figure 15). ApoA-2 could be used as loading control, since its average number of peptides was not statically different between control and GDM HDL. Immunoblot semi-quantitative analyses of apoE, PON1 and SAA1 confirmed the results obtained by MS peptide counting, while apoA-1 levels were lower. ApoA-1 HDL concentration was also decreased when measured by immunoturbidimetry (Table 8).

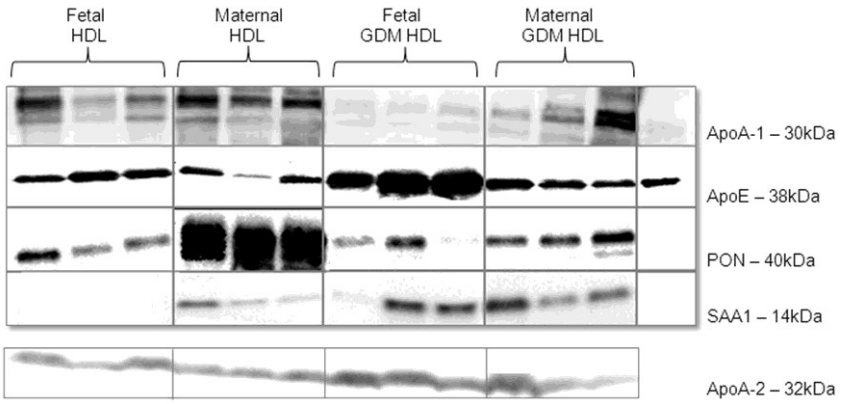


Figure 15: Immunoblot of proteins associated with HDL
To validate the results obtained by LC-MS/MS maternal and their corresponding fetal HDLs from control and GDM pregnancies were subjected to immunoblot analyses. ApoA-2 was used as loading control, since its average peptides counted were similar in GDM and control samples (see Table 7).

	Maternal		Fetal	
Characteristics of HDL	**HDL**	**GDM HDL**	**HDL**	**GDM HDL**
Cholesterol (mg/mg protein)	0.81 ± 0.23 *	0.57 ± 0.15	0.78 ± 0.24	0.64 ± 0.37
Triglycerides (mg/mg protein)	0.27 ± 0.17 ###	0.37 ± 0.13 $	0.08 ± 0.01	0.11 ± 0.03
Non-esterified cholesterol (mg/mg protein)	0.13 ± 0.06	0.14 ± 0.02	0.27 ± 0.09 #	0.23 ± 0.15 $
Esterified cholesterol (mg/mg protein)	0.68 ± 0.18 *	0.43 ± 0.12	0.51 ± 0.15	0.41 ± 0.23
Phospholipids (mg/mg protein)	1.41 ± 0.42	1.27 ± 0.29	1.42 ± 0.39	1.22 ± 0.48
ApoA-1 (mg/mg protein)	2.21 ± 0.58 ##	1.35 ± 0.47 *	1.19 ± 0.31	1.04 ± 0.36
CETP mass (µg/ml)	3.75 ± 1.13 ***	1.50 ± 0.65	3.05 ± 1.52 ***	1.57 ± 0.86
CETP activity (pmol/µl/30 min)	97.95 ± 22.4 ###,***	57.49 ± 26.97 $$$	51.39 ± 13.45 *	32.24 ± 11.45
CETP activity (nmol/µg protein/30 min)	29.42 ± 8.30	33.11 ± 13.16	18.64 ± 7.68	28.59 ± 18.27
PON1 (nmol/min)	75.12 ± 18.21 ###,**	34.65 ± 9.45 $$	13.88 ± 3.34 *	5.6 ± 2.56

Table 8: HDL characteristics
****p<0.001, **p<0.01, *p<0.05* differences control vs GDM HDLs; *###p<0.001, ##p<0.01, #p<0.05* differences maternal vs fetal, control HDL; *$$$p<0.001, $$p<0.01, $p<0.05* between maternal vs fetal, GDM HDL

3.2.3. Apolipoprotein distribution remodels fetal HDL

Lipid concentrations of maternal and fetal, control and GDM, HDLs are shown in Table 8. While HDL TGs were lower in control fetal HDL, PL levels were about similar, whereas non esterified cholesterol was higher in fetal as compared to maternal HDL, respectively. Interestingly, all these differences remained in GDM, but were less pronounced. GDM was associated with an enrichment of maternal and fetal HDL with TG paralleled by decreased total HDL-cholesterol levels ($p<0.05$). Notably, apoA-1 HDL concentration was decreased in GDM vs control controls when measured by immunoturbidimetry.

As already shown by native gel electrophoresis, fetal HDL migrates slower than maternal HDL because of its larger size. In GDM the proteome and lipid modifications enlarged both maternal and fetal HDL particles resulting in slower migration on the gel. As a consequence of higher apoE expression (GDM HDL particles were larger compared to control HDL (Figure 16). Fetal HDL size increased from 11.4 ± 0.2 nm to 12.6 ± 0.2nm, while maternal HDL diameter changed from 9.6 ± 0.3nm to 10.3 ± 0.3nm. The altered protein composition led to a slower migration of GDM HDL.

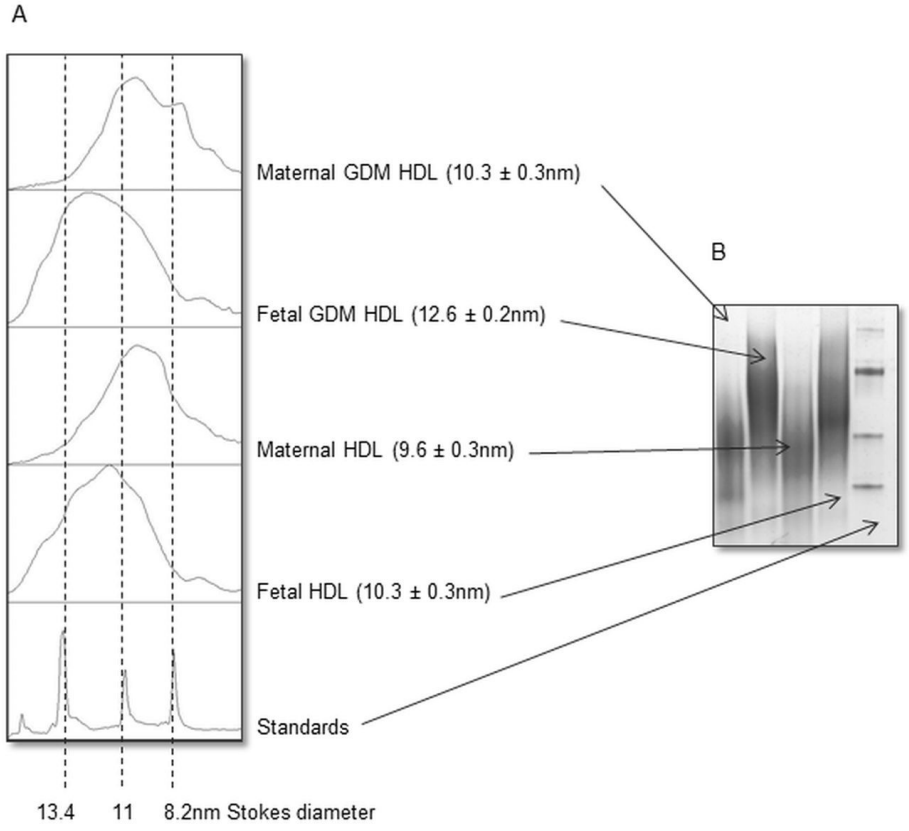


Figure 16: Effect of GDM on the remodeling of maternal and fetal HDL shown by densitometric scanning of native gradient gel electrophoresis gels

A) Densitometric scanning. The numbers represent mean ± sd of the Stokes diameter. B) Representative gel of 3 independent experiments. Standard proteins used were lactate dehydrogenase (8.2nm), B-phycoerythrin (11.0nm, Ficner et al., 1992), apoferritin (13.4nm, de Haen, 1987).

3.2.4. CETP levels and activity in GDM group

CETP mass ($p<0.001$) and activity were diminished in maternal ($p<0.001$) and fetal GDM plasma ($p<0.05$) as compared to control plasma (Table 8).

3.2.5. Anti-oxidative capacity of HDL is altered in GDM

Maternal HDL associated PON1 protein content was 3.5-fold lower in GDM than in the control controls (2.12 ± 1.2 vs 7.3 ± 1.8, $p<0.01$) (Table 8) associated with a lower PON1 activity (maternal GDM: 34.65 ± 9.45 nmol/min; maternal control: 75.12 ± 18.21 nmol/min, $p<0.001$; Table 7). Fetal PON1 was not detectable as part of the HDL proteome in GDM due to threshold of a minimum of 4 spectral counts for the protein to be quantified, while PON1 activity was still measurable in fetal GDM serum (fetal GDM: 5.6 ± 2.56 nmol/min; maternal control: 13.88 ± 3.34 nmol/min, $p<0.05$).

