Dissertation

The role of HDL- associated sphingosine-1-phosphate at the feto-placental vasculature: signaling and metabolism in pregnancy physiology and pathophysiology

submitted by

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Statutory Declaration

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

Ilaria Del Gaudio

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Disclosures

This thesis has been published in the following original papers:

- *Del Gaudio I, Sreckovic I, Zardoya-Laguardia P, Bernhart E, Christoffersen C, Frank S, et al*. **Circulating cord blood HDL-S1P complex preserves the integrity of the feto-placental vasculature.** Biochim Biophys Acta - Mol Cell Biol Lipids. 2020;1865(4).

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Co-authors contribution:
Sreckovic I. carried out preliminary cell culture experiments with S1P and HDL and performed data analysis
Zardoya-Laguardia P. performed wire myograph experiments and data analysis
Bernhart E. contributed with ECIS experiments and performed data analysis
Christoffersen C. provided S1P measurements in plasma and HDL and contributed to manuscripts editing
Frank S. Supervised myograph experiments and contributed to manuscript editing
Marsche G. Supervised calcium flux experiments and contributed to manuscript editing
Illanes S. contributed to data interpretation and manuscript editing
Hendrix S. established cell culture experiments with pro-inflammatory treatments and performed data analysis
Sasset L. performed SPT activity assay on placental chorionic arteries and data analysis

Di Lorenzo A. supervised the experiments on sphingolipid metabolism performed during the stay abroad and contributed to manuscript editing
Wadsack C. supervised all the projects and co-wrote the manuscripts.

All co-authors declare that they have no conflicts of interest with the content of this thesis and have explicitly agreed to use their data in the thesis.

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Zusammenfassung

Bereits während der Schwangerschaft bedingt eine ordnungsgemäße Funktion der Gefäße der Plazenta die Gesundheit der Nachkommen und kann bei Störungen langfristige, nachteilige Auswirkungen haben. Ein pathologisches intrauterines Milieu, bedingt durch mütterliche Stoffwechselstörungen, wirkt sich auf die plazentare mütterlich-fetale Interaktion aus, was zu fetalen Gefäßstörungen führt. Veränderungen am feto-plazentaren Endothel bilden hier die Grundlage für eine fetale Programmierung mit einhergehendem Risiko für den Fötus, im Erwachsenenalter chronisch zu erkranken.

Bioaktives Sphingolipid Sphingosin-1-phosphat (S1P), welches mit High Density Lipoprotein (HDL) im Blutkreislauf assoziiert ist, weist im Erwachsenen und in Tiermodellen vaskuloprotektive Eigenschaften auf. HDL-Partikel im fetalen Nabelschnurblut besitzen jedoch einen eigenen Stoffwechsel und erfüllen besondere Funktionen. Die hier vorgestellten Studien untersuchen die Rolle von aus der Nabelschnurblut isolierten HDL-S1P-Komplexen, in der Regulation der feto-plazentaren Gefäßfunktion. Zudem wird das Wissen über die Auswirkung von Präeklampsie (PE) auf den Sphingolipidstoffwechsel in der feto-plazentaren Gefäßversorgung erweitert. Initial wurde der Nabelschnurblut-HDL-S1P-Komplex charakterisiert, der im Folgenden als neonatales HDL (nHDL) bezeichnet wird. Auch biologischen Funktionen, die durch den Komplex an der feto-plazentaren Endothelschranke vermittelt werden, wurden aufgeklärt. *In vitro* wurde gezeigt, dass nHDL, indem S1P an seinen Rezeptor bindet, die Proliferation und die Barrierefunktion fetaler plazentarer arterieller Endothelzellen (fPAECs), sowie die Kalziummobilisierung fördert und Umstrukturierungen des Zytoskelettes bewirkt. Darüber hinaus demonstrieren *ex-vivo*-Studien zur vaskulären Reaktivität an Chorionarterien die Rolle von nHDL als potenter Vasodilatator, wobei die Rolle des S1P-Signals bei dieser spezifischen Wirkung noch untersucht wird. Es liegt nahe, dass nHDL eine Schlüsselrolle bei der Erhaltung der Integrität der feto-plazentaren Endothelbarriere spielt. Zudem wurde die entzündungshemmende Wirkung von nHDL-S1P untersucht. Da sich schwangerschaftsassoziierte Störungen durch eine Entzündung und Dysfunktion der Plazenta kennzeichnen, wurde die Fähigkeit von gesundem nHDL-S1P der Entzündungsreaktion entgegenzuwirken, getestet. In Gegenwart von nHDL-S1P konnte eine Reduktion der Produktion von pro-inflammatorischen Zytokine sowie der durch den Tumor-Nekrose-Faktor α (TNF-α) induzierten Adhäsionsmoleküle beobachtet werden, was auf eine Hemmung der Entzündungsmechanismen hindeutet. Dies wurde auch durch die reduzierte Expression des nukleären Transkriptionsfaktor-Kappa B (NF-κB)-Signalwegs bestätigt. Darüber hinaus war nHDL in der Lage, den durch Angiotensin II induzierten oxidativen Stress von fPAEC über das S1P-Signal abzuschwächen, was die gefäßschützenden Wirkungen des Komplexes

verdeutlicht. Schließlich wurde der Einfluss von PE auf den Sphingolipid-Stoffwechsel in der feto-plazentaren Vaskulatur untersucht. Die präeklamptische intrauterine Umgebung während der Schwangerschaft, führt demnach zu einer pathologischen Veränderung des feto-plazentaren Gefäßsystems. Die PE induzierten Veränderungen sind durch eine erhöhte *de-novo*-Sphingolipid-Biosynthese und eine Sphingomyelin (SM)-Anreicherung in den Choriongefäßen gekennzeichnet. Das gestörte S1P-Signal in fPAECs, ist hier mit der beobachteten vaskulären Dysfunktion konsistent. Zusammenfassend zeigen diese Daten die zentrale Rolle des bioaktiven Sphingolipids S1P insbesondere bei der Regulierung der vaskulären Homöostase und damit Barrierefunktion der Plazenta in der Physiologie und Pathophysiologie der Schwangerschaft.

Abstract

Long-term adverse outcomes on offspring health are strictly dependent on proper placental vascular function during pregnancy. Maternal metabolic derangements promote the establishment of a pathological intrauterine environment, which can affect placental maternal-fetal interaction, leading to fetal vascular disturbances. In particular, alterations at the feto-placental endothelium set the bases for a fetal programming with concomitant risk for the fetus to develop chronic diseases in adulthood.

Cumulative evidences have shown that, in adult and animal models, the bioactive sphingolipid sphingosine-1-phosphate (S1P) associated with high density lipoprotein (HDL) in the systemic circulation, exerts vasculoprotective properties. However, circulating HDLs in the cord blood possess a peculiar metabolism and function. The thesis presented here, investigated the role of cord blood derived HDL-S1P complexes in the regulation of feto-placental vascular function. Moreover, the thesis broadened the knowledge on the effect of preeclampsia on sphingolipid metabolism at the feto-placental vasculature.

In the first part of this thesis, we characterized the cord-blood HDL-S1P complex, to which we referred to as neonatal HDL (nHDL), and we clarified the biological functions mediated by the complex at the feto-placental endothelial barrier. We could show in vitro that nHDL, by delivering S1P to its receptor, promotes proliferation of fetal placental arterial endothelial cells (fPAECs), calcium mobilization, cytoskeleton rearrangements and barrier function. Moreover, ex-vivo vascular reactivity studies on chorionic arteries, highlight the role of nHDL as potent vasodilator, although the role of S1P signaling in this specific effect is still under investigation. This suggest that nHDL plays a key role in preserving the integrity of the feto-placental endothelial barrier.

Secondly, the anti-inflammatory action of nHDL-S1P was investigated. Since most of pregnancies-associated disorders are characterized by placental inflammation and dysfunction, we wanted to test the capability of healthy nHDL-S1P to counteract the inflammatory response. In presence of nHDL-S1P, we observed a reduction of pro-inflammatory cytokine expression as well as adhesion molecules, induced by tumor necrosis factor α (TNF-α), suggesting a potent inhibition of inflammatory mechanisms. This evidence was also corroborated by an observed downregulation of the nuclear transcription factor-kappa B (NF-κB) pathway. Furthermore, nHDL was able to attenuate oxidative stress induced by angiotensin II via S1P signaling in fPAECs, highlighting the vasculoprotective actions of the complex.

Lastly, sphingolipid metabolism at the feto-placental vasculature in preeclampsia, which is a serious pregnancy complication characterized by high blood pressure and vascular dysfunction, was examined. Our data revealed that the exposure to a preeclamptic intrauterine environment, leads to a pathological remodeling of the feto-placental vasculature. The alterations induced by preeclampsia consist of increased de novo sphingolipid biosynthesis and sphingomyelin (SM) accumulation in placental chorionic vessels. Of note, we observed an impaired S1P signaling in fPAECs, which is consistent with vascular dysfunction.

These data demonstrate the pivotal role of bioactive sphingolipid and specifically S1P, in the regulation of vascular homeostasis and barrier function of the human placenta in pregnancy physiology and pathophysiology.

1. Introduction

1.1 Vascular biology of the human placenta

The placenta is a unique vascular organ which mediates the communication between mother and fetus. Proper placental perfusion provides nutrient and oxygen to the developing fetus and it ensures that the waste products are effectively eliminated from the circulation. The human placenta consists of a maternal side, which is referred to as basal plate and it is constituted of fetal derived tissue (extra villous trophoblast) as well as maternal tissue (decidua basalis). The fetal side is referred to as chorionic plate, which consists of the amnion, the extra-embryonic mesodermal connective tissue and the syncytiotrophoblast (1).

This highly specialized organ has two separate circulatory systems: the maternal–placental (utero-placental) circulation, and the fetal-placental (feto-placental) circulation (Figure 1a) (2). To maximize the transfer from maternal to fetal circulation, the placenta is organized in branching tree-like structures called villus tree (3). Each villus constitutes a functional unit, where nutrient and gas exchange between the mother and the fetus takes place (Figure 1b).

Through the utero-placental circulation, maternal blood enters (via spiral arteries) to the intervillous space where it surrounds the villi. At the fetal side, the umbilical cord connects the placenta to the fetal circulation. The umbilical cord contains two umbilical arteries and one umbilical vein, surrounded by Wharton's jelly and is surrounded by an outer layer of amnion (4). Deoxygenated and nutrient-depleted fetal blood flows via the umbilical arteries from the fetus to the intervillous space. Thereafter, the umbilical vein carries the oxygenated and nutrient-rich blood circulating back to the fetal systemic circulation. Of note, the two circulations are in close contact but distinctly separated from each other.

Uncontrolled bleeding and mixing of the fetal and maternal blood may result in fetal-maternal hemorrhage (5).

The placental barrier is composed of multiple specialized cell types which sustain maternal-fetal physiology (6). Thus, nutrients and gases, which diffuse from the maternal to the fetal circulation, must cross different cell layers. The syncytiotrophoblast (SC) covers the entire surface of the villous tree by forming a continuous lining which keeps separated maternal and fetal blood within the intervillous space (7). The syncytium encloses the villous core stroma where stromal cell such as placental macrophages (Hofbauer cells) and fibroblasts are located (8). Inside the villi, fetal vascular cells (vascular smooth muscle cells, pericytes and endothelial cells) constitute the architecture of fetal arteries and veins (9,10).

The work of my thesis focuses on the role of the feto-placental endothelial barrier therefore the specific relevance of this placental compartment is further reviewed in section 1.2

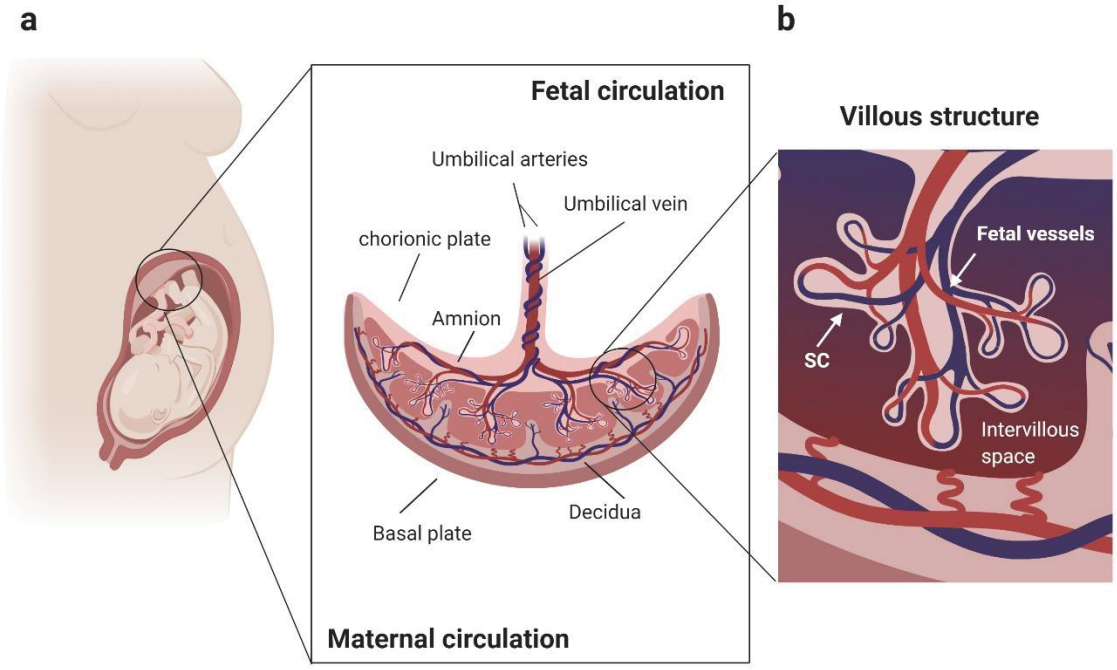

Figure 1: Schematic drawing of the human placenta architecture. a) On the fetal side (chorionic plate), the placenta is covered by the amnion, whereas on the maternal side (basal plate) the most external layer is the decidua. The feto-placental circulation is reversed to the adult one: oxygen- and nutrient-rich blood flows within the umbilical vein (red) towards the fetus, whereas oxygen and nutrient-depleted blood returns to the placental vasculature via two umbilical arteries (blue). b) The human placenta can be compartmentalized into functional units called villous tree, which are surrounded by a syncytiotrophoblast (SC) layer. The SC is in direct contact with maternal blood and prevents the mixing of the two circulations. Amidst the villi, there is the intervillous space. Fetal vessels are located within each villous branch, where the nutrient and gas exchange take place. The figure has been created with BioRender.com.

1.2 The role of the endothelium at the feto-placental vasculature

Peri- and post-natal fetal health is largely dependent on adequate feto-placental perfusion. However, fetal growth abnormalities may develop even in the presence of proper maternal utero-placental perfusion (11), suggesting that the functionality of the feto-placental vasculature is crucial to fetal well-being. The assessment of the feto-placental vascular state is usually performed on the umbilical artery by Doppler ultrasound of fetal blood flow (12).

However, it is at the level of small chorionic arteries branches and in the capillary system that placental function takes place. Chorionic plates arteries and veins branch from the umbilical cord insertion and cross the fetal surface of the placenta until they enter the villi. This complex vascular network plays a key role in vascular physiology by regulating vasomotor tone and blood flow to the fetus (13). Unlike other vascular beds, the feto-placental vasculature is not subjected to autonomic regulation (14). Thus, the balance between vasodilators and vasoconstrictors, which are mostly synthesized by vascular endothelial cells, is critical for maintaining placental vascular homeostasis. Chorionic vessels are responsive to several vasoactive compounds such as angiotensin II (AngII) (15), prostaglandins (16), endothelin (17) and nitric oxide (NO) (18), which regulate placental vascular resistance. Several studies have reported the association between an imbalance in the production of these vasoactive mediators in the placenta and the development of pregnancy-associated syndromes (19,20). The mechanisms underlying these placental pathologic features, although still poorly understood, are mostly dependent on the dysfunction of the fetoplacental endothelium.

In general, the endothelium represents a cellular monolayer located at the lumen of blood and lymphatic vessels (21). Because of its strategical location it creates an interface between the circulating blood and the surrounding tissue (22).

The local environment promotes morphological and functional endothelial cells heterogeneity, resulting in tissue-specific phenotypes (23,24).

Feto-placental endothelial cells (fPECs) are non-fenestrated endothelial cells, which play an important role in fetal development (25). Heterogeneity of the fetal endothelium in the macro- (umbilical cord blood) and micro- (chorionic villi) circulation has been reported (26,27). Besides their localization in large vessels or capillaries, fPECs phenotype and function largely depend on whether the cells line venous or arterial vessels (28). Endothelial cells isolated from veins (fPVECs) are spindle-shaped and display great cell plasticity. Moreover, genes associated with transport activity mainly characterized fPVECs profile, suggesting a role in the regulation of nutrients trafficking. Conversely, arterial-derived endothelial cells (fPAECs) express genes associated with signal transduction such as vascular endothelial growth factor A (VEGF), which regulates placental vascularization and angiogenesis (28). This suggest that the fPAECs play a distinct role in feto-placental vasculature formation and maintenance compared to fPVECs.