To examine whether lower PON1 activity affects the anti-oxidative capacity of fetal HDL, we measured the total anti-oxidant capacity of maternal and fetal HDLs from six control and GDM pregnancies (Figure 17). TAC of control, fetal HDL was lower than maternal ($p<0.05$) in line with our previous results demonstrating lower anti-oxidative capacity of fetal as compared to maternal HDL. In GDM a tendency for reduced TAC of maternal and fetal HDL was seen without reaching statistical significance.

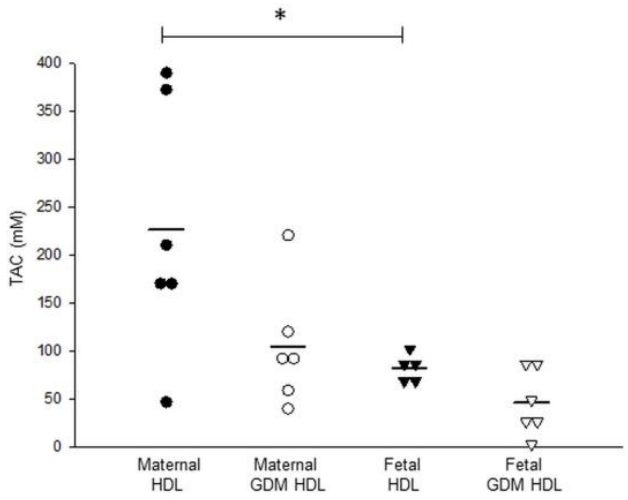
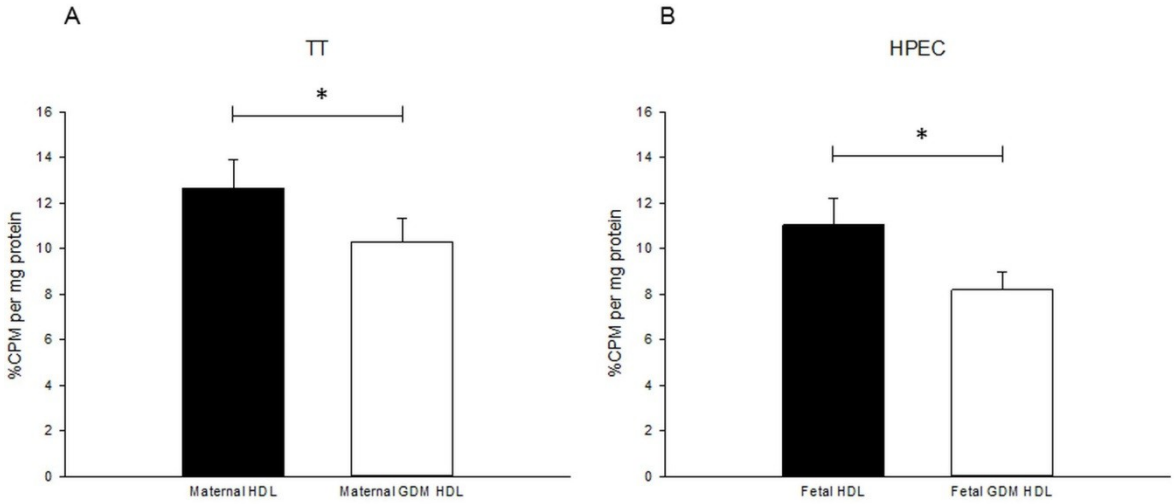


Figure 17: Altered anti-oxidative properties of HDL in GDM
Total anti-oxidative capacity (TAC) of HDL was quantified in maternal and corresponding fetal HDL from control and GDM pregnancies (n=6). *$p<0.05$

3.2.6. GDM decreases HDL efflux capability

The efflux rate of free cholesterol from term trophoblasts to maternal GDM HDL was 25% lower when compared to maternal health, GDM (*p<0.05*) (Figure 18A). Efflux capacity of maternal HDL positively correlated with the specific PON1 activity (r^2=0.677, p=0.045), TAC (r^2=0.865, p=0.026), apoA-1 (r^2=0.803, p=0.009) and apoE (r^2=0.684, p=0.042) (Figure 18C). Also in the fetal compartment fetal HDL from GDM was a less efficient cholesterol acceptor from human placental endothelial cells compared to control fetal HDL (*p<0.05*) (Figure 18B).



C

	efflux	
	r^2	p
PON1 (nmol/min/sc)	0.677	0.045
TAC (mM)	0.865	0.026
ApoA-1 (sc)	0.803	0.009
ApoE (sc)	0.684	0.042

Figure 18: GDM attenuates HDLs capacity to promote cholesterol efflux from trophoblasts and feto-placental endothelial cells
Cholesterol efflux to HDL from A) human term trophoblasts (TT) was measured using maternal HDL and from B) human placental endothelial cells (HPEC) using fetal HDL each isolated from control and GDM pregnancies (n=3). Data are mean ± sd of 3 different cell preparations performed in triplicate wells. *$p< 0.05$ C) Efflux capacity of maternal HDL correlated positively with PON1 activity, total anti-oxidative capacity of the HDL particle and peptide number of apoA-1 and apoE detected as part of maternal HDL proteome.

3.3. Gestational diabetes mellitus impairs fetal HDL-apoM-S1P vasoprotective action on the feto-placental endothelium

3.3.1. Fetal HDL is the major carrier of apoM-S1P complex

We previously described that apoM is a part of fetal HDL proteome like it is of a maternal HDL and that GDM attenuates association of apoM with fetal and maternal HDL. Figure 19A summaries the proteomics apoM analysis of maternal and fetal GDM HDL compared to control HDL (Figure 19A). Moreover results were confirmed independently by immunoblotting. Since apoM anchors S1P it is comprehensible that fetal HDL indirectly also associates with S1P (Figure 19B). Concentration of S1P per mg of fetal HDL protein is 0.332 ± 0.255 nM while in GDM it drops to 0.177 ±0.097 nM ($p<0.05$) which suggests S1P is directly related to HDL in normal and GDM pregnancies. Control fetal HDL-S1P correlated positively with apoM levels associated to HDL (r^2=0.923, p=0.001), while this correlation is absent in GDM. Opposite to S1P-HDL levels, concentration of total, circulating S1P in GDM was increased (0.568 ± 0.167 μM) when compared to S1P concentration in control, fetal serum (0.396 ± 0.107 μM, p<0.05).

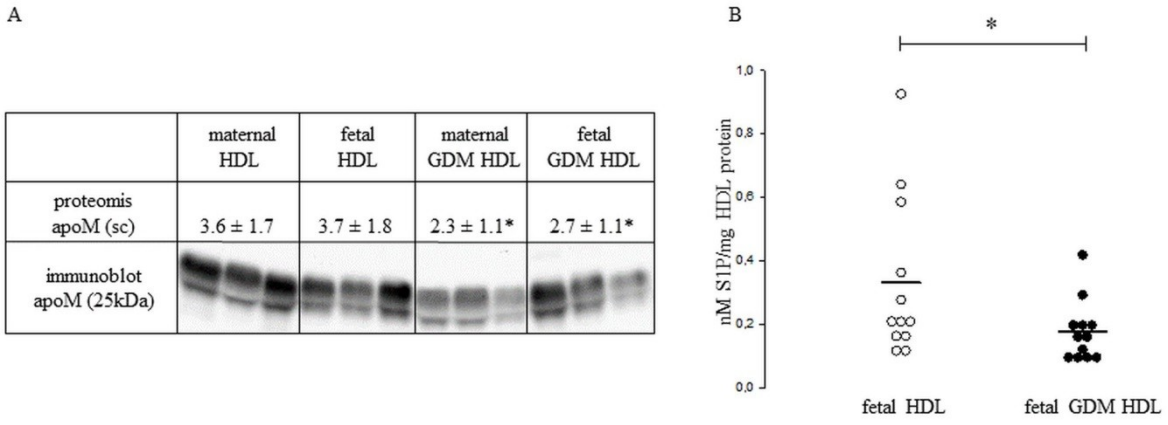


Figure 19: ApoM determines S1P levels
A) Maternal and fetal HDL associates with similar protein levels of ApoM, while GDM HDL carries less apoM. Analyses performed by proteomics (upper table, n=9) were confirmed by immunoblots (lower picture, n=3) B) S1P concentration on control and GDM fetal HDL (n=12) was determined by ELISA and expressed relative to total protein. Solid grey line represents the mean.

3.3.2. GDM diminishes fetal HDL protective effect on feto-placental endothelium

The S1P binding ability of apoM has biological impact. ApoM/S1P containing HDL is important to preserve endothelial function (61). To examine the effect of fetal HDL on the feto-placental endothelium, transendothelial electrical resistance measurements of HPAEC were performed. Barrier integrity of HPAEC was increased after treatment with S1P associated to BSA, fetal control and GDM HDL, when compared to control (medium – 5% FCS in EBM) (Figure 20A). Resistance of endothelial barrier, induced by fetal HDL, was higher over all time points then resistance achieved with fetal GDM HDL and S1P-albumin. Calculations of Δimpedance, which represents difference in impedance before and after treatment (Figure 20B), revealed that fetal GDM HDL could achieve only 75% of control, fetal HDL effect ($p<0.01$). These differences remained 1, 2 and 3 hours after treatment.