1.3 Inflammation and endothelial barrier dysfunction: features of pregnancy disorders

The endothelium acts as a semi-selective barrier which can modulate cellular and nutrient transport, regulate vasomotor tone, drive angiogenesis and prevents the egress of circulating pathogens and harmful materials into the tissue (29).

Perturbation of the vascular homeostasis by mechanic, chemical or immunologic mediators elicits a pro-inflammatory status which ultimately results in endothelial dysfunction (30). Under physiological conditions, the endothelial barrier prevents uncontrolled paracellular molecules transport due to cell-cell connections formed by transmembrane proteins attached to cell cytoskeleton (31). Hence, endothelial cells can regulate barrier permeability through rearrangement of junctional proteins. Adherens junction (AJ) is the most abundant type of cell-cell connection in the endothelium and prevent the crossing of molecules larger than ~ 3 nm (32). The stability of adherens junctions is provided by vascular endothelial cadherin (VE-cadherin), which plays a key role in the maintenance of vascular integrity (33). *In vitro* studies have shown that inflammation causes a displacement of VE-cadherin from the endothelial cells junctions, which results in barrier disruption and leukocytes transmigration (34,35). Increased vascular leakage and altered distribution of junctional VE-cadherin have been reported in feto-placental vessels from diabetic and preeclamptic subjects (36,37), suggesting that a pathological maternal environment can affect the feto-placental barrier integrity during pregnancy.

When the endothelium undergoes inflammatory activation, several cytokines such as tumor necrosis factor-α (TNFα) and interleukine-1β (IL-1β), induces the activation of inflammatory intracellular signaling pathways, which mainly trigger the nuclear transcription factors NF-κB and activator protein 1 (AP1) (38). Upon this stimulatory response, the augmented expression of adhesion molecules such as endothelial-leukocyte adhesion molecule 1 (E-selectin), vascular adhesion molecule-1 VCAM-1, and intercellular adhesion molecule-1 (ICAM-1) promotes the chemotaxis of adherent leukocytes and increases vascular permeability (39). Circulating levels of pro-inflammatory cytokines as well as enhanced placental NF-κB expression are increased in women with gestational diabetes (GDM) and preeclampsia (PE) (40–43). Hence, the placenta itself contribute to the systemic inflammation by releasing inflammatory markers, which promotes fibrotic response and vascular remodeling (44).

Endothelial cells are source of a variety of vasoactive mediators (45). For instance, NO and prostacyclin are released in response to hormones or physical stimuli to promote vessels relaxation and anti-thrombotic function (46). Conversely, substances such as endothelin, thromboxane A2, angiotensin II (AngII), can elicit smooth muscle cells hyperpolarization and

promotes vascular contraction (47,48). An imbalance in the relative contribution of endothelium-derived vasodilators and vasoconstrictors results in reduced vessel relaxation and an increased pro-inflammatory and pro-thrombic state, which favors endothelial dysfunction (49).

Reduced NO bioavailability is considered as an hallmark of endothelial damage and it is associated with widespread vascular diseases (hypertension, coronary artery disease, heart failure, diabetes, chronic kidney failure) and adverse cardiovascular events (50). Evidence from *in vivo* studies suggest that the activation of the renin-angiotensin system disrupts the NO balance (51). Mice lacking endothelial-derived nitric oxide synthase (eNOS) manifest hypertension and increased leukocyte adhesion to vascular endothelium (52). In addition to its vasoconstrictive properties, Ang II causes oxidative injury of endothelial cells through activation of NADH/NADPH oxidase and the generation of reactive oxygen species (ROS) (53). The oxidative stress induced by Ang II contributes to the pathogenesis of vascular diseases by inhibiting the production and release of NO, promoting vascular smooth muscle cells proliferation, impairing endothelial function, and stimulating pro-atherogenic, pro-inflammatory, and adhesion molecules expression (54). Studies in humans and animals support the concept that the antagonistic effect between NO and Ang II is a crucial determinant of cardiovascular events (55).

Additionally, altered NO synthesis/production and oxidative stress are common features of human pregnancy disorders (56). KO mice for eNOS exhibit impaired utero-placental and feto-placental perfusion (57). Moreover, human umbilical vein endothelial cells (HUVECs) from PE (58) and intrauterine growth restriction (IUGR) (59) pregnancies, show decreased eNOS expression and activity. Interestingly, increased expression of ROS and inhibition of NO-pathway in the feto-placental vasculature and villous stroma of preeclamptic and diabetic placentas was observed (60,61). These findings highlight an additional involvement of oxidative stress in the pathophysiology of inflammation induced placental injury. Maternal and placental oxidative stress has been implicated also in the regulation of the systemic blood pressure (62). AngII blunts endothelium-dependent vasodilation partially through the generation of ROS (63). The generation of Ang II by angiotensin converting enzyme (ACE) in the feto-placental vasculature leads to a strong vasoconstrictor activity in the feto-placental circulation (64,65). Elevated levels of AngII in fetal plasma (66) as well as increased renin activity (67) were reported in PE and IUGR. It is well accepted that inflammatory activation and oxidative stress associated with placental endothelial damage may affect multiple organs systemically and contribute to adverse pregnancy outcomes including maternal death, preterm birth and fetal death (68).

1.4 Preeclampsia: long-term consequences for neonatal vascular health

PE is currently one of the leading pregnancy complications (2 to 8 % of pregnancies worldwide) (69). It accounts for the death of approximately 80,000 women and 500,000 fetuses per year (68,70,71). This syndrome is a multisystemic disease which jeopardizes the function of different organs (e.g the liver, kidneys, placenta and brain), resulting in hypertension, proteinuria, vascular dysfunction, chronic immune system activation and renal dysfunction (72).

Genome-wide association studies (GWAS) have been identified candidate-genes linked with the development of the disorder (73,74). Several SNPs of genes (e.g eNOS, angiotensinogen, TNFα, and prothrombin) involved in maternal regulation of cardiovascular and inflammatory responses were found to correlate with PE development (75–78). However, the genetic contribution and its exact proportion to this pathology has not been proved conclusively (79).

Determining the etiology of PE still represents a challenge for the medical and scientific community, due to the complex interplay between maternal constitution and placental factors (80–83).

Nowadays, the treatment for PE consists in managing the clinical symptoms and termination of the pregnancy, which additionally increases the rate of preterm birth (84,85).

Given the key role of the placenta to development and remission of preeclampsia, researchers have been focused their investigations on placental vascular development and function in the onset of the disease (86).

During the first trimester of human pregnancy, the extra villous cytotrophoblasts of fetal origin invades the uterine spiral arteries and replace the endothelial layer of the maternal vessels, transforming them from small, high-resistance into low-resistance and large diameter vessels (87), which ensure adequate feto-placental perfusion (88). In PE, the cytotrophoblast fails to invade the spiral arterioles effectively, leading to abnormal placentation and concomitant placental under perfusion and ischemia, which finally results in the release several pro-inflammatory placental factors into the maternal circulation causing the clinical syndrome (89–91).

Women who are diagnosed with PE have an higher risk to develop cardiovascular diseases in future (4-fold increase in heart failure and 2-fold increase in coronary heart disease, stroke) (92) and thromboembolic events within 5 to 10 years subsequently after their pregnancy (93). Hence, although PE is confined to pregnancy, a lifelong monitoring of maternal health is absolutely required.

Additionally, in pregnancies affected by PE, studies of long-term outcomes have reported an higher proportion of children with increased susceptibility for morbidities later in life beyond that one's rising in their peri-natal life or due to the low birthweight (94). In particular, children exposed to PE during pregnancy have a higher risk to develop cardiovascular diseases, childhood hypertension, metabolic syndrome and diabetes (95,96).

It has been proposed that, the adverse outcomes in the offspring prenatally exposed to PE, strongly reflect a fetal programming mediated by the placenta (97). Elucidating the mechanisms by which preeclampsia induce feto-placental perturbations has become a challenge of the scientific community. However, it appears that processes regulating endothelial and immune function, angiogenesis and oxidative stress synergistically modify vascular risk of the fetus *in utero*, leading to altered vascular phenotype later in life (98).

When the endothelial dysfunction establishes alongside with systemic oxidative and inflammatory state, the placenta releases ROS, cytokines and antiangiogenic factors which exacerbate placental ipofusion, hypoxia, inflammation and systemic endothelial dysfunction (43,89,99,100). Hence, offspring of preeclamptic mothers are exposed to a pathological intrauterine environment, which has a long-term impact on their vascular health, most likely by activating mechanisms that impairs endothelial function or alter vascular gene expression.

1.5 High density lipoprotein (HDL) and vascular protection

HDLs are highly heterogeneously composed group of particles which differ in size, density, electrophoretic properties, lipid and apolipoprotein content (101). Such heterogeneity depends on the relative distribution of proteins, lipids and enzymes within or associated with the particle. HDL proteins can be classified into subgroups which include apolipoproteins, enzymes, and proteins regulating several biological processes such as lipid transfer, acute-phase response, complement activation, immune response and hemostasis (102).

Regarding the protein component, ApoA-I and ApoA-II are the major apolipoproteins (Apo) constituting HDL particle (70% and 20 % respectively) (103). Whereas 10-15% of HDL protein mass is composed of 12 minor proteins (including ApoA-4, ApoC, ApoE, ApoD, apoM), end enzymes such as lecithin:cholesterol acyl transferase (LCAT), cholesterol ester transfer protein (CETP) and paraoxonase-1 (PON1), which are not only structural components but regulates lipid metabolism as well as cell function (104).

Of note, the composition of the HDL proteome is strongly dependent on the technique used for the particle isolation (105). Ultracentrifugation-based HDL isolation may potentially deplete

the particles from some proteins due to the high ionic strength of concentrated salt buffers used during centrifugation (106). On the other hand, by using liquid chromatography approaches, the HDL isolation result extremely contaminated with abundant plasma proteins, such as albumin (106). Nowadays, an integrative approach based of both methods is highly recommended. However, from which core-proteins the different HDL particles are assembled is still under discussion.

In addition to proteins, lipidomic analysis have identified several lipid species associated with the HDL particle including free cholesterol, cholesteryl esters (CE), steroids, triglycerides, diacylglycerides, monoacylglycerides, free fatty acids (FFA), phospholipids (phosphatidyl-choline, phosphatidyl-ethanolamine, lysophosphatidyl-choline) and sphingolipids (ceramide, sphingomyelins and sphingoid base-derived sphingolipid) (107).

HDL plays a pivotal role in reverse cholesterol transport (RCT), by which the cells get rid of excessive and accumulated cholesterol, which may be toxic. RCT removes cholesterol from the peripheral vessels and tissues by transport back to the liver for catabolism (108).

The inverse correlation between cardiovascular events and plasma HDL-C (high density lipoprotein cholesterol) has been first described in “The Framingham Heart Study” more than a century ago (109). Subsequently, several epidemiological and clinical studies confirmed the association between low plasmatic levels of HDL and increased risk of coronary artery disease (CAD), myocardial infarction (MI) and atherosclerosis (110).

The cardioprotective function of HDL was initially mainly attributed to the capability of the particle to interfere with atherogenesis and plaque rupture (111). However, it became evident that, given the heterogeneity of the particle and its biological function, HDL functionality cannot be restricted only to the cholesterol measurement.

In addition to its role in RCT, HDL has several beneficial pleiotropic properties, which contribute to its protective vascular function. Indeed, HDL can directly regulate endothelial cell function thereby sustaining vascular health. Numerous *in vitro* and *in vivo* studies have reported multiple vasculoprotective properties of HDL which include: cytoprotective, antioxidative, anti-inflammatory, vasodilatory, anti-thrombotic and functions (112). In particular, HDL protects the endothelium from apoptosis by activating survival pathways and reducing cellular generation of superoxide (113).

Furthermore, HDL can attenuate oxidative damage induced by free radicals, by inactivating and removing pro-inflammatory oxidized lipids from low-density lipoprotein (LDL), which retained the vessel wall (114). HDL anti-oxidative property is closely related to its anti-inflammatory action.

It has been shown that infusion of reconstituted HDL is able to decrease the expression of adhesion molecules thereby, reducing the adhesion of monocytes the vascular wall (115). HDL inhibits the production of pro-inflammatory cytokines by modulation of the NF-κb and peroxisome proliferator-activated receptor gamma (PPARγ) (116).

The endothelium-protective action of HDL is also a result of its vasodilatory capability. HDL binds with high affinity to the scavenger receptor class B type I (SR-BI) on the endothelium, elicits eNOS cascade activation and subsequently NO release (117). The generation of ROS within the vasculature counteracts NO production in smooth muscle cells. Oppositely, HDL treatment can lower oxidative stress, resulting in increased NO availability and improved vasodilation (118).

HDL can be accountable for several anti-thrombotic functions exerted on platelets as well as on endothelial cells. *Ex vivo* studies have demonstrated a direct effect of HDL on reduction of platelet activity as well as anti-thrombotic function toward the endothelium via enhanced NO production (119–121).

It was widely believed that mainly alterations in HDL proteome could impaired its protective functions. Under inflammatory or tissue injury condition, decreased levels of ApoA-I and increased level of serum amyloid A (SAA) and ApoC-III, characterize the proteome of the HDL particle, resulting in impaired RCT (122,123).

Subjects with type 1 and type 2 diabetes (T1DM/T2DM) show low levels of ApoA-I and antioxidant protein paraoxonase-3 (PON3), which affect the anti-inflammatory function of HDL and increases the risk of cardiovascular diseases in these populations (124–126).

Furthermore, the proteomic profile of HDL isolated from coronary artery disease (CAD) patients was enriched in apoE, ApoC-IV, complement C3 and ApoA-IV, suggesting a remodeling of the particle to inflammatory features (127). Profiling of HDL protein constituents could therefore represent a useful tool for biomarkers identification in several disease.

However, increasing attention has been paid to the lipid component of the particle. Interestingly, phospholipids on HDL particles may regulate cholesterol efflux capacity (128). Moreover, phosphatidyl-choline species are involved in HDL-mediated anti-inflammatory properties (129). More recently, innumerable studies have been also focusing on the vascular function driven by HDL associated sphingolipid (130), corroborating the concept that, besides proteins, HDL lipid composition and distribution might be the crucial for HDL function.

1.5.1 Lipoprotein metabolism in pregnancy physiology and pathophysiology

Physiological changes that occur in the mother during pregnancy, greatly affect lipid metabolism. In normal pregnancy, during the first two trimesters, an increase in lipid synthesis is observed as result of hyperphagia and increased lipogenesis (131). Thus, between 10 and 30 weeks of pregnancy most women show a highly atherogenic profile characterized by a 2.5-fold increase in triglycerides (TG) levels, 1.5-fold increase in total cholesterol, with concomitant higher levels of very-low density lipoprotein (VLDL), HDL and small dense LDL (132).

This anabolic state in early pregnancy is also promoted by hormonal and metabolic changes such as increased insulin sensitivity and progesterone levels, cortisol and leptin stimulation which all together contribute to maternal fat accumulation (133).

During late pregnancy, lipid metabolism changes to catabolic a phase in which the breakdown of fat depots takes place, in order to meet the demand of the developing and growing fetus. The maternal hyperlipidemia of the third trimester is caused by increased insulin resistance and augmented estrogen and placental lactogen, which aggravates the insulin resistance (134).

Consequently, an enhanced lipolysis of triglycerides takes place in the adipocytes, accompanied by reduced lipoprotein lipase (LPL) activity. As a result, the inefficient clearance of triglycerides-rich lipoproteins, favors the increased in HDL and LDL triglyceride/cholesterol ratio.

Whereas VLDL and LDL steadily increases during pregnancy, HDL levels increases (45%), during mid gestation, followed by a drop in their levels at term (15%) (135). Interestingly, Apo-AI levels are stable, suggesting that a remodeling of the particle takes place in late pregnancy (136).

In particular, the HDL_2 subfraction, rich in triglycerides, is increased during the third trimester at the expense of HDL_3, which is enriched in cholesterol (132). Moreover, the increased activity of the cholesteryl ester transfer protein (CETP), contributes to the triglycerides enrichment of HDL and LDL particles, which are normally not deputed to triglycerides transport (132,137).

The human placenta express different lipoprotein receptors (138), including LDL receptor (LDLR) and SR-BI, which ensure uptake of maternal triglycerides-rich LDL particles (139) and cholesterol transport from the maternal circulation to fetal tissue (140), respectively. Changes in placental lipoprotein receptors expression have been associated with impaired uptake or efflux functions in pregnancy disorders. For instance, placental LDLR decreases during maternal hypercholesterolemia (141) whereas IUGR placentae are characterized by LDLR overexpression and SR-BI downregulation compared to healthy controls (142).

Although hyperlipidemia is a required physiological and transient change during gestation, excessive or reduced cholesterol supply to the fetus, can result alterations of lipoprotein with long-term consequences for the fetus (143).

A large body of literature suggest that an abnormal lipoprotein profile, characterized by increased total cholesterol, triglycerides, LDL and decreased HDL levels, correlates with increased risk of PE and GDM during pregnancy (144,145). Moreover, the loss of HDL-mediated atheroprotection result in augmented level of oxidized LDL in the maternal circulation which is corroborated by a higher risk of cardiovascular events (146,147).