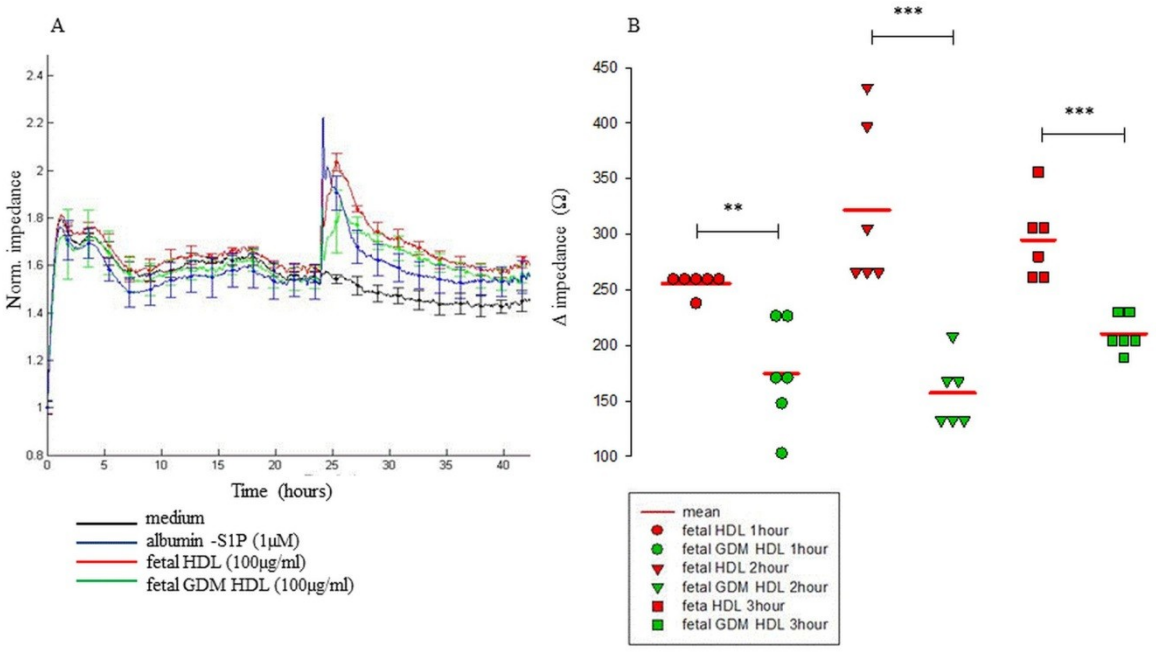


Figure 20: GDM diminishes permanently fetal HDL protective effect on feto-placental endothelial cell barrier integrity

A) Transendothelial electrical resistance measurements of HPAEC were performed with an electrical cell–substrate impedance sensor (ECIS). Cells were seeded and left for 24h until they reached resistance steady state. Cells were treated with 100μg/ml HDL protein (fetal control and GDM) and 1μM S1P associated to BSA in 5% FCS EBM, in duplicates. Graph shown is representative of three different independent experiments. Medium - 5% FCS in EBM B) Δimpedance shows differences in resistance measured before and one, two or three hours after treatment. Data shown is obtained from three different independent experiments. **p<0.01; ***p<0.001*

S1P ligation with its receptors elicits a range of cell-type-specific adhesive and motility responses. For example, when administered to endothelial cells, HDL with S1P stimulates assembly of adherens junctions, induces formation of actin stress fibers and cortical actin (139), and promotes endothelial cell migration better than apoM-free HDL (140). To test effect of fetal HDL on cytoskeletal rearrangement of arterial endothelial cells of placenta, cells were treated with S1P-albumin, fetal control and GDM HDL for 10 min, fixed, permeabilized, and stained with phalloidin Texas Red for F-actin staining. As shown in Figure 21A, when cell were treated with medium actin fibers were diffusely arranged in the cells. After incubation with S1P-albumin and fetal HDL, cells were wide spread and actin fibers rearranged to form prominent cortical ring (arrows pointing in Figure 21B and C). In contrast, cells were more round shaped when fetal GDM HDL was applied and, while actin fibers showed some reorganization, formation of cortical ring was not as pronounced (Figure 21D).

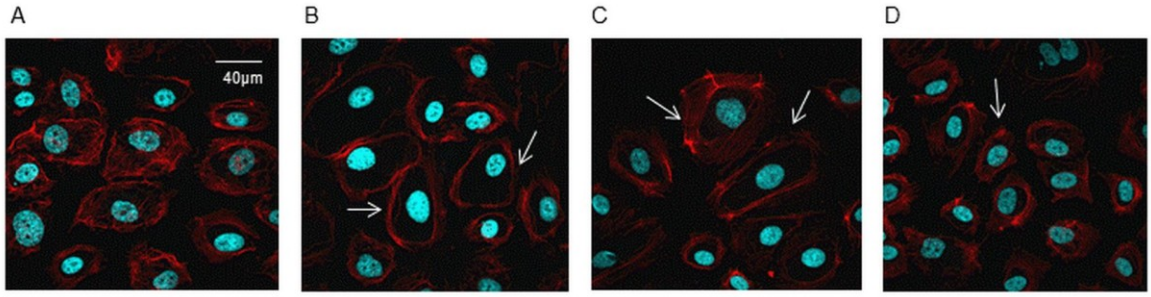


Figure 21: Assembly and organization of F-actin fibers in HPAEC after treatment with different fetal HDL
Cells (75.000/well) were cultured on 1% gelatin coated slides for 24h. After several washing steps cells were incubated with vehicle control (A), 1µM S1P-BSA (B), 100µg/ml fetal control (C) and GDM HDL (D) for 15min, fixed, permeabilized, and stained with phalloidin Texas Red for F-actin staining (red fibers). Nuclei were stained with DAPI (blue). Scale bar 40µm

S1P acts as a potent chemoattractant for endothelial cells, which is essential for wound-healing response and angiogenesis (139,141). S1P-BSA, fetal control and GDM HDL stimulated chemotaxis of HPAEC (Figure 22). Fetal HDL induced migration of 4549.1 ± 629.3 cells, while 3155.2 ± 715.8 cells migrated towards fetal GDM HDL ($p<0.001$). Interestingly, S1P-albumin was able to cause migration of 1751.0 ± 284.8 cells which represents only 1/3 of cells migrated to fetal HDL ($p<0.001$).

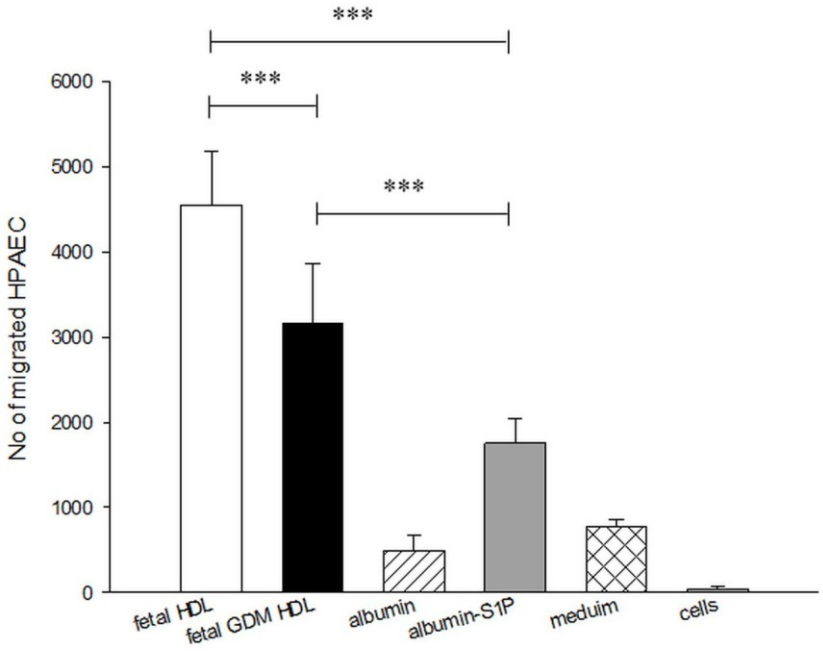


Figure 22: Induction of migration HPAEC by fetal HDL is impaired by GDM
HPAEC were serum starved for 3h and subjected to migration assay with 1 µM S1P-BSA, 100µg/ml protein of fetal, control (n=6) and GDM HDL (n=6). After 4h migration period HPAEC were washed, fixed and stained with DAPI. Pictures were taken from each well of the filter and cells were quantified using Dot count software. Data are mean ± SD and showing one representative of three experiments performed in triplicates. ****p <0.001*

3.3.3. The protective effect on feto-placental endothelial barrier integrity of fetal HDL is mediated by S1P and mainly S1PR1 dependent

Since recently published data indicating that apoM is important for delivery of HDL-bound S1P to S1PR1, we were interested to check if fetal HDL promotes placental endothelial barrier enhancement via S1P signaling dependent on the S1P1 receptor. To answer this question HDL-S1P was firstly specifically hydrolyzed by SPL. After one hour incubation of fetal HDL with SPL approximately 50% of HDL-S1P was depleted (Figure 23A). Displacement of S1P did not affect size of the hydrodynamic radius of fetal HDL particles which was estimated by densitometric scanning of native gradient gel electrophoresis gel (Figure 23B, n=3). Due to the lower S1P concentration, depleted HDL attenuated permeability of HPAEC barrier up to 80% when compared with corresponding, not-depleted HDL (Figure 23C and D). SPL alone did not have any effect on the cells integrity.

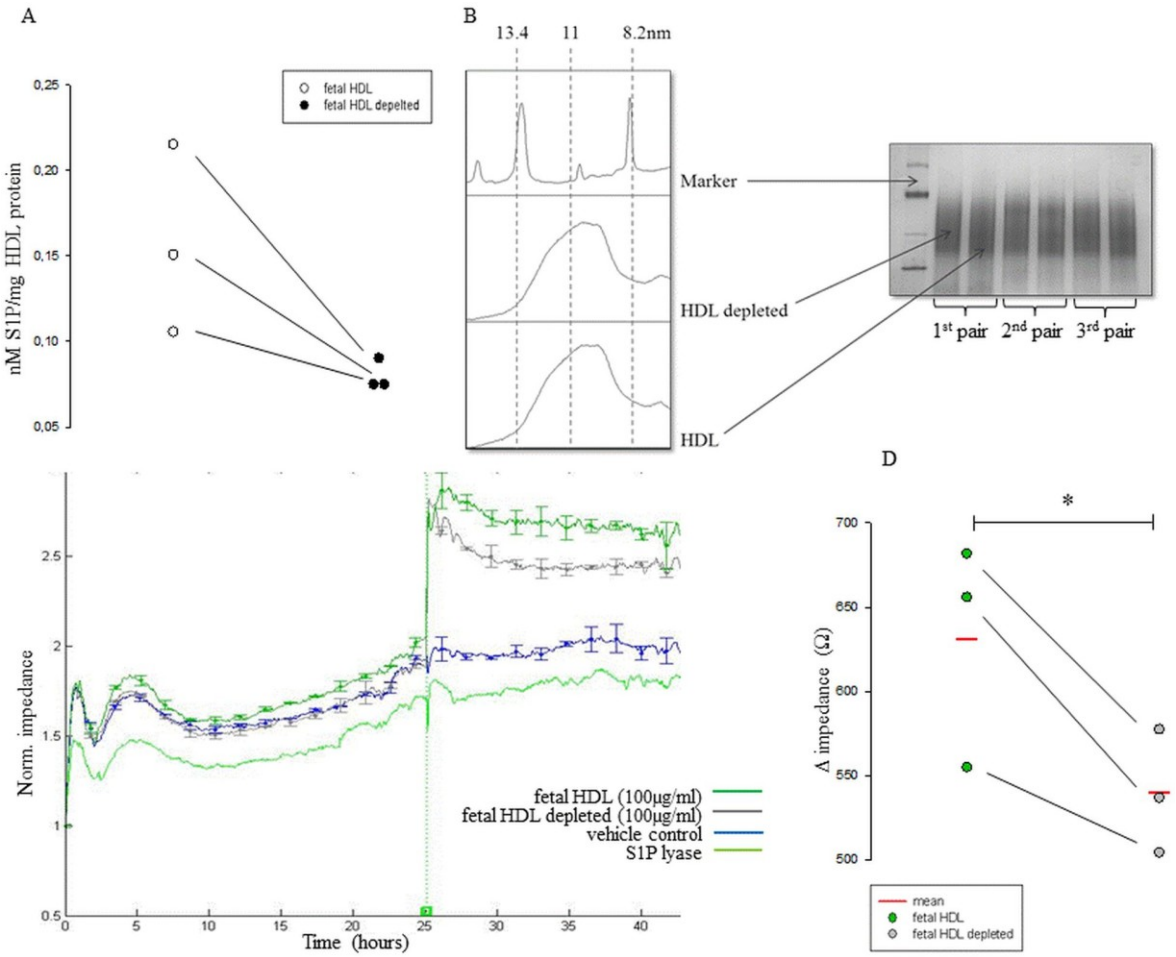


Figure 23: S1P depletion of fetal HDL by S1P lyase (SPL)
A) Three deferent fetal HDL were incubated with SPL at 37ºC for 60min (1nmol HDL-S1P was treated with 5µg human active SPL). S1P concentration was estimated with ELISA. Black lines are connecting S1P concentrations of the same fetal HDL before and after incubation with SPL. B) Effect of S1P depletion on the remodeling of fetal HDL shown by densitometric scanning of native gradient gel electrophoresis gel. C) ECIS measurements of HPAEC treated with 100µg/ml protein of control and corresponding S1P depleted HDL in duplicates, and with SPL (same amount used for depletion). Graph shown is representative of three different experiments. D) Δimpedance shows differences in resistance measured before and one hour after treatment. Data shown is obtained from three different experiments. Black lines are connecting Δimpedance measurements of control and corresponding S1P depleted fetal HDL. *$p<0.05$

In further set of experiments, HPAEC were firstly pre-treated with selective S1PR1 antagonist W146 (Figure 24A) or with S1PR1 and S1PR3 antagonist VPC23019 (Figure 24C) for 30min and then treated with S1P-albumin and fetal HDL. Calculations of Δimpedance showed that effects of both molecules were completely abolished when S1PR was blocked (Figure 24B and D). Importantly, it seems that protective effect on placental endothelial barrier integrity of fetal HDL is mostly S1PR1 dependent due to the observation that both antagonists alone caused a similar, huge drop of the impedance. In addition, relative S1PR1 mRNA levels were 3-fold higher ($p<0.001$) than S1PR3 mRNA levels in HPAEC (Figure 25) which argues for the previous statement.

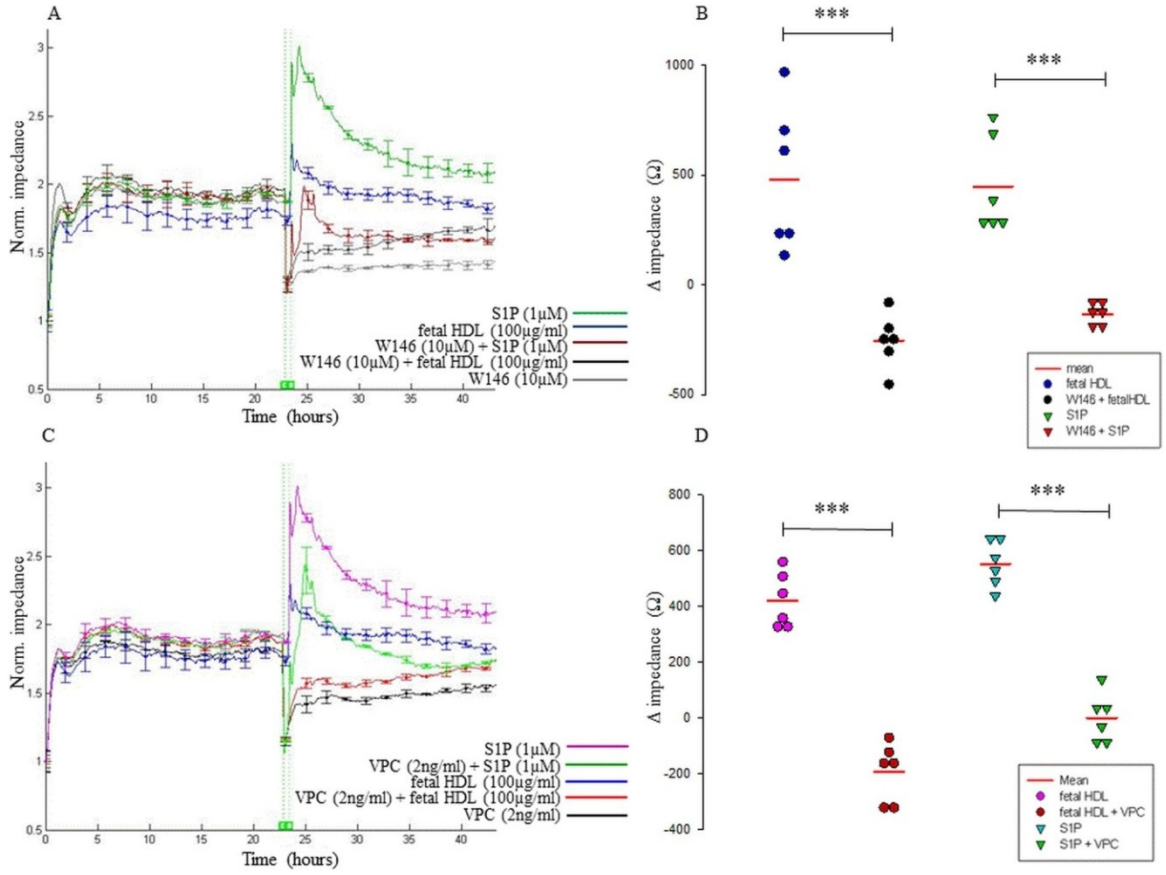


Figure 24: Fetal HDL increases barrier integrity of HPAEC via S1PR1
Cells were firstly pre-treated with selective S1PR1 inhibitor – W146 (10µM) (A) or with S1PR1 and 3 antagonist – VPC 23019 (2ng/ml) (C) for 30min and then with 100µg/ml fetal HDL protein and 1µM S1P-BSA, in duplicates. Graph shown is representative of three different experiments. B), D) Δimpedance shows differences in resistance measured before and one hour after treatment. Data shown is obtained from three different independent experiments. ****p<0.001*