A maternal dyslipidemic pattern is strongly linked to adverse fetal vascular outcomes. The offspring of hypercholesterolemic mothers showed fatty streaks in the aorta and atherosclerotic lesions (148). Other studies have corroborated the concept that, dyslipidemia associated with maternal obesity and diabetes has long-term consequences on offspring life expectancy (149). Several animal studies have shown that epigenetic changes may be responsible for the fetal programming and increased atherosclerotic susceptibility (147).

1.5.2 Characteristics of cord blood-derived HDL

Cord-blood lipid levels are reported to be significantly lower compared to those in adults (150). Strikingly, the lipoprotein profile differs: while LDL is the major class of lipoprotein in maternal circulation, more than 50% of total cholesterol in the cord blood is carried by HDL (150). Thus, the cord-blood lipoprotein profile during pregnancy is peculiar and stays unchanged till the first week after delivery (151,152).

The composition of cord-blood derived HDL which we referred to as neonatal HDL (nHDL) is substantially different from that in adults (153). Several studies have reported that the concentration of most of the apolipoproteins (ApoA-1, ApoA-2, ApoC-3, and ApoD) are considerably lower in nHDL, some (ApoF and ApoL) are not found at all, whereas only ApoE was present at level comparable with the adult particle, suggesting enhanced cholesterol transport function (154,155). Interestingly, nHDL is enriched in proteins associated with coagulation and transport, whereas displays reduced anti-oxidative capacity (154).

We have previously shown that a pathological intrauterine environment during pregnancy affects neonatal lipoprotein profile and function (156), pointing out the key role of the particle for future cardiovascular health of the offspring.

1.6 Metabolism and function of bioactive sphingolipids

Sphingolipids (SLs), firstly discovered by J. L. W. Thudichum in 1876, were initially believed to play a role in energy metabolism and as structural components of cellular membranes. However, in the past 20 years great attention has been paid to the capability of SLs to mediate signal transduction and cellular interaction as well as to regulate protein sorting (157).

Structurally, SLs are composed of a hydrophobic region consisting of a long-chain sphingoid base backbone (with generally 18 carbons), which is linked to a fatty acid via an amide bond (with 16-24 methylene groups), and a hydrophilic region, which vary according to the SL species (hydroxyl for ceramide, phosphorylcholine for sphingomyelin, or carbohydrate residues of varying complexity for glycosphingolipids). Fatty acids can differ in the composition but palmitic (C16:0) and stearic (C18:0) are the most abundant.

In particular, SL species such as ceramide (Cer), sphingosine (Sph), sphingosine-1-phosphate (S1P), and sphingomyelin (SM), acts as bioactive molecules regulating several cellular processes, including proliferation, migration, apoptosis and inflammation (158–160).

During the last two decades, numerous studies have been carried out to describe the biochemical pathways which govern SL biosynthesis, degradation, transport and distribution, with the ultimate goal to decipher their function (161). Figure 2 shows a schematic overview of the mammalian SL metabolism.

The *de novo* biosynthesis takes place in the endoplasmic reticulum (ER), starting with the condensation of serine and palmitoyl-CoA catalyzed by serine palmitoyl transferase (SPT) to generate 3-keto-dihydrosphingosine. SPT activity can be negatively regulated by the action of reticulon 4B, also known as Nogo-B (162), or pharmacologically inhibited by myriocin (163), thereby affecting the *de novo* synthesis.

The 3-Keto-dihydrosphingosine is subsequently reduced to Sph, and further on acylated by (dh)Cer synthase to generate dhCer or Cer (164). Cer represent a metabolic hub for SLs production. It can be converted to produce bioactive second messenger's sphingosine, S1P or ceramide-1-phosphate (C1P). However, Cer is primarily transported to the Golgi complex, where it can be used as a substrate for production of complex SLs (165), such as glycosphingolipid and SM.

Complex SL species are redistributed within the different cellular component and undergo degradation and production cycles to ensure SLs turnover (166). For instance, through the salvage pathway, long-chain sphingoid bases derived from complex SLs are used to re-generate Cer (167). It has been reported that the salvage pathway contributes for 50%-90% of SL synthesis (168), suggesting a major role of sphingolipid breakdown in biosynthesis/turnover homeostasis as well as in cellular function. Indeed, impaired SL

metabolism is associated with several human disorders (cardiovascular, metabolic and neuronal), which are mainly the result of alterations of enzymes involved in SL degradation (169,170).

Production and or accumulation of SLs can be stimulated by cytokines (158), oxidative stress (171), chemotherapeutics (172) as well as by heat stress condition (173) or ischemia/reperfusion injury (174), and ,in turn, SLs can activate downstream pathway and regulate cell physiology.

Complex SLs may act as mediators of cell-cell adhesion, as regulators of signal transduction and they can also bind toxins and growth factors. Whereas, among the simplest SLs species, the most studied are Cer and S1P, which have been reported to be involved in a variety of cellular processes such as proliferation, migration, growth, senescence, and apoptosis (175). It was initially proposed that Cer and S1P play opposing roles, based on the evidence that Cer accumulation promotes apoptosis, while S1P stimulates cell survival and antagonizes Cer effect (176). This mechanism has been defined as “sphingolipid rheostat”, by which cells can regulate their fate through interconversion of SLs species. However, more recent studies have suggested that this sensing mechanism is not only based on relative levels of Cer and S1P, but is strongly dependent on the production, secretion and signal transduction of specific SLs metabolites in a tissue-dependent manner. A more detailed description of the biology of SM, Cer and S1P is discussed in the following paragraphs.

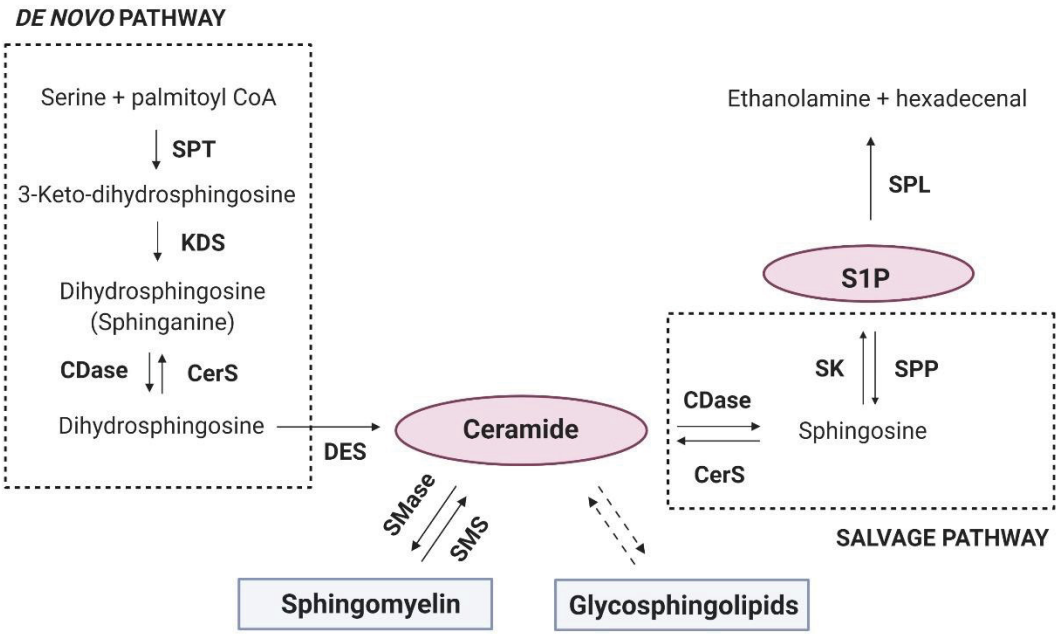

Figure 2: Overview of cellular sphingolipid metabolism. SPT, Serine palmitoyl transferase; KSD, 3-keto-dihydrosphinganine reductase; DES, Dihydroceramide desaturase; SMase, Sphingomyelinase; SMS, Sphingomyelin synthase, CerS, Ceramide synthase; CDase, Ceramidase; SK, Sphingosine-1-phosphate kinase; SPP, Sphingosine-1-phosphate phosphatase; SPL, Sphingosine-1-phosphate lyase. The figure has been created with BioRender.com.

1.6.1 Sphingomyelin

SM are the most abundant complex SL in eukaryotic cells (85% of all SLs in humans). The majority of Cer produced in mammalian cells are converted to SM by the action of the sphingomyelin synthases (SMS) at the trans-Golgi (177). Hence, plasma membranes which are mostly enriched in SM are mainly trans-Golgi derived (178).

The presence of SM at the plasma membrane has several biological implications. SM can directly regulate cholesterol availability, by increasing its synthesis and interfering with the binding of LDL to its receptor on the cell surface (179). Moreover, SM possesses the capacity to form lipid rafts, which serve as platform for signal transduction (180).

The production of SM leads also to a generation of diacylglycerol (DAG), which has also been described as bioactive molecule. Considering that SMS activity can regulates the production of several lipid mediators including SM, DAG and Cer, it has been proposed that SMS might represent a crucial player in cell fate regulation (181).

Accumulation of SM due the inherited deficiency of sphingomyelinase (SMase) causes the Niemann-Pick disease (NPD), characterized by impaired cholesterol trafficking (182). Moreover, evidence of the association between increased plasma levels of SM and the incidence of cardiovascular diseases and diabetes has been reported (183–186).

1.6.2 Ceramide

Besides having a central role in SL metabolism, Cer have been implicated in the regulation of several cellular processes such as apoptosis, autophagy, cell differentiation, inflammation as well as insulin resistance and atherosclerosis (187–189).

In mammals, Cer can be generated by the action of ceramide synthases (CerS) and catabolized by ceramidases (CDase). To date, 6 different CerS have been identified, which can selectively produce Cer with specific chain length and double bonds, thus with different function (190). Indeed, the loss of a specific CerS in KO mice, cannot be overcome by the activity of any another CerS, resulting into distinct disorders (191).

CerS activity appears to be involved in pro-apoptotic effect mediated by inflammatory cytokines (192), glucolipotoxicity (193) and negatively affects tumor growth (194).
Conversely, CDase is involved in the pro-survival effect mediated by growth factors (195). Moreover, CDase is overexpressed in cancer cells and tumor derived tissues, resulting in decreased levels of Cer and increased levels of S1P (196).
Despite, the well-described pro-apoptotic role of Cer, is becoming more and more evident that Cer action is pleiotropic and dependent on the peculiar Cer species in a specific tissue or model system (197).
Recent studies reported a strong correlation between specific plasmatic Cer ratios (Cer-C16:0/C24:0, Cer-C22:0/C24:0), and mortality in three independent cohorts of CAD patients (198). Furthermore, obese patients with T2D show higher levels of Cer C18:0, C20:0 and C24:1 compared to control subjects (199). Moreover, lipidomic analysis of colorectal cancer tissue, revealed an increase of C16:0, C24:0, and C24:1 species, with a concomitant decrease in C18:0 and C20:0 species compared to non-tumor tissue (200). In addition, in PE specific ceramide species have been found augmented in placenta or maternal plasma (201,202). Thus, increasing number of evidences point to a potential use of ceramide as prognostic or diagnostic markers.

1.6.3 Sphingosine-1-phosphate

Cer can be deacylated by CDase to produce sphingosine, which can be converted in S1P through the action of sphingosine kinases (SK), localized in the cytosolic compartment or associated with membranes (203). The cellular level of Cer, sphingosine and S1P significantly differ from each other, with Cer being the most abundant, while S1P is presents at the lowest level. Interestingly, small variations in Cer content could clearly affect the level of sphingosine or S1P. Two subtype of kinases (SK1 and SK2), which can be differentially located but catalyzing the same reaction, have been identified. While SK2 is mainly localized in the nucleus (204), SK1 can be associated with membranes, translocate to the nucleus and it can also be secreted by the cells (205). Animal studies have shown that mice lacking both isozyme are embryonically lethal do to impaired vascular development, suggesting that S1P is necessary for proper vascular function (206). Moreover, abnormal decidualization was observed in female $SphK1^{-/-}$, $SphK2^{+/-}$ (207).
At the ER S1P can be dephosphorylated by phosphatases (SPPs), which are considered rate-limiting enzymes in the salvage pathway (208). Indeed, SPP1 overexpression, leads to

increased intracellular Cer levels (209). Hence, SPPs activity plays a key role in the downregulation of extracellular S1P signaling.

As final step in the SL metabolism the S1P lyase (SPL), localized at the ER, catalyzes the irreversible conversion of S1P in phosphoethanolamine and hexadecenal (210). SPL is highly expressed in the intestine and thymus, whereas is poorly present in brain and skeletal muscle (210). Hence, alteration of SPL activity is associated with intestinal tumor progression and impaired immune system homeostasis (211,212). S1P is mainly produced by erythrocytes and vascular endothelial cells (213). However, SPL activity is not detectable in platelets and red blood cells, suggesting that downregulation of the enzyme may be crucial for S1P secretion (214). In fact, SPL activity is blunted in endothelial cells in response to shear stress, resulting in increased S1P release (215). Hence, SPL can dynamically regulate S1P levels at the vascular interface.

1.7 S1P signaling: key player in vascular health

Intracellularly produced S1P gets transported out of the cells through the action of specific transporters. Spinster homologue 2 (SPNS2) was identified as specific transporter in endothelial cells. In fact, mice lacking of SPNS2 in the endothelium, show ~ 50% less circulating S1P levels (216), suggesting that the endothelium is a major source of S1P.

ABC transporters were also reported as potential S1P transporters in erythrocytes but this findings are still under discussion (217). In mammals, S1P levels are the highest in plasma (~ 1 μM) followed by lymph (~ 100 nM), whereas S1P concertation is significantly lower in the tissues, thereby generating a gradient (218).

Once released into the blood stream, S1P binds to carrier proteins. Approximately, 65% of S1P is bound to HDL, whereas around 25% to albumin and only a minor fraction to LDL and VLDL (219). S1P is associated with HDL through ApoM. Indeed, HDL particles isolated from ApoM deficient mice have undetectable levels S1P and fail to activate S1P-mediated signaling (219).

Several studies showed that the type of carrier can affect S1P half-life: particularly, 15 to 30 min when S1P is bound to albumin, while its half-life is 4-fold longer when it is associated with HDL (220).

S1P can activate five cell-surface receptors (S1PR1-5) in an autocrine or paracrine fashion (221). Endothelial cells express S1PR1-3, with the type 1 receptor being the most abundant one (Figure 3), S1PR4 is mostly present in the lymphoid system, whereas S1PR5 in the central nervous system (222). As G protein coupled receptors, S1PRs can activate different types of

Gs thus different signaling cascade: S1PR1 couples with Gαi, while S1PR2 and S1PR3 elicit couple Gαi, Gαq and Gα12/13, although S1PR2 preferentially Gα12/13, whereas S1PR3 Gαq. S1PR4-5 also couple with Gαi and Gα12/13 (222).

The S1P/S1PR1/Gαi signaling plays a key role in vascular homeostasis. Mice constitutively KO for S1PR1 die between E12.5 and E14.5 due to defect in vascular development and stabilization (223). Furthermore, studies employing conditional KO mice demonstrated that S1PR1 signaling can regulate angiogenesis, immune cell trafficking, endothelial barrier function and vasomotor tone (224–226).

Binding of S1P to S1PR1 on endothelial cells stimulates mitogen-activated protein kinase MAPK/ERK, phospholipase C (PLC), ras-related C3 botulinum toxin substrate (Rac) and phosphatidyl inositol 3-kinase cascade (PI3K/Akt/eNOS), inducing cell proliferation, migration, barrier function and vasodilation (227). Moreover, S1PR1 activation promotes intracellular receptor crosstalk, resulting in transactivation of vascular endothelial growth factor (VEGF) and PDGF receptors (228).

Oppositely to S1PR1, S1PR2 signaling is pro-inflammatory and activates Rho resulting in smooth muscle cell contraction and increased barrier dysfunction (229). Thus, changes in the expression of S1PR1 and S1PR2 plays an important role on vascular permeability. The role of S1PR3, on the other hand, is more controversial. It has been shown that S1PR3, mostly overlaps S1PR1 signaling, however, in inflammatory conditions it induces vascular permeability through stress fiber formation (230).

The type of S1P carrier, besides its half-life, affects also the interaction with its receptors and thus the signaling. For instance, the binding of HDL to SR-BI via ApoA-I may facilitate the transfer of S1P to its receptors due to spatial proximity (231).

Of note, S1P bioavailability, its receptors expression as well as the downstream coupling can result in broad range of molecular events.

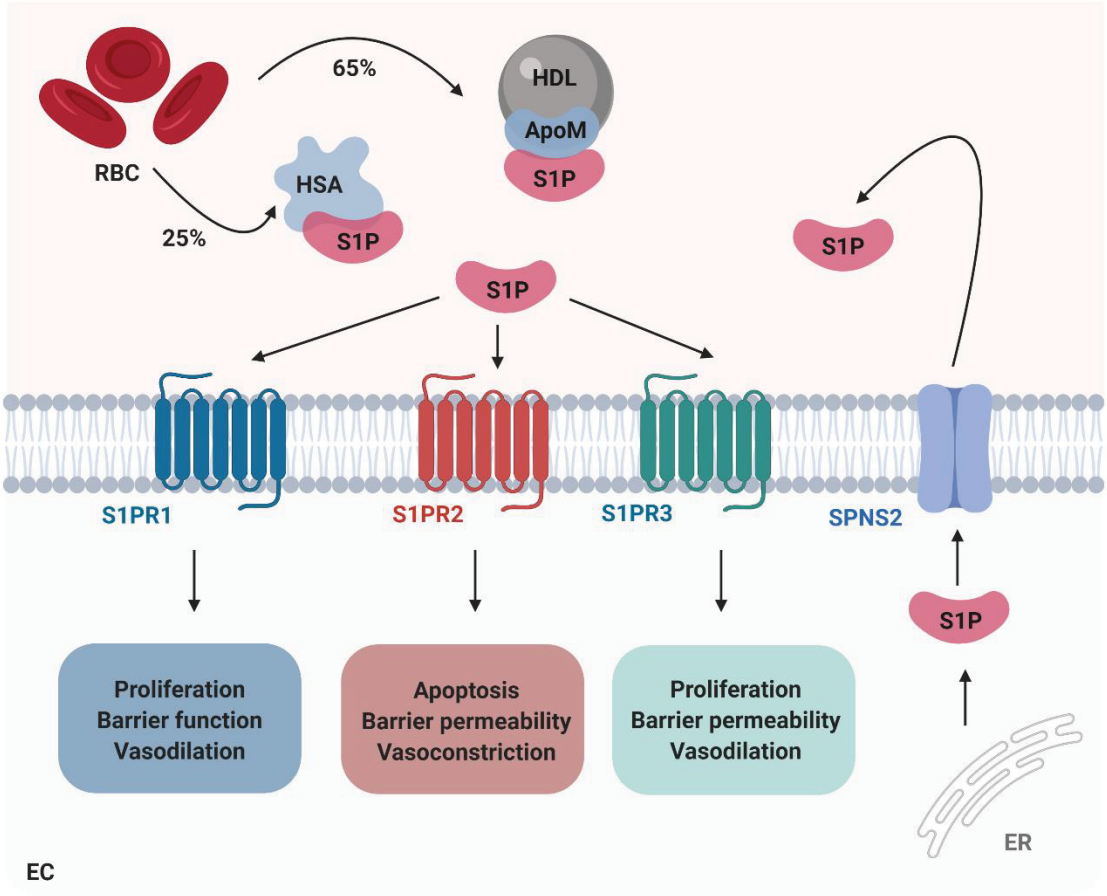

Figure 3: Schematic representation of S1P signaling in endothelial cells. RBC, Red blood cell; HDL, high density lipoprotein; ApoM, apolipoprotein M; EC, endothelial cell; HSA, human serum albumin; ER, endoplasmic reticulum; S1PR, S1P receptor; SPNS2, S1P transporter spinster 2. The figure has been created with BioRender.com.

1.7.1 S1P and endothelial cell proliferation

Proliferation of endothelial cells is crucial in the angiogenic response as well as in vascular remodeling processes during vascular inflammation/injury. S1P can induces DNA synthesis in endothelial cells via ERK activation (232). In fact, S1P-mediated endothelial cells proliferation is strongly reduced in presence of ERK inhibitors (232). Furthermore, several studies have shown that this signaling in endothelial cells is S1PR1-mediated (233).

1.7.2 S1P and the enhancement of endothelial barrier function

As mentioned above, S1P can regulate endothelial barrier function. Upon S1P/S1PR1 axis, vascular and lymphatic endothelium undergo to cytoskeleton reorganization and VE-cadherin redistribution to tighten cell-cell contacts and enhance barrier function (234).

Although, this is the case whether S1P is associated to HDL or albumin, the effect is prolonged when S1P is bound to HDL (235). Moreover, a recent study showed that HDL particles containing apoM-bound S1P, but neither apoM-deficient HDL nor albumin-S1P, can attenuate lymphopoiesis and neuroinflammation (236), pointing out that HDL-S1P may have peculiar functions compared to S1P associated with other carriers. Indeed, alteration of circulating HDL-S1P complex and impaired S1P signaling have been implicated in diseases characterized by increased vascular leakage and permeability (237–240).

1.7.3 S1P and the regulation of vascular tone

It is well-established that S1P can regulate vascular tone by stimulating endothelial cells to release bioactive substances, which can in turn affect, smooth muscles cell function. S1P mainly acts by increasing NO production in endothelial cells via eNOS (241). In vitro silencing experiments have shown that the knockdown of the S1PR1 drastically blunts eNOS cascade in response to S1P (242). Interestingly, the S1P-mediated eNOS activation is comparable to the one induced by VEGF or bradykinin (241), suggesting the great contribution of the S1P/S1PR1 signaling in the modulation of vascular tone.

1.7.4 S1P signaling in placenta

Recent progresses in lipid research have highlighted the role of bioactive sphingolipid and in particular S1P, in several aspects of reproduction. For instance, S1P signaling sustains ovarian (243) and endometrial function (244), favoring implantation and embryonic growth (245).

In the interest of placental development and function, a tight regulation of trophoblast proliferation/differentiation, as well as angiogenesis and proper vascular function are required to allow nutrients and oxygen transfer across the placenta.

Because S1P is a pleiotropic messenger in vascular development and stability (246), several studies have been carried out to unveil the role of S1P in placental biology during pregnancy. It has been described that S1PRs are differentially distributed in the human placenta (247,248), as well as the enzymes involved in S1P anabolism and catabolism (249).

In vitro studies reported that S1P can regulate trophoblast activity by inhibiting trophoblast differentiation (248), whereas downregulations of SPHK1 activity is associated with poor placental angiogenesis (244). Interestingly, the decidua of Sphk1−/−Sphk2+/− females is characterized by higher numbers of apoptotic cells with concomitant vascular dysfunction of decidual blood vessels (207). Moreover, S1P regulates vascular tone of uterine arteries in a concertation-dependent manner (250), thereby affecting blood flow within the utero-maternal circuit. Over the last decade, several studies have demonstrated that dysregulation of sphingolipid and S1P metabolism and signaling may lead to adverse pregnancy outcomes. In fact, impaired S1P signaling has been reported in term placentae of PE and IUGR subjects compared to controls (251,252). However, little is known about the physiological and pathological role of S1P at the feto-placental interface.

2. Results

This section is summarizing the results presented in the following published papers:

Del Gaudio I, Sreckovic I, Zardoya-Laguardia P, Bernhart E, Christoffersen C, Frank S, et al. Circulating cord blood HDL-S1P complex preserves the integrity of the feto-placental vasculature. Biochim Biophys Acta - Mol Cell Biol Lipids. 2020;1865(4).

Del Gaudio I, Hendrix S, Christoffersen C, Wadsack C. Neonatal HDL Counteracts Placental Vascular Inflammation via S1P–S1PR1 Axis. Int J Mol Sci. 2020 Jan 25;21(3):789.

Del Gaudio I, Sasset L, Di Lorenzo A, Wadsack C. Sphingolipid Signature of Human Feto-Placental Vasculature in Preeclampsia. Int J Mol Sci. 2020 Feb 4;21(3):1019

Perturbance of the feto-placental vascular homeostasis is associated with long-term adverse outcomes for the offspring (25,253). In adults, HDL are considered as biomarker of systemic vascular function. Specifically, circulating HDL-apoM-S1P complexes preserve the integrity of the vascular endothelium, which is impaired in the majority of cardiovascular diseases (237). However, whether cord blood derived HDLs (nHDL), which are a unique HDL fraction, play a vascular protective role during pregnancy, is still an open question. In our first work, we demonstrated that these complexes are also present in the cord blood circulation (254). By delivering S1P to S1PR1 (expressed on the feto-placental endothelium), the nHDL particles promote the activation of ERK and PLC pathways with concomitant increase of endothelial cell proliferation and calcium mobilization (254). S1P signaling was also involved in cytoskeleton reorganization and improved placental endothelial barrier function. In addition, placental chorionic arteries showed vasorelaxation upon nHDL stimulation (254). All in all, these results prove a functional role of circulating nHDL-S1P in feto-placental vasoprotection. In order to discover a potential anti-inflammatory and antioxidant effect of the nHDL *in vitro*, we exposed primary fetal placental arterial endothelial cells (fPAECs) to TNFα and AngII (255). We observed that nHDL-S1P interferes with NF-κB activation and with the expression of pro-inflammatory mediators. Moreover, the complex was able to attenuate the production of reactive oxygen species promoted by the AngII, suggesting a pivotal role of S1P signaling at the feto-placental unit in pathological conditions (255). Having proven that S1P signaling plays a functional role in the pathophysiology of the feto-placental vasculature, we decided to investigate the clinical relevance of this finding. Hence, we investigated how preeclampsia affects sphingolipid as well as S1P metabolism and signaling at the feto-placental interface. We reported a pathological sphingolipid remodeling of preeclamptic placental chorionic arteries and isolated cells, which consists in increased dihydrosphingosine and sphingomyelin

production and concomitant decrease of S1P synthesis and endothelial signaling (256). These data corroborate the concept of a diseased vascular state of the feto-placental vasculature during preeclampsia.

3. Discussion

Pregnant women undergo deep cardiovascular, anatomical and metabolic changes, which ensure nutrient and oxygen supply to the growing fetus. Failure of maternal adaptation to pregnancy may result in several complications, including GDM, PE and IUGR, which further jeopardise the mother as well as the health of the fetus.

The human placenta, which represents the functional connection between the maternal and fetal circulations, is of critical importance for mediating maternal adaptation to pregnancy. In particular, the feto-placental interface plays a major role in fetal development, as feto-placental vascular dysfunction can lead to fetal anomalies despite proper uteroplacental perfusion (11). Thus, a deeper understanding of the mechanisms which stustain feto-placental homeostasis might be of high relevance for fetal outcomes.

In adults, HDL are considered as biomarker of vascular health (101). It attracts particular attention because, in contrast with other lipoproteins, many physiological functions of HDL influence the systemic circulation in favourable ways unless HDL is modified pathologically. Besides its role in RCT, HDL mediates anti-inflammatory, anti-oxidative, anti-apoptotic and anti-atherogenic functions in the vasculature. Indeed, low levels of circulating HDL and high levels of LDL are associated with CAD and atherosclerosis (112).

In pregnancy, HDL is active in the maternal as well as in the fetal circulation. However, in pregnant women, as well as in non pregnant individuals, LDL represents the most abundand lipoprotein and the main cholesterol carrier, while in the fetal circulation HDL is the major carrier of cholesterol (153). Employing shotgun proteomics studies, our lab could demonstrate that cord blood-derived HDL, which we referred to neonatal HDL (nHDL), possesses a distinct protein cargo which differ from maternal HDL, and hence may attain other functions (154). In addition, we have previously shown that GDM affects nHDL composition and functionality. Our data revealed that, beside lower levels of PON-1 and CETP, which affect antioxidant and cholesterol efflux capacity respectively, nHDL isolated from GDM plasma samples carry significantly lower levels of ApoM (156).

Evidence from animal studies suggest that the loss of ApoM promotes vascular inflammation and leakage (257). In addition, patients with systemic lupus erythematosus (SLE), CVD or diabetes have lower levels ApoM plasma levels (238,258).

ApoM has been described as the main chaperone of the bioactive sphingolipid S1P on the HDL particle (219). In the circulation, the majority of S1P is bound HDL, while only to a lesser extend to albumin (219). Interestingly, several studies have demonstrated that S1P accounts for many of the vascular protective functions mediated by HDL (231). We therefore speculated that S1P might play an essential role in the fetal circulation as S1P-HDL complex preserving

the homeostasis of feto-placental vasculature. Thus, we measured ApoM and S1P content on nHDL isolated from female and male offspring. We demonstrated that S1P levels strongly correlate with ApoM protein on HDL, suggesting that ApoM is the main binding partner of S1P in the feto-placental circulation as well.

It has been reported that S1P production can be regulated by estrogen levels. Thus, women within the reproductive age have higher levels of S1P in the plasma compared to males with the same age (259). However, we did not find any difference in S1P content between males and females, which is not surprising given that the estrogen levels in the umbilical cord is not affected by the sex of the fetus (260). Interestingly, we detected higher plasma levels of S1P in mothers of female's newborn compared to males, suggesting a sexual dimorphism in the regulation of S1P metabolism in the placenta. A growing number of evidence is highlighting the relevance of fetal sex in placental programming, which differentially regulates fetal response to the intrauterine environment, and in turn affects maternal physiology by releasing specific proangiogenic, hormonal, and immune mediators (261,262).

The identification of neonatal HDL-S1P complex in the cord blood arises the question whether and how the complex may affect feto-placental endothelial function. To this end, we used an experimental model based on primary endothelial cells isolated from chorionic arteries (fPAECs). These cells reside in the feto-placental vasculature, thus in direct contact with fetal circulation and exposed to the action of nHDL. The pleiotropic action of S1P on the endothelium includes cell proliferation and motility, and regulation of angiogenesis, barrier function and vascular tone (232,234,241). Most of these biological processes have been attributed to the activation of S1PR1 and S1PR3 on the vascular bed (222,223).

S1PR1 is the most prominent expressed receptor on the endothelium and, to some extent shares redundant signaling functions with S1PR3, which is expressed on endothelial as well smooth muscle cells. Our data revealed that both receptors are also expressed on the feto-placental vasculature with S1PR1 being the most abundant.

The binding of S1P to its receptor elicits the activation of signaling pathways including MAPK/ERK, PI3K/Akt and PLC. Looking at the downstream effectors of S1PRs, we could detect the activation of ERK and PLC pathways upon nHDL-mediated fPAECs stimulation. While ERK activation leads to cellular proliferation, phosphorylation of PLC promotes calcium-dependent vasoregulatory signals. Both pathways are indeed crucial for proper endothelial function.

Unexpectedly and in contrast to current literature demonstrating HDL-dependent activation of Akt, we could not confirm this finding with our primary endothelial cells (219,263). However, this discrepancy could be attributed to a highly specific cellular response to HDL, which in our

case, is not only due to the specificity of the nHDL but also on the type of vascular bed, which the particle gets in contact with. A possible explanation for this might be that in our used cellular system ERK signaling takes over Akt signaling, considering that a crosstalk between the two pathways has already been described (264).

To prove that observed effect on downstream signaling was S1P-mediated, we used two inhibitors: the selective S1PR1 antagonist W146 and the non-selective S1PR1/3 antagonist VPC23019. Importantly, we found out that, in the presence of the inhibitors, the nHDL-induced ERK and PLC activation was drastically abolished. In addition, our data identified S1PR1 as major contributor in the nHDL signaling cascade, due to the evidence that pharmacological inhibition led to a similar effect. Corroboratively, analysis on transcriptional level showed 3-fold higher expression of S1PR1 compared to S1PR3 in fPAECs.

To further evaluate, the cellular response following ERK and PLC activation we employed BrdU and calcium mobilization assays. In agreement with previous observation, nHDL-S1P could induce endothelial cell proliferation, which is linked to placental angiogenesis, and elevation in intracellular calcium concentration, which affects regulation of vascular tone as well as barrier promoting functions (263,265).

During gestation, a complex interplay between pro- and anti-angiogenic factors and pro and anti-inflammatory mediators, sustain the physiological function of the feto-placental endothelial barrier (25). Structurally, the integrity of the endothelial barrier depends on the formation of cell–cell contacts as well as on the dynamic regulation of actin and myosin-induced cellular rearrangements (32). Hence, it has been demonstrated that hyperglycemia during pregnancy increases the permeability of placental endothelial cells by affecting adherens and tight junctions connection, thereby perturbing barrier integrity (36,37). Christoffersen and colleagues reported that HUVECs stimulated with apoM-containing HDL or albumin-S1P, undergo reorganization of actin and adherens junctions towards a barrier promoting phenotype (219). In agreement with this study, treatment of fPAEC either with nHDL or S1P associated with human albumin (S1P-HSA) led to a reshape of the actin cytoskeleton to cortical actin ring formation and strengthening of the adherens junction through VE-cadherin assembly. Importantly, these effects were prevented by S1PRs antagonists W146 and VPC23019, suggesting that S1P triggers the nHDL-induced cytoskeleton remodeling of the feto-placental barrier function.

To further investigate whether, the morphological changes observed in fPAECs could result in an improved mechanotransduction and barrier function, we employed an electrical cell-substrate impedance sensing system (ECIS). A confluent monolayer of fPAECs, was treated with nHDL or S1P-HSA alone, or in presence of inhibitors, and endothelial barrier function was

assessed in real-time. We demonstrated that both nHDL and S1P-HAS extended fPAEC endothelial barrier enhancement compared to S1P-HSA alone, which is in line with the work from Wilkerson et al (235). Indeed, S1P associated with nHDL particles maintains cellular resistance longer than albumin, highlighting a carrier-dependency in the S1P signaling in the placenta as well. Although, our work did not aim to compare the carrier effect on cellular response, we did observe that nHDL could longer and steadily sustain barrier impedance, despite the different levels of S1P on the two carriers (1µM associated with albumin vs 100nM associated with nHDL).