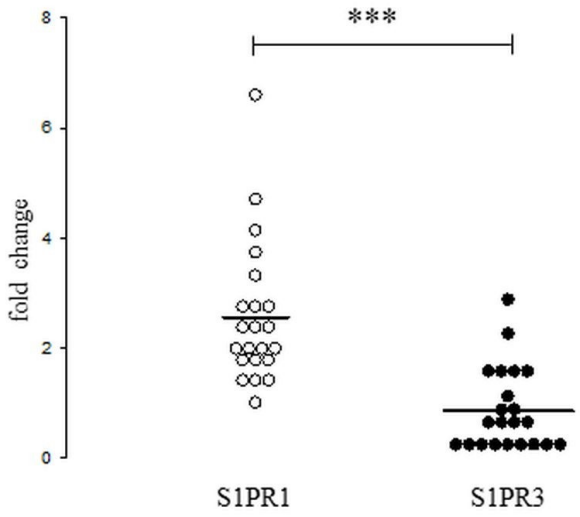


Figure 25: Relative expression of main S1P receptors on feto-placental endothelium
Dot plot shows 2.8 times higher fold change of S1PR1 expression compared to S1PR3 calculated from 22 separate isolated HPAEC. Receptor expression is normalized to expression in the whole term placenta tissue and set as 1.

4. DISCUSSION

4.1. Distinct composition of human fetal HDL attenuates its anti-oxidative capacity

In pregnancy HDL is present not only in the maternal but also in the fetal circulation. Its role in RCT is established in both circulations (96,142,143) and in fetus HDL is the main cholesterol carrying lipoprotein (85). While it is appreciated that fetal HDL differs from maternal HDL by its higher apoE content (142), nothing is known about the full HDL proteome in both circulations and the associated functions. However, blood lipoprotein profile in early life are known to be related to and predictive of those in adults, but little is known about these determinants (77). Here we employed shotgun proteomics to test the hypothesis that fetal HDL carries proteins at different amount than maternal HDL, which contributes to distinct HDL functionality in both circulations. Based on our results, we propose that fetal HDL composition is accompanied by changes in HDL function, as reflected by its impaired anti-oxidative capability, and by fewer proteins related to innate immunity. To the best of our knowledge this is the first study to compare HDL-particles isolated from individual mothers and their corresponding neonates.

In general, each apolipoprotein except apoE and apoA-2, was significantly lower on fetal than maternal HDL, while apoF and apoL were not even detectable. Studies have already shown that cord blood levels of apoE and apoA-2 were similar to those in adults (68,144). ApoE is a key HDL-apolipoprotein which enables binding of HDL to cellular receptors of the LDL-receptor family (145). Moreover, apoE rich, large HDL particles are implicated in the RCT pathway as ligands of scavenger receptor class B type-I (SR-BI) (96) and ATP-binding cassette transporter G1 (ABCG1) (146). Also, it has been recently shown that inhibition CETP activity specifically enhances SR-BI and ABCG1-dependent efflux to larger HDL_2 subspecies (147). Taking together, the lower CETP activity and apoE-rich HDL particles, which we observed in the fetal circulation, supports the idea of

an atheroprotective role of fetal HDL in the feto-placental vasculature. This notion is supported by our recently published results, demonstrating that phospholipid transfer protein, a further key enzyme in lipid transfer and cholesterol heomeostasis, also contributes to the remodeling of fetal HDL and increases paticle's efflux capacity (143).

The existence of qualitative differences between maternal and fetal HDL was also corroborated by differences in apoA-4 enrichment of fetal HDL particles. Normally in adults, apoA-4 is carried by lipoprotein classes smaller than typical HDL_3 (148), but here we found an increased size of fetal HDL (Figure 11). Among the many postulated physiological roles of apoA-4, it activates LCAT, an enzyme that converts free cholesterol into the more hydrophobic cholesterylester (149), which is then sequestered into the core of HDL particles. The 4-fold higher LCAT activity in the fetal circulation is consistent with the capacity of apoA-4 to activate the enzyme. Since fetal HDL contains 2-fold higher levels of apoE than adult HDL, apoE may additionally contribute to fetal LCAT activation (150). Structural similarity between apoA-4 and apoA-1 (151) paralleled by significant lower apoA-1 levels and apoA-4 enrichment on fetal HDL, make it tempting to assume that these apolipoproteins may act exchangeable in fetal plasma.

ApoL, co-isolating with dense HDL particles (d=1.21–1.24 g/ml) is a major active protein of the trypanosome lytic factor (TLF) which neutralizes among others the protozoan Trypanosoma brucei brucei (152). Evidence has emerged that HDL, but not immunoglobulin, harbors the cytolytic activity (153,154). Besides apoL, apoA-1, apoA-2, apoC-1, apoC-2, apoC-3 and haptoglobin related proteins (Hrp) are also involved in regulating the TLF activity. Interestingly, fetal HDL is deficient of all of these apolipoproteins except apoA-2. Also PON1, with its anti-oxidant activity, plays also a role in innate immunity. PON1 transgenic flies were protected from Pseudomonas aeruginosa lethality, and protection depends on the lactonase activity of the enzyme (155). Collectively, the distinct apolipoprotein composition along with the lower PON1 activity of fetal HDL leads one to speculate about an effect of maternal HDL on innate immunity but, interestingly, not in the fetus.

The pattern of analyzed proteins, which were statistically elevated on fetal HDL, suggests a particle characteristics expected for the light HDL_2 sub-fraction, while other apolipoproteins enriched on maternal HDL have been described almost exclusively on small dense HDL_3 (156). Indeed, as GGE showed, fetal HDL has a shift towards pre-β

mobility while maternal HDL with its higher apoA-1 levels migrates mainly as α1- and α2-particle. This shift in particle mobility can be explained by the differences in particle size as a consequence of HDL remodeling, by which fetal apoE-rich HDL shifts towards HDL2. This is consistent with observations by others (157,158). Lower CETP activity in the fetal circulation, along with TG poor LDL and VLDL, may account for larger fetal HDL particles, since plasma CETP transfer activity primarily depends on the availability of its substrates, TG rich lipoproteins and HDL, but not on CETP mass (159).

Many studies have indicated that the anti-oxidant function of HDL depends upon apoA-1 and related enzymes, such as PON1 (160). PON1, an enzyme associated with HDL inhibits LDL and HDL oxidation (161). Moreover, PON1 binds directly to apoA-1 associated to HDL; the high affinity binding significantly stabilizes the protein and stimulates the lactonase activity of the enzyme (162). Strikingly, our results revealed that the apoA-1 content of fetal HDL was more than 30% lower than maternal HDL. Moreover PON1 mass and activity were profoundly decreased in fetal HDL. Lack of these anti-oxidative proteins caused higher susceptibility of fetal HDL particles to Cu^{2+}-induced oxidation, as demonstrated by a shorter lag time of oxidation. This is in line with the strong positive correlation of Tlag and Tmax exclusively found with PON1 mass and activity in the maternal circulation. Some proteins (e.g., albumin and apoM) also protect HDL against Cu^{2+}-induced oxidation by binding metal-ions (163-165). It seems likely that these proteins account for remaining anti-oxidative capacity of fetal HDL.

It is noteworthy that we did not observe several complement factors and enzymes that were documented in earlier proteomics studies as being associated with HDL (166-168). We identified apoB in trace amounts on both maternal and fetal HDL, which likely represents contamination (10,49,156). High levels of albumin were found especially on the fetal HDL fraction. Albumin binds free fatty acids and other hydrophobic compounds with high affinity. A recent study isolated albumin biochemically or by affinity chromatography and found apoA-1 associated with it, suggesting that the HDL may interact with albumin (167,168). Importantly, we identified novel proteins as part fetal HDL proteome such as IGHG-1, IGLC-2, hemoglobin α and γ chain.