Activation of S1P/S1PR1 cascade has been describe to promote endothelial barrier function (227). Conversely, S1PR3 signaling is associated with increased barrier permeability (229,230). Our data showed that S1PR1 inhibition abolished the barrier-promoting effects induced by nHDL, corroborating the beneficial role of S1PR1 signaling on feto-placental endothelial barrier function. However, these findings are restricted to physiological pregnancy conditions. Additional studies are warranted to understand whether S1PR3 is involved in the regulation of vascular permeability in pathological conditions. In pregnancy, placental vascular homeostasis is strictly dependent on proper endothelial function, barrier integrity and vascular tone.

In particular, the feto-placental vasculature lacks autonomic innervation, thus the vascular tone is modulated mainly by the endothelium (14). This concept is of high importance considering that the vascular resistance at the feto-placental interface influences the blood flow to the fetus. Endothelial-derived hormones and vasoactive substances such as NO, prostaglandins, and endothelium-derived hyperpolarizing factor (EDHF) are major players in the regulation of vascular tone. An inbalance in synthesis and metabolism of these vasomodulators can result in increased feto-placental vascular resistance, due to altered tissue sensitivity in response to stimuli (14). For instance, it has been demostrated that chorionic arteries from PE and hypertensive pregnancies show increased vasoconstriction in response to AngII and endothelin-1, thereby contributing to placental vascular dysfunction and reduced blood flow to the fetus (15,17). On the other hand, reduced NO levels in the maternal as well as in the feto-placental circulation have been reported in pathological pregnancies (18,19).

Since S1P is capable to promote NO production in endothelial cells, we hypothesized that nHDL could acitivate eNOS signaling in fPAECs in a S1P-dependent manner and thus regulate vessels vasodilation. Our results demonstrated that nHDL could efficiently induce eNOS activation via S1P. In addition, chorionic arteries stimulated with nHDL showed a pronounced vasorelaxation and may lead to the identification of new points for eNOS regulation in placental vascular endothelial cells.

Nofer et al. described that the vasodilatory action of HDL in mice rings partially relies on the signaling of bioactive lysophospholipids associated with HDL via S1PR3 (263). Despite our data corroborating the role of HDL as potent vasodilator, we did not observe any significant difference in vessels relaxation after pharmacological inhibition of S1PRs, suggesting only a mild contribution of S1P signaling in nHDL-mediated vasodilation. Of note, enzymatic removal of S1P from nHDL, significantly impaired the capability of the particle to promote vasorelaxation, suggesting that S1P plays a role in nHDL-induced regulation of vascular tone in the placenta. However, the specific involvement of S1P signaling in the regulation of feto-placental vascular reactivity is still elusive. Moreover, it needs to be considered that HDL can also induce vasodilation via SR-BI, independently of S1P (117). Thus, although nHDL can elicit eNOS activation via S1P, the vasodilation of chorionic arteries is the result of a complex interplay between different contributing pathways.
These findings have significant implications for the understanding of how the placental vascular integrity is locally regulated by fetal circulating mediators as nHDL-S1P in normal pregnancy.

Given the well-established anti-inflammatory and anti-oxidative effect of HDL, we next investigated whether nHDL could counteract feto-placental inflammation and endothelial dysfunction. Systemic low-grade inflammation in pregnant mothers is part of a physiological phase during pregnancy. Indeed, an inflammatory environment is required for an healthy maternal adaptation to pregnancy, especially in the first trimester (266). However, much research has been done identifying the adverse effects on fetal development caused by a derailed maternal inflammatory response in pregnancy (267,268). Of particular importance, researchers have been devoted to shed a light on the involvement of the placenta in abnormal inflammation during pregnancy. Nevertheless, the mechanisms regulating placental inflammatory response and dysfunction are still poorly understood.
Since the feto-placental endothelium is directly connected to the fetal circulation, any dysfunction of endothelial homeostasis might result in impaired fetal growth. Aberrant inflammatory response at the feto-placental interface can lead to altered endothelial cell phenotype and impaired vascular tone, features which have been associated with pregnancies complicated by diabetes, IUGR and PE (269,270). In particular, PE has been associated with elevated levels of the pro-inflammatory cytokine TNF-α, which promote endothelial activation and damage (99).
Using a PCR-based gene panel we demonstrated that nHDL and HSA-S1P could suppress the induction of several pro-inflammatory mediators (genes clustering with TNF-receptor

superfamily, apoptosis and NF-κB pathway) in fPAECs. In contrast, both complexes could rescue the expression of nitric oxide synthase 3 (NOS3), which contributes to the production NO in the vascular bed. Although, both nHDL and HSA-S1P were able to restrain the expression of endothelial inflammatory markers, nHDL treatment was more effective, suggesting a carrier-driven regulation of S1P anti-inflammatory effect. TNF-α evokes the production of cytokines and adhesion molecules for leukocytes' enrolling during vascular inflammation (38,39). In more detail, we could show that in fPAECs exposed to inflammatory condition and treated with nHDL could suppress the expression of inflammatory mediators such as VCAM, ICAM, E-selectin, MCP-1 and IL-8. In addition, fPAECs pre-treatment with W146 and VPC23019 inhibitors, blunted the protective action of nHDL against TNF-α-induced inflammation. Moreover, the results obtained from S1PRs inhibition, revealed that most likely S1PR1 is the main player in the nHDL-dependent anti-inflammatory function, as already claimed by others (226).

The work of Ruiz and colleagues has previously shown that human adult HDL^{+}ApoM was able to suppress the TNF-α-induced expression of VCAM, ICAM and E-selectin, whereas HDL^{-}ApoM (with undetectable amount of S1P), lacked this capability (239).

In agreement with their findings, our flow-cytometry experiments showed that expression of VCAM and ICAM on fPAECs was attenuated in presence of nHDL. However, E-selectin expression was only reduced at mRNA but not at the protein level. Nonetheless, this discrepancy could result from the different settings in the experimental approach including type of vascular bed, type of HDL and also the used incubation time to induce the inflammatory status.

TNF-α as well as oxidative stress have long been known to induce NF-κB signaling. In fact, placentae from PE pregnancies show an aberrant activation of the pathway compared to healthy ones (42–44). It has been shown that TNF-α- induced NF-κB phosphorylation is reduced by WT mice-derived HDL, whereas is mostly preserved in presence of HDL isolated from ApoM KO mice (226), arguing for an S1P-dependent HDL effect. Furthermore, they could demonstrate that in HUVECs, human adult HDL is equally effective. Nevertheless, for these set of experiments, the authors used native adult HDL particles, thus they could not confirm a direct link between HDL effect on NF-κB inhibition and the involvement of S1P signaling.

In agreement with their work, our findings corroborate the concept that nHDL limits feto-placental endothelial inflammation by blocking NF-κB signaling activation. In addition, the pharmacological inhibition of S1PRs, confirmed the direct participation of S1P action in the observed effect.

Perturbation of cellular oxygen balance and increased ROS production is considered a hallmark of inflammation-associated endothelial damage (45). Several reports demonstrated a causative role of placental dysfunction and increase of ROS in correlation with high blood pressure during pregnancy (271,272).

The enzyme NAD(P)H oxidase is a key player in the regulation of ROS in the vascular bed, and its activity can be affected by different stimuli (53). Tölle et al., for example have shown that HDL-associated bioactive lipid could downregulate NAD(P)H oxidase activity upon thrombin treatment in smooth muscle cells (273). Here, we show that nHDL limits AngII-induced ROS production in fPAEC through S1P signaling. In addition, we further clarify a potential mechanism by which S1P signaling could preserve feto-placental endothelium from oxidative stress. In fact, we observed that nHDL and HSA-S1P treatment reduce the expression of the NAD(P)H oxidase catalytic subunit Nox1.

These findings indicate that nHDL–S1P complexes represent a powerful and efficient anti-inflammatory mechanism to regulate feto-placental inflammatory response and thereby protecting the fetus in normal pregnancy. It is important to bear in mind that these findings cannot be directly translated to the complex pathophysiological scenario of all pregnancy's complications. Hence, further studies investigating the effects of specific pregnancy-related disorder on nHDL and placental function are needed.

Although the HDL associated S1P is considered to be the biologically active in the systemic circulation, locally produced S1P is also of great importance for vascular physiology (162,274). Thus, the understanding of the mechanisms regulating sphingolipid metabolism and concomitant endogenous S1P bioavailability, may have a number of important implications for future studies. Dysregulation of sphingolipid metabolism has been linked to the onset of many cardiovascular and metabolic diseases (169). Interestingly, several studies have pointed out a key role of sphingolipids in different features of reproduction including placental angiogenesis, decidualization and trophoblast differentiation (207,244,248).

Sphingolipid profile analysis in plasma of pregnant women diagnosed with PE have been performed with the goal of finding potential biomarker for this disorder. Nonetheless, these studies led to conflicting results (202,275). Most of the studies have approached the disorder from a maternal point of view. However, little is known about the consequences on placental physiology and fetal outcomes.

Based on these considerations we focused our investigations on the feto-placental vasculature, aiming to understand how PE affect metabolism of sphingolipids in placental blood vessels, which are a crucial part of the fetal systemic circulation. Our study showed a pathological sphingolipid remodeling as well as an altered gene profile in placental chorionic

arteries from PE pregnancies. Ceramide species which are composed of sphingosine and a fatty acid exert different biological functions depending on their acyl chains (276). Generally, accumulation of C16:0, C18:0, and C20:0 have been linked with anti-apoptotic function (277), whereas C24:0 (very long chain ceramide), is anti-apoptotic (197). In agreement with the analysis of Romanowicz et al. in umbilical cord arteries (UCA), we found that PE was associated with a mild reduction in total ceramide levels, whereas only C20:0-Cer levels were statistically significant reduced (278). Conversely, the study by Melland-Smith and colleagues reported an increase in C18:0, C20:0, and C24:0 ceramide species in PE placentae compared to control (201). This could be explained because they analyzed total placental tissues, whereas we specifically isolated chorionic arteries. Furthermore, it is well recognized that sphingolipid synthesis is differentially regulated depending on the cell type (170).

Of note, several studies reported a strong correlation between plasma ceramide ratios and the incidence in cardiovascular events. The study by Peterson et al. revealed a negative correlation between C24:0/C16:0 ratio in plasma and the risk of cardiovascular events in a CAD cohort (198), indicating that the ceramide profile might be representative for pathological outcomes. However, in PE and on cellular level this observation could not be confirmed.

We found that chorionic arteries of PE placentae show increased in SPT activity, dhSPh and SM levels, indicating that a peculiar sphingolipid remodeling takes place during PE compared to CAD. Elevation of dhSPh measured by LC/MS was supported by the increased SPT activity in PE. Accumulation of intracellular dhSph have been associated with lipotoxicity in diabetes and cardiovascular diseases (279,280). Therefore, it is reasonable to think that it might also play a role in the vascular dysfunction observed in PE.

Adverse cardiovascular outcome can also arise from an imbalance in the SM homeostasis (281). Moreover, inflammation and insulin resistance positively correlate with SM levels (282). Interestingly, PE placentae showed increased levels of SM in UCA as well as in microvesicles of syncytiotrophoblasts (283,284).

In agreement with these previous studies, we reported an elevation of C16:0-, C18:0-, and C24:0-SM in PE chorionic arteries compared to healthy subjects. Interestingly, the C18:0-SM species was also found to be increased in placental lipid rafts and plasma of PE subjects (285,286), suggesting that this specific species might be involved in the pathogenesis of the disorder. Despite all sphingolipids have been implicated in pathophysiological processes, S1P is the most studied and well described for its pleiotropic function and its potent biological activity.

Recently, it has been shown that SPHK1/S1P/S1PR1 signaling plays a crucial role in pregnancy by promoting placental angiogenesis (287). Moreover, PE blunts S1P signaling in

term placentae by downregulation of SPHK1 and S1PR1/3 expression (251). In line with these findings, we observed in PE endothelial cells isolated from chorionic arteries an increased in the expression of SGPL1, SGPP1, and S1PR2, accompanied by a transcriptional as well as protein decrease of S1PR1. These findings indicate a reduced production of endothelial-derived S1P, along with the shifting of the signaling transduction from S1PR1 to S1PR2. S1PR2 is reported as a pro-inflammatory receptor involved in endothelial dysfunction and increased vascular resistance (288,289). This might explain the overexpression of S1PR2 in endothelial cells as well as in chorionic artery from PE pregnancies, which are characterized by hypertension and systemic inflammation.

Additionally, SPTLC1 and SPTLC2 expression was increased, suggesting an upregulation of the *de novo* biosynthesis, as previously described in an inflammatory and hypertensive environment (173). Consistently, SPT enzymatic activity was higher in PE chorionic arteries compared to healthy pregnancies. Importantly, the upregulation of SPT activity did not lead in accumulation of all the sphingolipid species. In fact, total ceramide content was only slightly affected. On the contrary, SM were significantly increased in PE arteries. However, this evidence is not unexpected considering that post-translational modification can differentially affect multiples enzyme involved in metabolic homeostasis. Nogo-B has been recently described as antagonist of SPT activity (162). Nogo-B KO mice in the endothelium are preserved from hypertension and heart failure (162,290) due to enhanced local S1P S1PR1 signaling activation. Our study reported that Nogo-B had a higher expression in the endothelium of chorionic arteries and in isolated cells of PE placentae. This evidence suggests that Nogo-B overexpression in fPAECs of PE placentae might play a role in the pathogenesis of the disorder. However, we cannot draw any conclusion about the function of Nogo-B in the overall vessel, considering that its expression in smooth muscle cells is much lower compared to the one in the endothelium.

Taken together our findings suggest that PE induces a sphingolipid dependent remodeling of the feto-placental vasculature which contributes to the pathological phenotype of the endothelium.

The data from this thesis uncover a pivotal role of S1P and sphingolipid biology in a human model organ. Many researchers employ animal models to investigates different aspects of placental function and dysfunction during pregnancy, due to the easier manipulation of system. However, placenta physiology strongly differs between humans and mice. The human placenta represents therefore a robust, easily accessible and more reliable model to investigate physiological and pathophysiological processes occurring during pregnancy.

The novel finding of protective HDL-S1P complexes within the cord blood circulation, argues for a potential mechanism by which the fetus protects himself from the maternal environment. Indeed, a pathological remodeling of the complex during GDM or PE might contribute to the observed higher risk of developing cardiovascular and metabolic disorders for the offspring later in life.

HDL and S1P infusion therapy have been proposed in the context of atherosclerosis and CAD. However, there are no currently available studies demonstrating a mortality benefit. This is most likely due to the complex interplay which links HDL, S1P and tissue and cellular responses.

An S1P mimetic (FTY720), has been approved by the FDA to treat multiple sclerosis. However, the immunosuppressive effect showed by the drug, might have a drawback effect on vasculature homeostasis, as reported by Cantalupo et al. (274). Thus, the potential use of this drug in the context of pregnancy disorders creates concerns.

In the light of our studies, a better therapeutic approach would be targeting S1P signaling and/or metabolism selectively on endothelial cells, which control and sustain feto-placental vascular homeostasis.

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Circulating cord blood HDL-S1P complex preserves the integrity of the feto-placental vasculature

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ABSTRACT

Perinatal and long-term offspring morbidities are strongly dependent on the preservation of placental vascular homeostasis during pregnancy. In adults, the HDL-apoM-S1P complex protects the endothelium and maintains vascular integrity. However, the metabolism and biology of cord blood-derived HDLs (referred to as neonatal HDL, nHDL) strikingly differ from those in adults. Here, we investigate the role of neonatal HDLs in the regulation of placental vascular function. We show that nHDL is a major carrier of sphingosine-1-phosphate (S1P), which is anchored to the particle through apoM ($r^s = 0.90$, $p < 0.0001$) in the fetal circulation. Furthermore, this complex interacts with S1P receptors on the feto-placental endothelium and activates specifically extracellular signal-regulated protein kinases 1 and 2 (ERK) and phospholipase C (PLC) downstream signaling, promotes endothelial cell proliferation and calcium flux. Notably, the nHDL-S1P complex triggers actin filaments reorganization, leading to an enhancement of placental endothelial barrier function. Additionally, nHDL induces vasorelaxation of isolated placental chorionic arteries. Taken together, these results suggest that circulating nHDL exerts vasoprotective effects on the feto-placental endothelial barrier mainly via S1P signaling.

1. Introduction

Sphingosine-1-phosphate (S1P) mediates, as a bioactive phospholipid, a variety of cellular processes and it is highly abundant in the plasma (~1 μM). Its major cellular sources are erythrocytes and the vascular endothelium, which critically contribute to maintain the S1P pool in the plasma [1]. Once released extracellularly, S1P mainly binds to high density lipoprotein (HDL) through interaction with apolipoprotein M (ApoM) and to a lesser extent to serum albumin [2]. S1P signals through specific G protein-coupled receptors (GPCRs), which are ubiquitously expressed and coupled to a variety of G proteins [3]. Nonetheless, S1P evokes distinct physiologic functions depending on the relative expression of S1P receptors (S1PRs) as well as G proteins. Findings from a growing number of studies indicate that S1P is a mediator of many of the cardiovascular-protective effects of HDL, such as anti-oxidative and anti-inflammatory activities, including the ability to enhance endothelial barrier function and to induce vasodilatation through the induction of nitric oxide and prostacyclin synthesis [4–6]. The positive effect of this complex and its regulatory function have been demonstrated both in vitro and in vivo. Indeed, the functionality of HDL-S1P complex is highly diminished in cardiovascular diseases and diabetes [7–9]. The complex bioactivity on different vascular beds was

Abbreviations: S1P, sphingosine 1-phosphate; nHDL, neonatal high-density lipoprotein; ApoM, apolipoprotein M; ApoA-I, apolipoprotein A1; ApoE, apolipoprotein E; GPCRs, G protein-coupled receptors; S1PRs, sphingosine 1-phosphate receptors; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; ERK, extracellular signal-regulated protein kinases 1 and 2; PLC, phospholipase C; HPLC, high-performance liquid chromatography; SGPL1, sphingosine 1-phosphate lyase1; SR-BI, scavenger receptor class B type I; HPAEC, human placental arterial endothelial cells

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broadly studied in animal models. However, little research has been conducted to understand how S1P regulates vascular function at the human feto-placental unit [10]. The placenta plays a crucial role during pregnancy, being the interface between mother and fetus. It anchors the conceptus, provides an interface for exchange of nutrients, lipids and gases, and possesses endocrine functions, but also acts as an immune barrier between the mother and the semi-allogeneic fetus [11]. The placental tissue is in a constant state of growth throughout gestation with a highly regulated development and adaption of its vasculature. Any alteration applied to placental vascular homeostasis may have an effect on subsequent placental function and hence fetal growth and development [12]. While apolipoprotein A1 (ApoA-I) is the major structural component in adult HDL, the nHDL are specifically rich in apolipoprotein E (ApoE), highlighting the peculiar biology of the particle [13]. Despite most of the apolipoprotein and lipids were found reduced in the neonatal particles, we previously demonstrated that ApoM is similarly present on neonatal and adult HDL proteome [14]. This could be seen as an indicator of the high relevance of S1P signaling not only in adults but also in fetal physiology. Based on these findings we hypothesize that nHDL is a carrier of S1P and that this complex could play a pivotal role in fetal development in late pregnancy by regulating placental vascular homeostasis. To the best of our knowledge this is the first study which investigates the role of nHDL-S1P complex in its own physiological environment, namely the endothelium of human placental vessels, which is in direct contact with neonatal blood. Here we aimed at uncovering the S1P biology in the fetal circulation by identifying the protective properties mediated by the nHDL-S1P complex and its underlying molecular mechanisms.

2. Materials and methods

2.1. Study population

Women without pregnancy complications (N = 20) were recruited at the time of delivery and gave written informed consent. The study design was approved by the ethical committee of the Medical University of Graz (29-319 ex 16/17). All women in the study were selected based on a negative oral glucose tolerance test at 24–28 weeks of gestation and absence of medical complications during pregnancy. Clinical characteristics of the subjects are summarized in Online Resource 1.

2.2. Blood sampling and plasma preparation

Umbilical cord blood was taken 10 min after delivery at the latest. The neonatal cord blood plasma was collected from 10 males and 10 female newborns as mixed blood from umbilical arteries and vein. EDTA-plasma was isolated by centrifugation for 15 min at 2000*g* at 4 °C and stored at −80 °C until further analyses.

2.3. Isolation and characterization of HDL

Neonatal HDLs were isolated by an adapted ultracentrifugation method [15,16]. Briefly, EDTA-plasma was centrifuged in an Optima XE-90 ultracentrifuge using the TI/70.1 rotor (Beckman Coulter, CA, USA). After adjusting plasma density to 1.24 g/ml with potassium bromide, 1.7 ml plasma was transferred to a Quick-Seal Bell-top tube (Beckmann Coulter, CA, USA), covered with a potassium bromide solution (density 1.006 g/ml) and centrifuged at 100,000 rpm for 3 h. 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. Isolated HDL was stored in salt at 4 °C, covered with argon gas to avoid oxidation. Prior to further analyses HDL was desalted with PD-10 columns with sephadex (GE Healthcare, Vienna, Austria). Characteristics and purity of isolated HDLs were confirmed by lipid diagnostics. Protein concentration was determined by BCA protein assay kit (Pierce, Thermo Scientific, Rockford, IL, USA), while HDL cholesterol was measured by colorimetric method (Greiner Diagnostic, Bahlingen, Germany). Due to the small amounts of plasma obtained from the umbilical cord, individual neonatal HDL isolations were pooled (mixed females and males) for the experiments.

2.4. ApoM ELISA

Quantification of ApoM was performed as previously described [17]. Briefly, Costar high binding 96 well plates (Corning, New York, NY, USA) were coated with capture antibody against human ApoM (M03, 50 µl/well Abnova, Taipei, Taiwan) diluted to 5 µg/ml in TBS and incubated overnight. The plates were blocked by adding 200 µl of blocking solution containing 2% BSA in TBS for 2 h. 90 µl of DTT solution (50 mmol/l DTT in a 0.2 mol/l sodium phosphate buffer) were added to 10 µl of sample at 30 °C for 15 min to unfold apoM. Thereafter, 100 µl of iodoacetamide solution (0.6 mol/l iodoacetamide in a 0.02 mol/l sodium phosphate buffer) were added to each well for 1 h, protected from light, to prevent reformation of disulfide bridges. Subsequently, the samples (diluted around 50 times in dilution buffer containing TBS with 1% Triton X-100 and 1% BSA) were transferred to the ELISA plate for overnight incubation. The plate was washed three times with TBS followed by at least 3 h incubation with 75 µl of detection antibody against human apoM (1:1000; ab91656; Abcam, Cambridge, UK) diluted in TBS containing 1% BSA and 2% Triton-X 100. The plate was washed three times with TBS and 0.1% Triton-X 100 and incubated for 2 h with 75 µl of HRP-conjugated anti-rabbit IgG antibody (diluted 1:2000 in TBS buffer containing 1% BSA and 0.1% Triton-X 100), followed by development with SIGMAFAST™ OPD system (Merck, Darmstadt, Germany). Absorbance was read at 492 nm by using an ELISA reader (SPECTROstar Nano, Bmg Labtech Ortenberg, Germany). The obtained ApoM concentrations were calculated from the standard curve included in each assay run.

2.5. S1P quantification

S1P content in plasma (75 µl) and in neonatal HDL fractions (150 µl), was measured by HPLC as described elsewhere [18]. In brief, the S1P extraction was performed with chloroform-methanol in a two-step procedure followed by derivatization with 2,3-naphthalenedicarboxaldehyde. Subsequently, derivatized samples were analysed with an Agilent 1290 HPLC (Agilent, Santa Clara, CA, USA) using a Synergi 4u Fusion-RP 80A column (30 × 2.0 mm; Phenomenex) with a flow of 0.5 ml/min. The used solvent phases were as follows: mobile phase composed of 85% acetonitrile with 15% methanol (HPLC grade; Rathburn Chemicals Ltd., Walkerburn, UK), and aqueous phase composed of 20 mmol/l H_2KPO_4 (pH 4.8) and 15% methanol (HPLC grade; Merk, Darmstadt, Germany). The separation was achieved by using a gradient of the mobile phase: 0–6 min, 47.5%; 6–9 min, 47.5–87.5%; 9–10 min, 87.5%; 10–12 min, 87.5–47.5%; and 12–15 min, 47.5%.

2.6. S1P depletion

S1P depletion on neonatal HDL was achieved by incubating 1 nmol S1P-HDL with 5 µg of active human sphingosine-1-phosphate lyase1 SGPL1 (SRP0191, Merck, Darmstadt, Germany) dissolved in PBS for 60 min at 37 °C. The above mentioned HPLC-based method was used to determine S1P content on HDL.

2.7. Human placental arterial endothelial cells (HPAEC) isolation and treatment

Primary HPAEC were isolated from 12 healthy term placentae using a standard protocol [19]. HPAEC were characterized by immunocytochemistry and internalization of acetylated low-density-lipoprotein (Biomedical Technologies, Stoughton MA). Isolated cells were cultured on 1% gelatin-coated T75 flask using Endothelial Basal

Medium (EBM) (Lonza, Basel, Switzerland) supplemented with the EGMTM-MV BulletKit (Lonza Basel, Switzerland). The purity of isolated cell populations was assessed by indirect immuno-fluorescence staining with the following markers: Von Willebrand Factor (vWF; A0082, Agilent, Santa Clara, CA, USA); CD31 (mon60021, Sanbio BV, Uden, Netherlands); CD90 (DIA100, Dianova, Hamburg, Germany); Actin smooth muscle (M0851, Agilent, Santa Clara, CA, USA); Vimentin (M0725, Agilent, Santa Clara, CA, USA); MsX Fibroblasts (CBL271, Merck, Darmstadt, Germany); Desmin (M0760, Agilent, Santa Clara, CA, USA). In all the experiments HPAEC were treated with the following compounds unless specified otherwise: (i) nHDL (200 μg/ml) dissolved in PBS (neonatal HDL particles contained ~0.507 ± 0.154 nmol of S1P per mg of total HDL protein, as measured by HPLC. Thus, HPAEC were exposed to (ii) ~100 nmol/l S1P); (ii) S1P complexed with human serum albumin named as S1P-HSA (360,492, Huzzah® S1P, Avanti Polar Lipids, Alabaster, USA) (used in a concentration of 1 μmol/l [20] as a positive control); (iii) selective inhibitor of S1PR1 - W146 (1 μmol/l) (857,390, Avanti Polar Lipids, Alabaster, USA) dissolved in PBS containing Na_2CO_3 and 20% (2-hydroxypropyl)-beta-cyclodextrin according to the product data sheet, and (iv) S1PR1/3 inhibitor VPC23019 (1 μmol/l) (4195, Tocris Bioscience, Bristol, UK), dissolved in DMSO. When cells were treated with W146 or with VPC23019, Na_2CO_3, (2-hydroxypropyl)-beta-cyclodextrin and DMSO respectively, were added as vehicle in the control settings.

2.8. Immunoblotting

Lysates of total cell (10 μg of proteins) were separated by 4–20% gradient gel for 60 min at 120 V, 400 mA and transferred by electrophoresis to 0.2 μm nitrocellulose membranes (Trans-Blot Turbo Mini Nitrocellulose Transfer Membrane, BioRad, Hercules, CA, USA) using the Trans-Blot Turbo Transfer System (BioRad, Hercules, CA, USA). Membranes were blocked at RT in blocking buffer (5% non-fat dry milk in 1× TBE buffer) for 1 h, and then incubated with primary antibody overnight at 4 °C. Proteins were detected by using specific primary antibodies against: S1PR1 (1:10000; ab125074, Abcam, Cambridge, UK);S1PR3 (1:1000; ab126622, Abcam, Cambridge, UK), Hsp90 (1:2000; 610,418; BD Bioscience; East Rutherford, New Jersey, NJUSA); p-ERK (1:2000; 9106; Cell Signaling, Danvers, MA, USA); ERK (1:2000; 9107; Cell Signaling, Danvers, MA, USA); p-PLC(1:500; 2481; Cell Signaling, Danvers, MA, USA); PLC (1:500; 14,247; Cell Signaling, Danvers, MA, USA); p-eNOS ((1:500; 9571; Cell Signaling, Danvers, MA, USA); eNOS ((1:500; D9A5L; Cell Signaling, Danvers, MA, USA). Signals were visualized using the ECL development method (SuperSignal West Pico or Femto Chemiluminescent Substrate; Pierce, Thermo Scientific, Rockford, USA) and quantified densitometrically using EvolutionCapt (Vilber Lourmart, Collégien, France) software.

2.9. RNA isolation and quantitative real-time PCR

Isolation of total cellular RNA was performed using RNeasy Mini kit (QIAGEN, Hilden, Germany). Quality and concentration of obtained RNA was determined by measuring 260/280 ratio using the Scandrop 250 (Analytik Jena AG, Germany). cDNA was generated using iScript cDNA Synthesis Kit (BioRad, Hercules, CA, USA,). For quantitative real-time PCR (RTQ-PCR) the AB7900–2 Syllabus thermal cycler (Life technologiesTM, Vienna, Austria) was used. The cycling conditions were: 2 min at 50 °C, initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and an annealing temperature of 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. Total term placental tissue was used as positive control. The results were normalized to the house-keeping gene cyclophilin A (PPIA) (Hs04194521_s1, ThermoFisher Scientific, Waltham, MA, USA).

2.10. Proliferation assay

HPAEC were seeded into a 96-well plate in 100 μl of complete medium and cultured overnight. Thereafter, the medium was replaced with 1% FCS medium and the cells were pre-incubated with W146 (1 μmol/l) and VPC23019 (1 μmol/l) for 30 min then treated with 1 μmol/l S1P-HSA and 400 μg/ml of total neonatal HDL protein for 24 h. The cells were labelled with 10 μl/well of BrdU solution for 3 h. Afterwards cells were fixed (200 μl/well of FixDenat), incubated with 100 ml/well of Anti-BrdU-POD working solution for 90 min and washed 3 times and subsequently the substrate solution (TMB) was added according to the manufacturer's instructions (colorimetric Cell Proliferation ELISA, BrdU- Roche, Basel, Switzerland). Absorbance was read at 492 nm with the ELISA plate reader SPECTROstar Nano.

2.11. Calcium mobilization assay

To measure changes in intracellular Ca^{2+} levels HPAEC were seeded in 96 black/clear bottoms well assay plates (Corning, New York, NY, USA). After reaching confluency or prior to Ca^{2+} measurements the cells were pre-incubated with W146 and or VPC23019 for 1 h, then the calcium dye (FLIPR® Calcium 6, Molecular devices, CA, USA) was added for 2 h at 37 °C 5% CO_2. After incubation, the assay plate (containing the cells) as well as the compound plate (with vehicle, S1P-HSA and nHDL) were transferred directly to the FlexStation® to be assayed. Thrombin (3u/ml) was used in each experiment as positive control. The fluorescence intensity was recorded with excitation wavelengths of 485 nm and an emission wavelength of 525 nm. Data were analysed using SoftMax® Pro software.

2.12. Electrical cell-substrate impedance sensing (ECIS Z system)

To determine the effects of neonatal HDL on the barrier function, impedance measurements were performed using an ECIS Z System (Applied Biophysics, Troy, USA). HPAEC were plated on gelatine-coated gold electrodes of 8W10E+ arrays and serum-starved. The impedance was recorded in real time at 1 min intervals at 4 kHz. After reaching stable impedance, they were treated with nHDL, S1P-HSA and S1P inhibitors W146 or VPC23019. Serum-free EBM was used as a control. Analysis of the data was performed using ECIS software (Applied Biophysics, Troy, USA).

2.13. Immunofluorescence staining

HPAEC were seeded in gelatin-coated chamber slides. After 24 h, the cells were serum starved for 3 h, then pre-incubated with W146 or VPC23019 inhibitors for 30 min and followed by treatment with 1 μmol/L S1P-HSA or 200 μg/ml nHDL protein for 1 h. 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 washed three times with PBS and then blocked with Ultra Vision Protein Block (TA125PBQ, ThermoFisher Scientific, Waltham, MA, USA) for 25 min at room temperature. After blocking, the primary antibody for VE-cadherin (monoclonal mouse anti human, sc-9989 Santa Cruz, CA, USA) was applied for 1 h at room temperature. Following secondary antibodies were used: DyLight633 (goat anti mouse 5 μg/ml, ThermoFisher Scientific, Waltham, MA, USA), Alexa Fluor® 488 Phalloidin (A12379, Life Technologies, Invitrogen, Carlsbad, CA, USA) and FITC (goat anti mouse 10 μg/ml, BD Pharmingen). Slides were counterstained with DAPI (4′,6 diamidino-2-phenylindole, Invitrogen, 2.5 μg/ml), mounted with ProLong Gold antifade reagent (P36930, Life Technologies, Invitrogen, Carlsbad, USA) and analysed by fluorescent microscopy using a Zeiss LSM 510 Meta microscope, objective Plan-Apochromat 639/1.4 Oil DIC, lasers 405, 488 and 633 nm and LSM Image Browser software.