Pregnancy is a dynamic process over a defined period of time. Here we analyzed the maternal and fetal HDL only at the end of this period i.e. at term of gestation. We do not know whether these changes are already present from the first formation of HDL in the

fetal circulation onward, or are the result of continued modifications and remodeling of fetal HDL over the course of gestation under maternal, placental or fetal influence. Further studies are warranted but will have ethical limitations. Secondly, pregnancy itself has huge impact on lipid metabolism and maternal serum lipid profile. It is likely that those changes affect HDL particles in pregnant women. From the data here one can draw the conclusion that fetal HDL particles differ from those of their pregnant mothers but not from adults in general. To answer this question longitudinal study should be performed in which HDL proteome and function of healthy females should be analyzed before conception, during pregnancy and after it. Finally, we did not find any sex differences in fetal HDL proteome and function, possibly due to the low subject number.

In conclusion (Figure 26), we clearly showed that fetuses exhibit major modifications of their HDL proteome, in addition to the quantitative decrease of HDL-triglycerides and increased HDL-cholesterol levels. When comparing amounts of HDL-associated proteins, 14 proteins, primarily involved in lipid metabolism, in the inflammataory pathway and in innate immunity, appear to be differentially expressed between the mother and the fetus. This unique protein composition of fetal HDL defines its particle size and attenuates its anti-oxidative capacity. As a speculation it may also reduce its protective role in innate immunity.

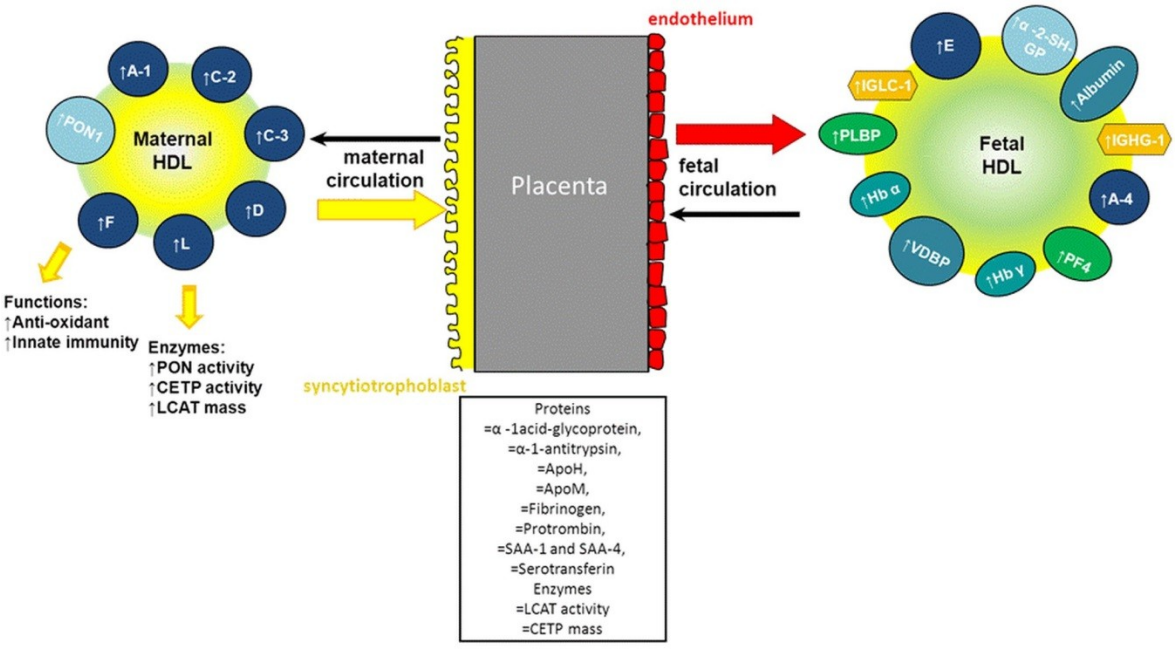


Figure 26: Feto-maternal HDL differences
Scheme emphasizes differences between fetal and maternal HDL in proteome, size and function. Human placenta separates maternal from fetal circulation by syncytiotrophoblasts facing the maternal side, while

endothelial cells are in contact with fetal blood. Fetal HDL is statistically enriched in apoE, proteins involved in coagulation and transport processes, which suggests a particle characteristic for the light HDL_2 sub-fraction. In contrast, proteins that were elevated on maternal HDL (apoL, apoF, PON1, apoD, apoCs) have been described almost exclusively in the dense HDL_3 fraction and relevant to its more pronounced anti-oxidative function and role in innate immunity. PON1 mass and activity, CETP mass and LCAT activity were higher in maternal circulation. Proteins and enzymes listed in square do not differ between circulations. Figure represents a modification of the scheme taken from Hiden et al. 2009 (169) with kind permission of Gernot Desoye. PLBP – ProPlatelet Basic Protein, VDBP – Vitamine D Binding Protein, PF4 – Platelet Factor 4, GP – glycoprotein.

4.2. Maternal gestational diabetes mellitus remodels fetal high-density lipoprotein and impacts its functional diversity

The incidence of GDM is increasing worldwide (170) exposing large numbers of infants to hyperglycemia in utero. This exposure is an important risk factor for fetal and neonatal obesity, which may persist into childhood and even adulthood ultimately increasing the risk for insulin resistance, type 2 diabetes and obesity. Although the association of high maternal glucose levels and offspring complications has been well documented (171), postnatal normalization of maternal glucose levels does not prevent fetal complications . GDM is a condition of low grade inflammation even in lean mothers (172), which may contribute to the risks for the growing fetus. Even more, this usually transient metabolic derangement of the mother can potentially convert in offspring´s later in life into a more permanent abnormality that may increase the likelihood of CVD development (173). In the present study we sought to identify some consequences of the GDM for both mother and fetus and have concentrated on the HDL proteome.

Our results clearly show that the intrauterine environment associated with maternal GDM not only affects the mother but also fetus, ie. fetal HDL. The GDM associated alterations of the fetal and maternal HDL proteome are accompanied by impaired anti-oxidative and anti-inflammatory functions.

In fetus, the availability of many substrates depends on their concentration in maternal circulation and to the extent that they are transported across the placenta (174). Both maternal hyperglycemia and hypertriglyceridemia are frequent in diabetic mothers as a consequence of their augmented insulin resistant conditions, and these changes would enhance the substrate availability to the fetus (175). In our study, glucose levels in GDM mothers were inconspicuous as reflected by HbA1c similar to the control mothers. Also the maternal and fetal concentrations of total cholesterol and TGs were not different. In line with this neither fetal mass nor fetal ponderal index, a proxy measure for fetal fat was increased. Despite this seemingly unaltered intrauterine environment of GDM, GDM caused modifications on maternal and fetal HDL proteome. Seven and eight important proteins were differently associated with maternal and fetal HDL, respectively, in GDM when compared to control HDLs. Remarkably four of these proteins were similarly altered in the maternal and fetal HDL proteome. HDL deficiency of apoM, PON1 and prothrombin in GDM and HDL enrichment with SAA1 was present in both circulations. SAA1 is an

acute-phase reactant, which is increased by 100–1000 fold during acute infection (176). It is essentially associated with HDL in which it can become the major protein by replacing apoA-1. In addition, levels of CRP, another marker of inflammation, correlated positively with HbA1c. This leads us to conclude that the low-grade inflammation associated with GDM in the mothers affects not only maternal HDL composition and function, but also modifies fetal HDL. In vitro the presence of SAA1 on HDL also decreases HDL's protective capacity against oxidation. It was suggested that these changes lead to an HDL particle with impaired atheroprotective potency (177,178). Indeed, PON1 mass and activity were three and two folds decreased, respectively, in maternal GDM HDL, while PON1 was barely detectable in GDM fetuses. Overall, the HDL particles had a lower total anti-oxidant capacity in GDM as compared to control controls. In addition to PON1, LCAT, PAF-AH and HDL associated plasmalogens account for anti-oxidative properties of HDL particle (177) which may represent an explanation for the remaining anti-oxidative capacity.

In general inflammation is accompanied by HDL changes resulting in a reduction of phospholipids and CE and an increase in TG (179,180). We observed similar changes in maternal and fetal HDL in GDM. Increased HDL-TG content can be a consequence of elevated CETP activity normalised per μg of CETP protein. CETP-mediated replacement of CE by TGs in the HDL core results in decreased plasma HDL cholesterol levels, which is a feature of the acute-phase response. In GDM subjects specific CETP activity per μg protein was elevated compared to control controls which might be one of the causes of modifications of the HDL lipid moiety in diabetes.

ApoE, one of the abundant proteins on fetal HDL, was found in higher levels associated with maternal and fetal HDL. ApoE enrichment shifted GDM HDL particles towards pre-β mobility as is shown by GGE. This shift in particle mobility may be explained by differences in particles size as a consequence of GDM-associated HDL remodeling, by which apoE-rich HDL shifts towards HDL_2.

Levels of the major HDL protein - apoA-1 were decreased in GDM when analyzed by immunoturbidimetry and immunoblotting, while proteomics did not reveal differences. These inconsistent observations may be explained by different principles of applied techniques. Biochemical analyses are based on antigen-antibody interaction, which is determined by the affinity between an epitope and a single combining site on the antibody. On the other side LC-MS/MS can identify proteins across a 10,000-fold difference in

concentration with sensitivities down to the nano- to picomole ranges. Furthermore, oxidation of apoA-1 which is more likely seen in GDM, might mask the target epitopes, prevent detection in vitro by antibodies and therefore yield false negative results .(181) GDM is often characterized by endothelial dysfunction with increased formation of reactive nitrogen and oxygen species (182) and decreased anti-oxidant defenses (183) which might cause oxidation of apoA-1. In addition, oxidation of apoA-1 by myeloperoxidase or reactive carbonyls leads to generation of dysfunctional HDL resulting in impaired cholesterol efflux (184). Moreover, advanced glycation end products, as accumulating in the diabetic state, interfere with SR-BI-mediated cholesterol efflux in vitro (185). These findings might represent a possible explanation for the reduced cholesterol efflux to maternal and fetal HDLs from GDM pregnancies. The capacity of HDL to promote cellular cholesterol efflux in an ex-vivo model has been reported to correlate more closely with carotid intima–media thickness than the HDL- cholesterol concentration (20). Accordingly, HDL efflux capability might represent a better parameter than HDL-cholesterol concentrations for estimating the postnatal (cardio)vascular risk.

Both, fetal and maternal GDM HDLs are lacking ApoM, a plasma protein which is mainly bound to HDL and serves as a carrier of sphingosione-1-phosphate (S1P) (61). HDL-associated apoM-S1P complex mediates vasoprotective actions on endothelium through activation of G protein-coupled receptor (186). More studies are necessary to check if lack of apoM on HDL contributes to the endothelial dysfunction in GDM.

Importantly, we identified novel proteins as part fetal HDL proteome in GDM such as complement C3 and hemopexin. C3 levels are increased in the atherosclerotic plaque (187), and elevated plasma levels of C3 are correlated with myocardial infarction (188). Hemopexin binds heme with the highest affinity of any known protein. Therefore, high hemopexin levels indicate significant degradation of heme containing compounds. The specific role hemopexin and C3 as part the HDL proteome in GDM remains to be determined.

Although our studies was carefully designed and performed, some limitations have to be acknowledged. The small volume of cord blood available allowed us only to isolate a limited amount of HDL by density gradient ultracentrifugation. Since we wanted to investigate paired mother-offspring samples as strength and pooling was not an option we were limited only to a small number of samples. A further limitation for the same reason

was that we were not able to compare HDL isolated from arterial and venous cord blood which precluded determination of a potential contribution of the placenta to the distinct composition of fetal HDL.

In conclusion, even well-controlled GDM is associated with HDL-proteome remodeling resulting in altered functional properties of maternal and fetal HDL (Figure 27). Eight proteins involved in lipid metabolism, inflammatory pathway and innate immunity are differentially associated with HDL in GDM compared to control pregnancies. Among these changes in apoM, PON1, SAA1 and prothrombin are similar on both maternal and fetal HDL. The HDL remodeling in GDM is associated with impaired anti-oxidative properties. These findings provide novel insights into potential mechanisms leading to altered (cardio)vascular effects of HDL later in life. Whether these changes are secondary to other processes occurring during disease progression or are accounted for by the HDL particles themselves, remains an important question for the future.

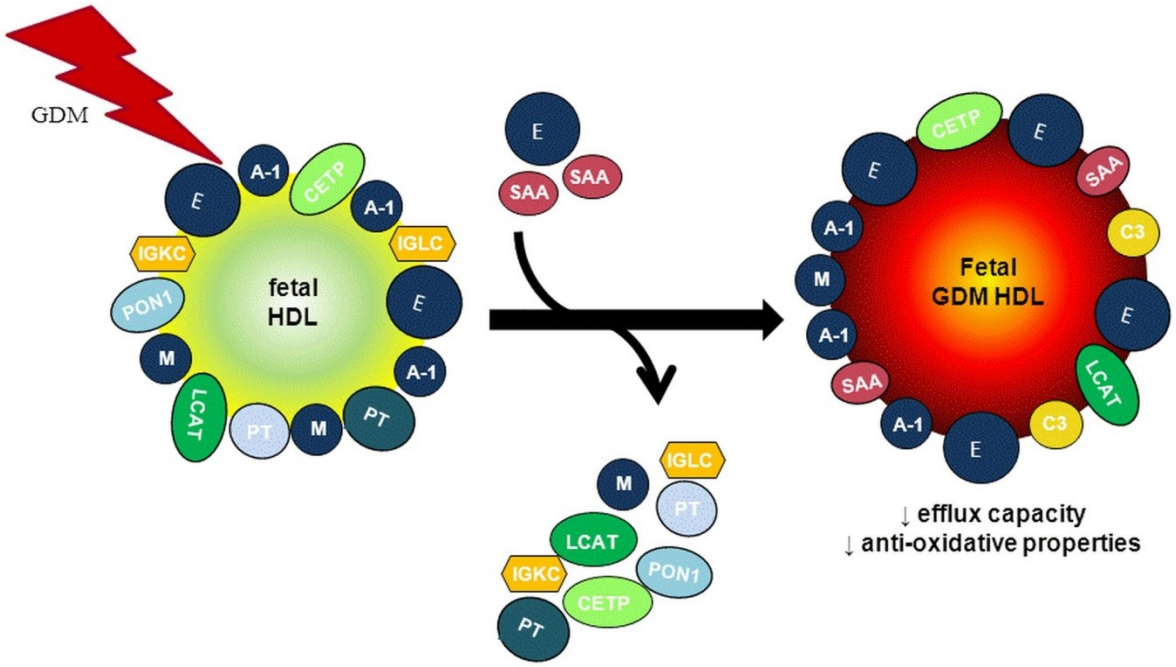


Figure 27: Maternal gestational diabetes mellitus remodels fetal high-density lipoprotein and impacts its functional diversity
HDL should be viewed as a phase whose constituents come and go with distinct half-lives. The remodeling that HDL undergoes is particularly amplified during GDM when SAA becomes a new HDL associated proteins. In parallel, HDL is losing apoM and proteins involved in immune response (IGLC and IGKC), while HDL associated enzymes (PON1, LCAT and CETP) show decreased mass and activity. Alterations occurring in HDL composition and metabolism in GDM are intimately associated with impaired anti-oxidative properties of this particle and its role in RCT.

4.3. Gestational diabetes mellitus impairs fetal HDL-apoM-S1P vasoprotective action on the feto-placental endothelium

Previously it was shown that fetal HDL is unique in respect to its proteome, size and function and under pathophysiological conditions, like in well-controlled GDM, HDL-proteome is sustained, resulting in altered functional properties of fetal HDL. Here we are emphasizing the importance of fetal HDL on mediating feto – placetal endothelial integrity. Results demonstrate clearly that 1) fetal HDL is the carrier of the apoM-S1P complex; 2) fetal HDL maintains migration and barrier stability of feto-placental endothelium predominately by S1PR1 3) that GDM diminishes fetal HDL protective effects on placental endothelium. To the best of our knowledge this is the first human study to describe fetal HDL-S1P effects on placental endothelial cells, supporting a direct role of apoM on diabetes.

The serum S1P concentration in adults it is approximately 0.6μM (189) while in fetal circulation it is 0.4±0.1μM. S1P content of HDL is confined to the apoM-containing particles in human plasma (61). Amount of S1P on fetal HDL strongly correlates with its apoM levels, suggesting that, as in adults (61), fetal HDL is a carrier of S1P through its interaction via apoM and approximately 58% of circulating S1P is bound to fetal HDL.

Recent evidence suggest that S1P may mediate many endothelium associated actions of HDL such as vasodilation, angiogenesis and barrier function, protection against atherosclerosis and ischemia/reperfusion injury (12,190). In endothelial cells, S1P exerts a variety of effects that affect vascular maturation and morphogenesis: it stimulates endothelial proliferation, migration, and angiogenesis, protects against apoptosis and controls vascular permeability (59,62). For a constantly operable function of the vasculature the maintenance of barrier integrity is essential. Barrier permeability is maintained by a complex balance of tethering forces at cell–cell and cell–matrix level as well as intracellular contractile forces mediated by actin and myosin. Fetal HDL increased barrier integrity of human placental arterial endothelial cells in a similar manner as S1P bound to albumin, but the fetal HDL-S1P sustained endothelial barrier function longer than albumin-S1P. Consistent with the observed increase in endothelial cell monolayer integrity, fetal HDL as well as S1P-albumin provokes rapid and dramatic rearrangement of polymerized F-actin that was spatially confined to the cortical cytoskeletal ring. This S1P

induced actin reorganization was also observed in many other endothelial cells (191-193). In addition, fetal HDL-S1P not only sustains stability of endothelial cell barrier but also acts as a potent chemoattractant for endothelial cells, which is in accordance with the recent literature (39).