2.14. Wire myography

Term placentas obtained within 30 min after delivery exclusively from normal pregnancies were used for experiments. A calibrated eyepiece micrometer was used to obtain chorionic plate arterial rings of approximately 2 mm in length and ~50 μm in diameter. Arterial rings were maintained in Krebs-Henseleit buffer (Sigma Aldrich, Schnelldorf, Germany) modified and prepared in deionized water (6.9 g/l NaCl, 0.35 g/l KCl, 0.16 g/l KH_2PO_4, 0.141 g/l $MgSO_4$, 2.1 g/l $NaHCO_3$, 2 g/l D-glucose, 0.97 g/l EDTA•$2H_2O$ and 2.77 g/l $CaCl_2$, penicillin/streptomycin 1% (*w/v*), pH 7.4) and constantly bubbled with 2.5% O_2/20% CO_2 at 37 °C as previously described [21]. Two pin-supports settled in each myography chamber allowed isometric tension recording (PowerLab, ADInstruments) and were utilized to mount carefully the arterial rings on the myography system 620 M (Danish MyoTechnology, Aarhus, Denmark). Optimal passive tension (luminal diameter) was determined by the classical normalization procedure for each arterial ring [22,23] followed by 30 min stabilization. Functional and mechanical reactivation of the tissue were accomplished by applying modified Krebs-Henseleit buffer containing 60 mM KCl (Potassium physiological salt solution: KPSS; 9.2 g/l KCl, 0.289 g/l $MgSO_4$•$7H_2O$, 0.161 g/l KH_2PO_4, 0.277 g/l $CaCl_2$, 2.1 g/l $NaHCO_3$, 0.01 g/l EDTA-Na_2•$2H_2O$, 0.991 g/l D-glucose). An agonist-mediated contraction curve was generated by applying cumulative doses of the thromboxane A2 analogue U46619 (10-9-10-6 M) (D81741, Merck, Darmstadt, Germany) to achieve the 50% of the maximum contraction obtained by KPSS, followed by several washes to the baseline with modified Krebs-Henseleit buffer. Arterial rings were pre-incubated with S1P receptor inhibitors W146 or VPC23019 for 40 min. A single-dose of U46619 (EC_{80}; D81741, Merck, Darmstadt, Germany) was used to reach 80% of the maximal KPSS contraction. According to the experimental target, arterial rings were either treated with a single dose of native (200 μg/ml) or S1P-depleted (100 μg/ml) neonatal HDL dissolved in PBS. Relaxation percentages obtained were related to the EC80-induced tone.

2.15. Statistical analysis

GraphPad Prism software (version 7.0, GraphPad Prism software, San Diego, USA) was used for the statistical analysis. One- or two-way ANOVA with Tukey's post hoc test were used for all statistical analyses except where Student's *t*-test was applied. Spearman correlation was performed for correlation analysis. Data are expressed as mean ± SEM. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. nHDL is a carrier of ApoM-S1P complex in the feto-placental circulation

S1P plasma concentration in the cord averaged between 1.068 ± 0.212 μM in the male and 1.08 ± 0.235 μM in the female offspring (Fig. 1A). Additionally, we found significantly higher plasma levels of S1P in mothers of female offspring, suggesting a sex-depended regulation of S1P production in pregnancy (Online Resource 2). Considering ApoM being an established anchor for S1P we assumed that nHDL acts also as a carrier of S1P. Particle-associated ApoM content was 0.771 ± 0.107 and 0.845 ± 0.164 nmol/mg of HDL protein in male and female HDL isolations respectively (Fig. 1B). Furthermore, the levels of S1P on nHDL-containing fraction were comparable in male and female offspring (0.441 ± 0.111 vs 0.474 ± 0.153 nmol/mg of HDL protein) (Fig. 1C). Notably, we found a strong correlation between S1P and ApoM levels on neonatal particles ($r^s = 0.95$, $p < 0.0001$ in males; $r^s = 0.90$, $p < 0.0008$ in females) (Fig. 1D-E). These data highlight the role of ApoM as main chaperone for S1P binding on nHDL in the umbilical cord blood circulation.

3.2. nHDL elicits ERK and PLC pathways activation via S1PRs

The diversity of S1P-mediated effects reflects differences in the G proteins coupling to the receptor as well as endogenous S1P receptor expression in different tissues. In line with results of previous studies our HPAEC express both S1PR1 and S1PR3 [24,25] with S1PR1 being more abundant at both protein (Fig. 2A) and RNA level (Fig. 2B). Previous studies have shown that S1P signaling in endothelial cells triggers PI3K/Akt, Erk1/2 and PLC activation which have been implicated in cell proliferation, survival, and motility [26]. To evaluate the impact of nHDL on these signaling pathways we compared the effect of nHDL with that of the positive control S1P-HSA. Interestingly, the Akt signaling was not triggered (data not shown) in response to nHDL stimulation, whereas activation of ERK and PLC pathways was observed. The extent of nHDL-stimulated phosphorylation of ERK and PLC was comparable to that caused by S1P-HSA and was markedly reduced by S1PR1/S1PR3 inhibitors, strongly arguing for the role of S1PR in nHDL signaling in HPAEC (Fig. 2C-D). Pharmacological inhibition of ERK and PLC phosphorylation after nHDL treatment was equally efficient with both S1PR antagonists.

3.3. nHDL associated S1P stimulates HPAEC proliferation and calcium flux

Lysosphingolipids carried on HDL mediate the mitogenic activity of the particle by stimulating DNA synthesis in endothelial cells [20,27]. Moreover, several studies demonstrated that S1P is able to regulate calcium flux through its receptors [28,29]. Since activation of S1PRs by nHDL leads to G protein-dependent signaling by ERK and PLC, we next examined the impact of nHDL on cell proliferation and intracellular calcium mobilization in HPAEC. Notably, nHDL enhanced HPAEC proliferation (Fig. 3A). Induction of cell proliferation by both nHDL and the positive control S1P-HSA could be significantly attenuated by S1PR antagonists (Fig. 3A). Furthermore, nHDL induced calcium mobilization and this effect could be diminished by S1PR antagonists (Fig. 3B-C). These results suggest a S1P dependent nHDL-mediated intracellular calcium release and mitogenic effect on the human placental endothelium.

3.4. The protective effect of nHDL on feto-placental endothelial barrier is largely mediated by S1P

The endothelial barrier integrity is mainly dependent on cell-cell adhesion and cell-matrix tethering. Under physiological conditions, actin forms circumferential bundles which provide structural stability and anchoring for junctional complexes. This complex network confers membrane shape and architecture to maintain a functional endothelial barrier [30]. S1P ligation with its receptors induces cell-type-specific adhesive responses such as assembly of adherent junctions, formation of actin stress fibres and cortical rings [31,32].

To evaluate whether nHDL affects cytoskeletal rearrangement and adherent junction assembly of HPAEC, cells were treated with nHDL in the absence or presence of W146 or VPC23019 and stained for F-actin and VE-cadherin. When the cells were exposed to nHDL the actin fibres rearranged and formed a prominent actin ring (Fig. 4C) compared to the vehicle control (Fig. 4A). Moreover, the blocking effect of S1PR antagonists prevented actin organization (Fig. 4D-E). Furthermore, nHDL treatment increased the expression of VE-cadherin at the cell-cell contact regions (Fig. 4B, C) which was different when compared to control conditions (Fig. 4A).

Since ring-like actin structures determine barrier function in EC, we examined whether nHDL promotes placental endothelial barrier enhancement via S1P signaling. nHDL, markedly augmented trans-endothelial resistance (Fig. 4F, upper panel) and Δimpedance (Fig. 4F, lower panel), which was abolished in the presence of S1PR antagonists. These data confirmed the hypothesis that nHDL enhances feto-placental barrier function via S1P.

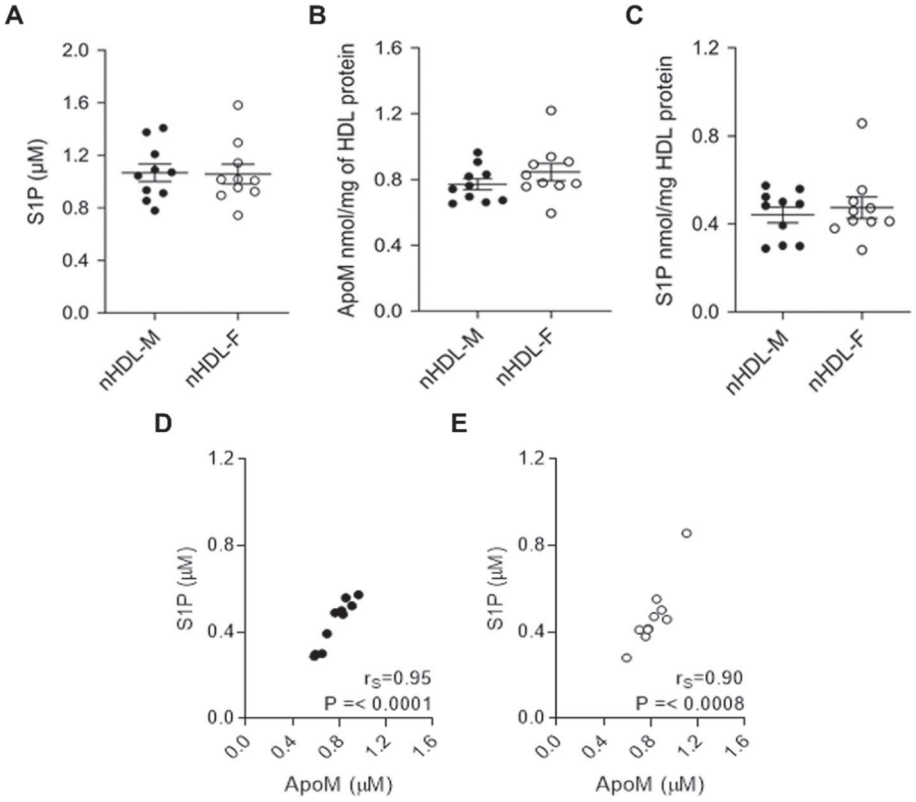

Fig. 1. Sphingosine 1-phosphate (S1P) and apolipoprotein M (ApoM) levels correlate in isolated high-density lipoprotein (HDL). A) S1P level in cord plasma from 10 male and 10 female offspring measured by HPLC. Cord blood-derived HDL referred as nHDL-M (male) and nHDL-F (female) were isolated and purified by ultracentrifugation. B) ApoM was measured by ELISA and C) S1P by HPLC. A correlation between HDL associated ApoM and S1P levels on isolated HDL from male D) and female E) offspring was performed. Correlation analysis was determined according to Spearman correlation coefficient (r_S).

3.5. Neonatal HDL induces placental arteries vasorelaxation

It has been reported that HDL acts as potent activator of endothelial nitric oxide synthase (eNOS) and regulator of vascular tone of conduit vessels [33,34]. In vitro studies have shown that HDL-associated S1P is crucial for HDL-mediated eNOS activation as the deletion of S1P receptors reduces 60% of eNOS phosphorylation [6]. To determine S1P involvement in the stimulation of nHDL-induced eNOS pathway, we incubated HPAECs with nHDL in the presence of S1PRs inhibitors. The levels of phospho-eNOS was significantly increased upon nHDL incubation (Fig. 5A). Moreover, blockage of S1PRs with W146 and VPC23019 significantly decreased the levels of phospho-eNOS, corroborating the pivotal role of S1P signaling in nHDL-mediated eNOS activation at the feto-placental endothelium. To investigate the effect of nHDL on vascular tone of placental arteries, we next examined the capacity of nHDL to relax U46619 pre-contracted arterial chorionic rings. nHDL markedly relaxed pre-contracted rings (≥50%) ($p < 0.001$). However, the vasodilatory effect of nHDL was not significantly reduced by pre-treatment of rings with the S1PR antagonists W146 (Fig. 5B) and VPC23019 (Fig. 5C). Interestingly, S1P depletion, which removed approximately 50% of nHDL S1P content, completely abolished the vasorelaxant capacity of nHDL, resulting in nHDL-induced vasoconstriction (Online Resource 3).

4. Discussion

The human placenta protects and support fetal growth while influencing maternal physiology according to the fetal demand. Specifically, the feto-placental barrier integrity is of critical importance to the fetal growth and well-being [35]. Placental endothelial dysfunction is a major hallmark of pregnancy-related disorders such as gestational diabetes mellitus (GDM) [36], preeclampsia and/or intrauterine growth restriction (IUGR) and one of the central features of inflammation which may affect the mother as well as the fetus health later in life [37–39]. In adults, plasma HDL particles are considered to act as key players in promoting vascular health and recent studies suggest that the bioactive sphingolipid S1P associated with the particles is specifically accountable for HDL-mediated protective effects [40]. We have previously shown that nHDL is unique in respect to its protein composition, size and function [14]. In addition, we have demonstrated that a pathological intrauterine environment during pregnancy impact the functionality of the neonatal HDL-particle [41]. The present study is the first to show that neonatal HDL particles represent a powerful mechanism of vascular protection during pregnancy. Particularly, two major findings of this study clearly highlight the importance of cord blood circulating HDLs and their capability to regulate vascular function at the fetal side of the human placenta: (1) nHDL serves as carrier of the apoM-S1P complex and (2) nHDL exerts vasculoprotective actions and sustains barrier integrity of the vasculature mainly through S1P signaling.

S1P content on HDL is confined to the apoM-containing particles in adult plasma and it strongly correlates with its apoM levels [18]. It has been estimated that S1P concentration ranges between 0.5 and 1.2 μM, in human plasma [42,43]. The majority of plasma S1P (~60%) is bound to HDL, whereas ~30% is bound to albumin and a minor fraction to other lipoproteins [44,45].

Notably, we showed that apoM is also associated with nHDL and acts as an S1P chaperon within the feto-placental circulation. A sexual dimorphism of S1P concentration has been suggested in the plasma of adults within the age of 19–55 due to different estrogen levels [46]. However, umbilical cord estrogen concentrations do not significantly differ between males and females [47]. Indeed, we could not detect any difference in S1P levels on nHDL from male and female offspring. However, we found higher S1P plasmatic concentration in mothers of female newborn, suggesting that the placenta might regulate S1P availability in the maternal circulation in a sex-dependent manner. Previous investigations also suggested a fetal sex-based difference in maternal hormones, angiogenic factors and immune mediators [48], most likely due to a differential programming of the placenta according to the fetal sex as shown in several pathways' enrichment analysis

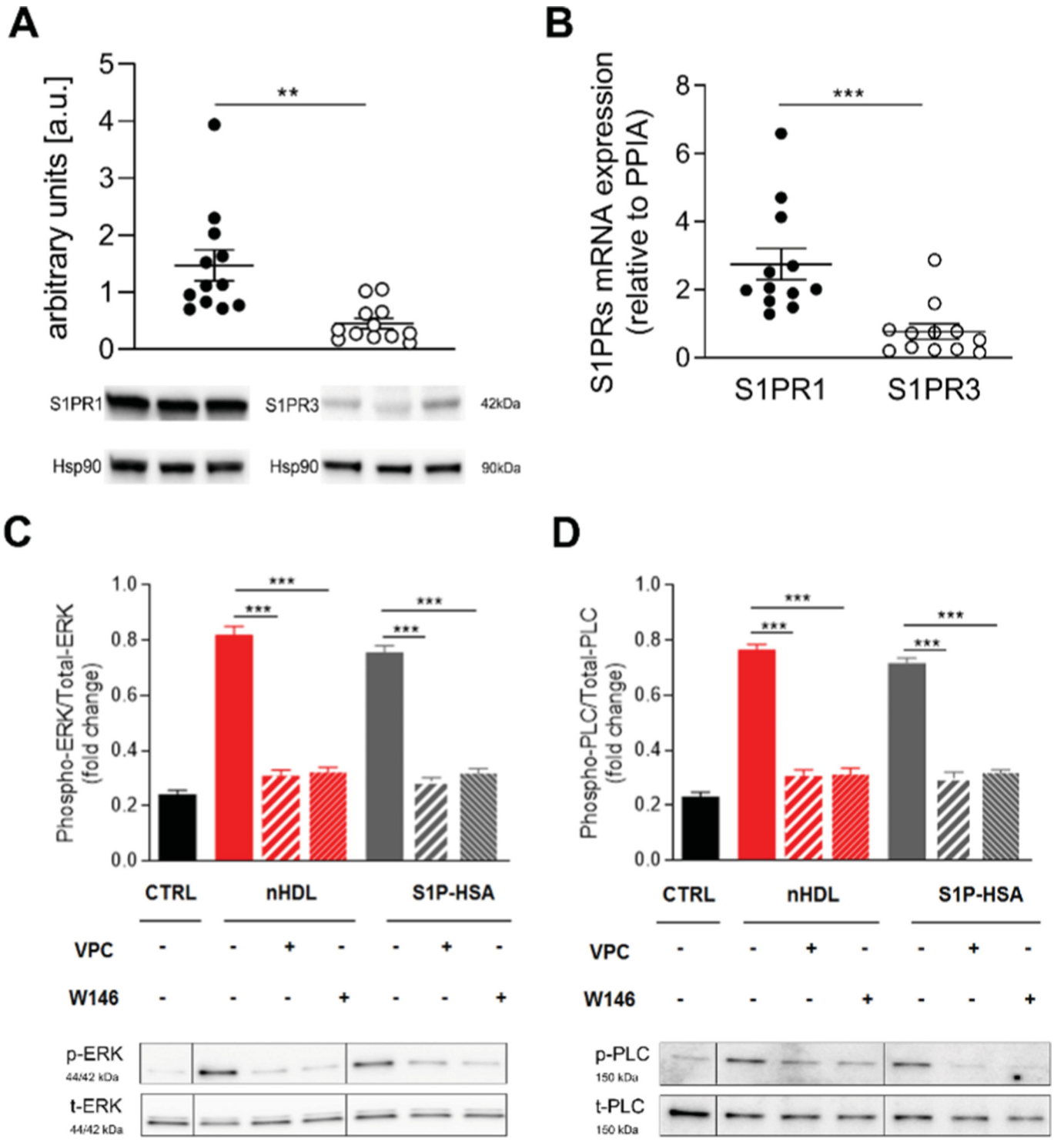

Fig. 2. nHDL activates S1PR1-mediated intracellular signaling pathways. (A) Primary arterial placental endothelial cells (HPAEC) were isolated from 12 individual term placentae and tested by Western blot analysis for S1PR1 and S1PR3 expression. (B) mRNA levels of S1PR1 and S1PR3 in HPAEC. HPAEC (n = 6) were serum starved and pre-treated for 30 min with W146 (1 μmol/L) or VPC23019 (1 μmol/l) respectively, before stimulation with nHDL (200 μg/ml; ~100 nmol/l S1P according to HPLC analysis), S1P-HSA (1 μmol/l) for 15 min. Activation of ERK (C) and PLC (D) were examined by Western blot using phospho-specific antibodies (p-ERK, p-PLC) and antibodies specific for the corresponding non-phosphorylated proteins (t-ERK, t-PLC). Results in C) and D) are presented as a ratio of phosphorylated and non-phosphorylated ERK and PLC, respectively. Data are shown as mean ± SEM. ***p < 0.001 by one-way ANOVA with Tukey's post hoc test.

studies [49,50]. In endothelial cells, S1P exerts a plethora of effects which regulates vascular maturation and morphogenesis. In particular, it stimulates endothelial proliferation, migration, and angiogenesis, protects against apoptosis and controls vascular permeability [51,52]. Many of these cellular functions have been shown to be dependent on S1PR1 and S1PR3. S1PR1 is highly abundant on the endothelium compared to S1PR3 [53], which is also expressed in vascular smooth muscle cells (VSMC) of different vascular beds [54,55]. We also found that S1PR1 is the predominantly expressed receptor type on feto-placental vasculature even though both receptors are present.