To prove that S1P on HDL directly functions on placental vascular permeability, we generated S1P depleted fetal HDLs by using S1P lyase, an enzyme which irreversibly hydrolases S1P to hexadecenal and ethanolamine-phosphate (194). Fifty percent displacement of S1P by SPL did not change the hydrodynamic radius of fetal HDL particles which were able to attenuate permeability of HPAEC barrier up to 80% time-constantly which is a clear proof that S1P drives fetal HDL endothelial barrier effects. Distinct biological functions of S1P are mediated by five different G-protein coupled receptors, for example enhancement of the vascular barrier integrity is mediated through agonism of the receptors S1PR1 and S1PR3 (195,196). Both receptors are expressed on the surface of placental HPAEC, while S1PR1 is more abundant. By using two different S1P receptor antagonists fetal HDL and S1P-albumin effects on endothelial integrity were abolished. Interestingly, both antagonists (W146-specific for S1PR1 and VPC23019 – unspecific antagonist for S1PR1 and S1PR3) showed the same membrane disintegration pattern when HPAEC were treated with antagonist alone and similar response when HPAEC were treated with antagonist plus fetal HDL. Additionally, it was shown *in vivo* that S1PR1-KO mice died during development due to a defect in vascular stabilization, suggesting that this receptor is essential for vascular development (197). In conclusion, several evidences confirm that S1P via S1PR1 is a potent regulator of vascular development, at least during embryogenesis (198).

Sphingolipid metabolism is altered in diabetic conditions, however, focus to date in this area has been on ceramide (199,200) and only a few studies have examined the involvement of S1P. Plasma S1P levels are elevated in two different animal models of type 1 diabetes, yet no changes in levels were detected in the livers of these animals (201), suggesting other sources of S1P. We saw that despite the statistically elevated S1P levels in fetal GDM circulation, amount of S1P bound to HDL is decreased. This observation is comprehensible since apoM levels on fetal GDM HDL are also reduced. Zhang et al. measured apoM levels and expression in diabetic rats rendered hyperglycemic by short-term glucose infusion (202). Serum apoM concentrations and hepatic apoM mRNA levels

were significantly reduced in the hyperglycemic rats, indicating that the low expression levels of apoM in these diabetic animals could be ascribed to hyperglycemia. Xu et al. investigated apoM expression and secretion in NMRI mice by inducing diabetes through the administration of alloxan (203). Plasma apoM concentrations decreased by 70%, hepatic apoM mRNA levels by 40%, and renal apoM mRNA levels by 20% compared to saline-injected controls. These results suggest that the reduction of apoM expression is a general phenomenon in animal models of diabetes. In humans apoM expression is reduced in some patients with diabetes but cannot be generally used as a reliable biomarker for the disease (204,205). However, we have showed that HDL associated apoM is decreased not only in well glucose controlled GDM mothers but also in their corresponding newborns which is in contrast to the animal studies. Since variable and different distribution of distinct lipoprotein subfractions between rodents and humans inhomogen apoM data should cautiously discussed. We are demonstrating that due to the apoM-HDL reduction, S1P association to the fetal GDM HDL is aberrant. Due to the lower levels of S1P, fetal GDM HDL was only able to increase placental endothelial barrier integrity up to 75% and to induce migration of HPAEC up to 70% when compared to control, fetal HDL. Additionally, actin rearrangement and formation of cortical cytoskeletal ring in HPAEC was not efficiently induced by fetal GDM HDL. ApoM-S1P containing HDL particle is well described as better in activating and inducing not only tight junction between endothelial cells and cytoskeletal rearrangement, but also as more efficient in inducing endothelial cell migration than apoM-free HDL (without S1P). This observation indicates that apoM is necessary for delivery of HDL-bound S1P to the S1P1-receptor (61) which might represent a possible explanation for our observations.

Although the association of high maternal glucose levels and offspring complications has been well documented (171), normalization of maternal glucose levels does not prevent fetal complications from occurring (206). GDM is a condition of low grade inflammation even in lean mothers (172), which may contribute to the risks for the growing fetus. The two main risks GDM imposes on the baby are growth abnormalities and chemical imbalances after birth, which may require admission to a neonatal intensive care unit. Untreated GDM also interferes with maturation, causing dysmature babies prone to respiratory distress syndrome due to incomplete lung maturation and impaired surfactant synthesis. Interestingly, S1P less mice have undetectable plasma S1P (207) and suffer from severe vascular leakage and endothelial dysfunction in the lung (208). Additionally, apoM-

/- mice also display vascular leakage in the lungs (61). Plasma apoM levels are reduced in patients with systemic inflammation (209), while mice with low plasma S1P levels are more prone to LPS infections and developed more severe lung symptoms than wild-type mice (210,211). Taking together these findings it is tempting to speculate that HDL-apoM-S1P complex plays an important role in development and maturation of fetal lungs.

Notably, despite the short duration of GDM, changes in the morphology of the feto-placental vasculature and its function have been demonstrated in women with GDM (212). In GDM, the feto-placental vasculature is characterized by increased branching (213) suggesting increased angiogenesis. This may be a result of changes in fetal metabolism such as hyperinsulinemia (169,214) and hyperleptinemia (215), which have pro-angiogenic properties, and of fetal hypoxia (216). Moreover, in GDM, the expansion of the feto-placental vascular tree is also paralleled by an increase in capillary volume (217) and diameter (218). Finally, the endothelial barrier function seems disturbed since the expression of junction molecules in the feto-placental vasculature is changed in GDM (219). In conclusion, previous knowledge in the field of GDM induced endothelial dysfunction strongly supports our speculation that fetal HDL-apoM-S1P induced stability of placental endothelium is jeopardized in GDM (Figure 28).

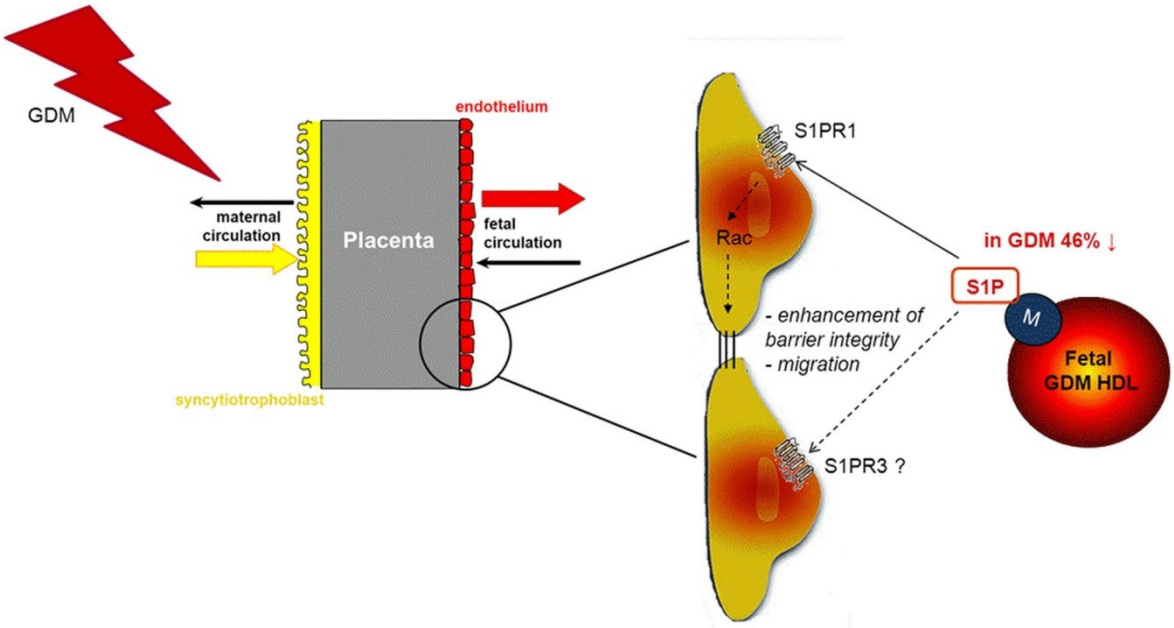


Figure 28: GDM impairs fetal HDL protective effects on placental endothelium
Fetal HDL exhibits S1P and mediates protective actions on the feto-placental endothelium e.g. migration and enhancement of barrier integrity. These effects of fetal HDL-S1P complex are induced through S1PR1activation. In GDM amount of S1P associated to HDL is 46% decreased due to the apoM-HDL reduction. Aberrations in S1P concentration are diminishing fetal HDL protective effects on placental endothelium.

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