It is well established that S1P binding to its receptors leads to an activation of downstream effectors such as Akt, ERK/MAPK and PLC

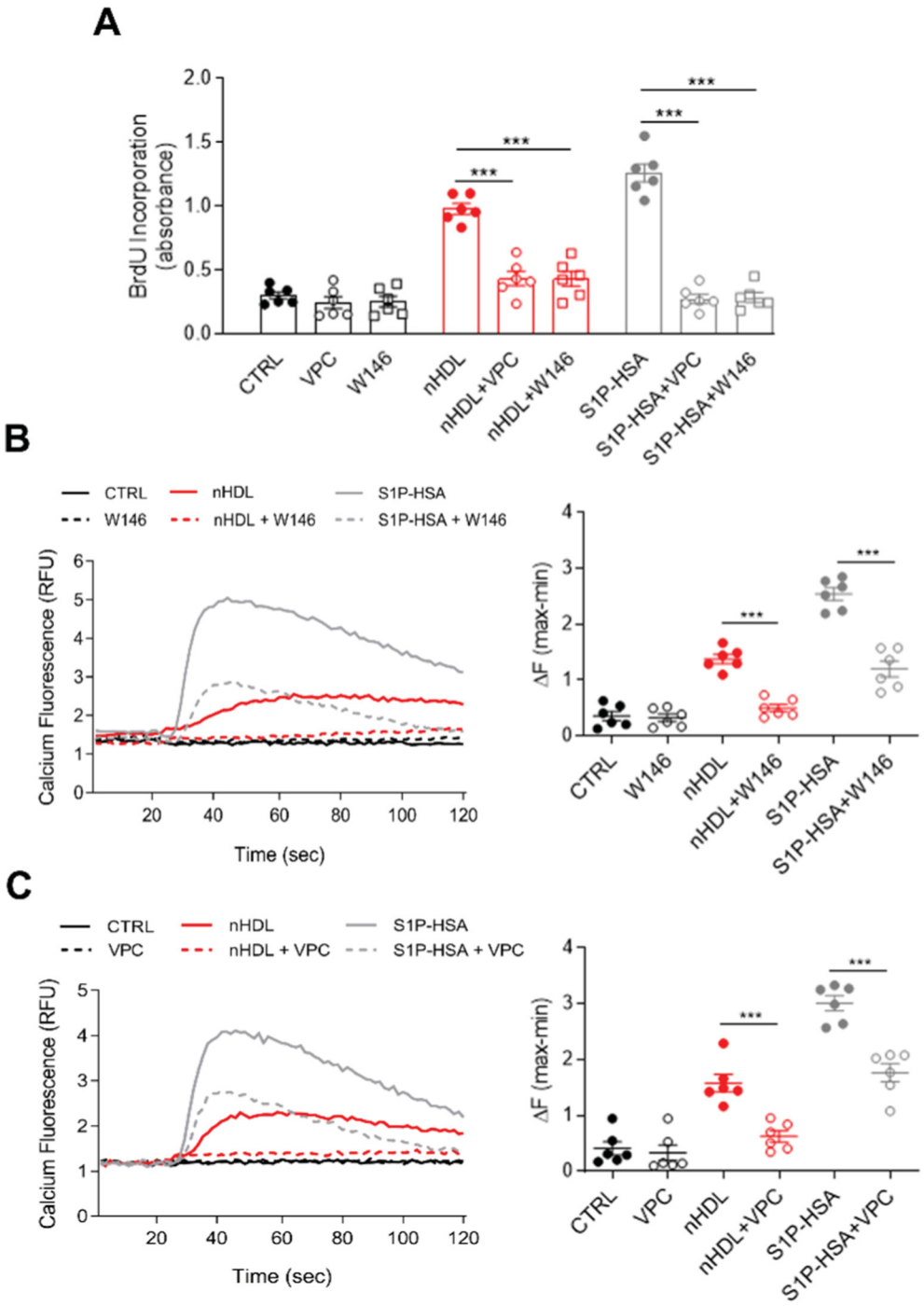

Fig. 3. nHDL promotes cell proliferation and calcium mobilization. A) HPAEC (n = 6) were treated with nHDL (400 μg/ml), S1P-HSA (1 μmol/l) in presence of W146 and VPC23019 for 24 h. Proliferation of HPAEC was quantified by BrdU incorporation. One representative time course of intracellular calcium flux in HPAEC pretreated with W146 (B; left) or VPC23019 (C; left) upon nHDL (200 μg/ml) or S1P-HSA (1 μmol/l) treatments are shown. Plates with treated cells were loaded into FlexStation and fluorescence intensity was recorded. ΔF (B right; C right) was calculated to determine changes in fluorescence intensity. Data represent mean ± SEM. ***p < 0.001, two-way ANOVA with Tukey's post hoc test.

[56]. When investigating downstream signaling targets of S1PRs, we observed that nHDL activates ERK and PLC pathways in primary human placental arterial endothelial cells (HPAEC). On the contrary to what was already shown in human endothelial cell lines [57,58], we could not detect any activation of Akt upon nHDL stimulation in primary endothelial cells. This suggests that the capability of the particle to elicit different signaling pathways is highly specific and dependent not only on the particle composition itself but also on the type of vascular bed. Importantly, it seems that S1PR1 is the main player in S1P signaling axis due to the observation that the selective S1PR1 antagonist W146 or the S1PR1/3 antagonist VPC23019 caused a similar effect on the placental endothelium. In addition, relative S1PR1 mRNA levels were almost 3-fold higher than S1PR3 mRNA levels in HPAEC, which argues for the previous statement. Consistent with the observed ERK and PLC pathways activation, nHDL promotes HPAEC proliferation and intracellular calcium mobilization in a S1PRs dependent manner. Our results nicely corroborate the general concept that an elevation in intracellular calcium levels after exogenous S1P stimulation is an important mechanism of nitric oxide (NO) production (7) and cell junction assembly (22) in order to maintain structural and functional integrity of the endothelium.

Vascular integrity and barrier permeability rely on the control of a complex interplay of tethering forces at cell–cell and cell–matrix level as well as intracellular contractile forces mediated by actin and myosin [30]. Wilkerson and colleagues elegantly showed that both HDL and albumin (containing an equal concentration of S1P) rapidly enhance the tightness of the barrier in human umbilical vein endothelial cells (HUVEC). Importantly, the HDL-S1P complex sustains cellular

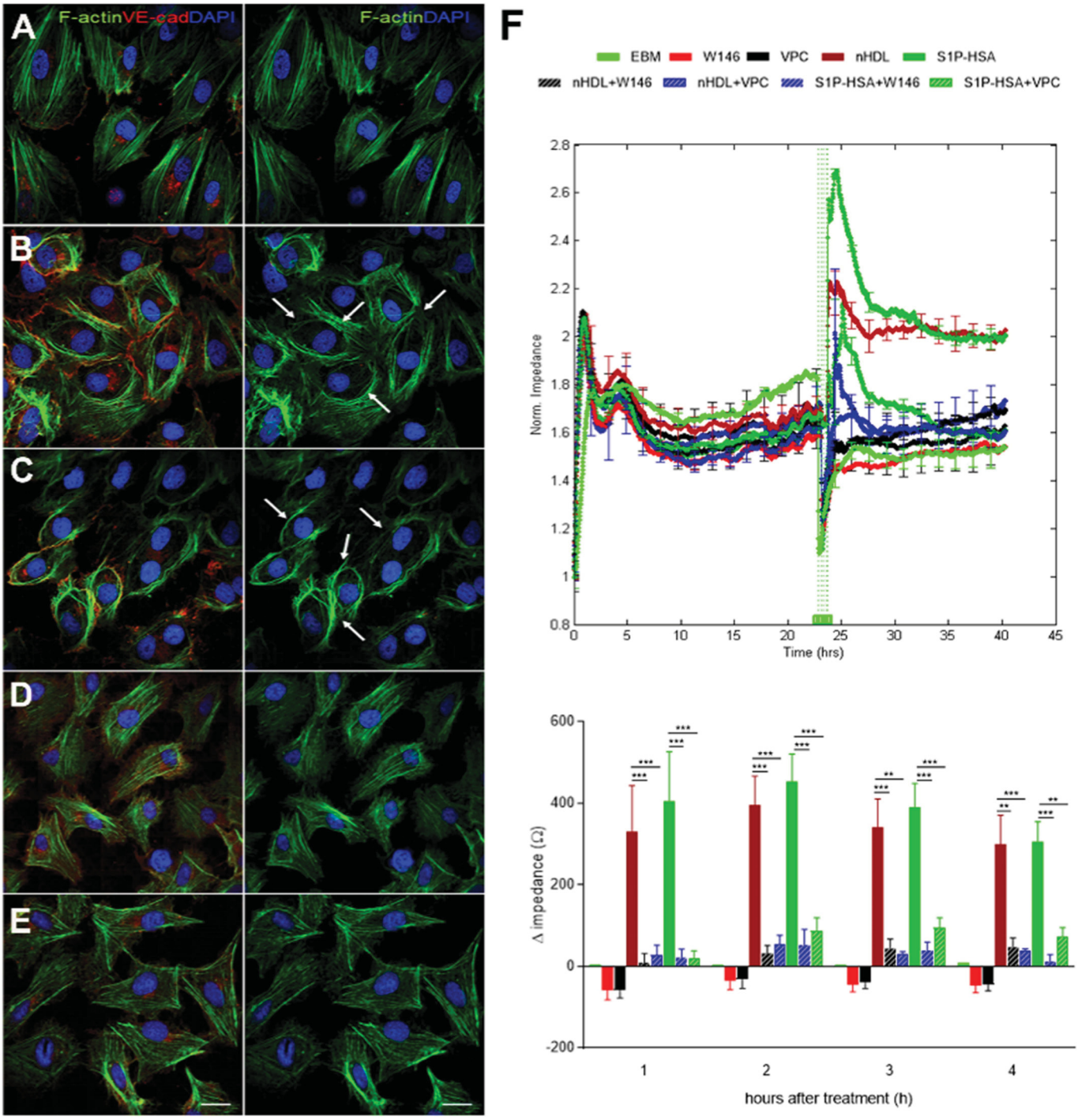

Fig. 4. nHDL induces actin reorganization and increases the endothelial barrier function in vitro. HPAEC were serum starved then incubated with vehicle control (A), S1P-HSA 1 μmol/l (B), nHDL 200 μg/ml (C) or preincubated with VPC23019 (D) or W146 (E) before nHDL stimulation. Cells were fixed, permeabilized and stained for F-actin (green), VE-cadherin (red) and nuclei (blue) and visualized with confocal laser scan microscopy. Scale bar 20 μm. F) Trans-endothelial electrical resistance of confluent monolayer of HPAEC exposed to S1P-HSA (1 μmol/l) and nHDL (200 μg/ml) in the absence or presence of VPC23019 or W146, was assessed over indicated time period (upper panel). One out of six representative experiments are shown. Δ impedance shows differences in resistance measured before and up to 4 h after treatment (lower panel). All data are presented as mean ± SEM. ***p < 0.001. Two-way ANOVA and Tukey's post-hoc test was used to test for significance.

impedance longer than albumin does [59]. Our studies show that nHDL reduces permeability as reflected by increased barrier impedance of HPAEC. Despite S1P bound to albumin has a higher initial maximal response, likely due to its higher concentration (1 μM with albumin vs 100 nM with nHDL), we observed that nHDL-induced enhancement of barrier integrity is stable over time compared with the response mediated by albumin. Activation of S1PR1 signaling has been associated with endothelial barrier-promoting function [60], whereas S1PR2 and S1PR3 activation leads to increased barrier permeability [61]. In our study, the evidence that S1PR1 inhibition blunted the sustained barrier effects induced by nHDL, suggests that nHDL-S1PR1 axis regulates this observed sustained placental endothelial barrier activity. However, we cannot rule out the possibility that in pathological conditions S1PR3 may play a role in endothelial barrier dysfunction. The increase trans-endothelial electrical resistance (TEER) by S1P occurs in association with rapid and dynamic actin rearrangements on

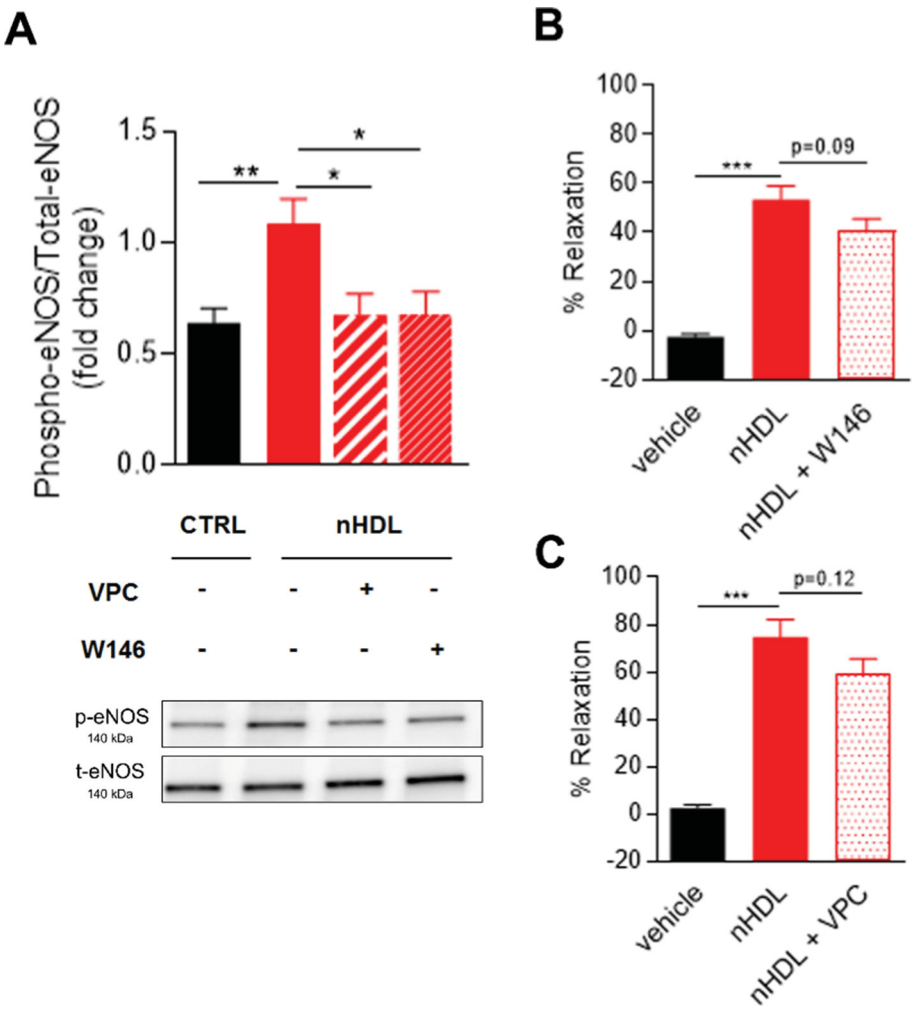

Fig. 5. nHDL promotes eNOS activation in HPAECs and placental arterial rings relaxation. HPAEC (n = 6) were serum starved and pre-treated for 30 min with W146 (1 μmol/l) or VPC23019 (1 μmol/l) respectively, before stimulation with nHDL (200 μg/mL; ~100 nmol/L S1P according to HPLC analysis), for 15 min. Activation of eNOS was examined by Western blot using a phospho-specific antibody (Ser1177-phospho-eNOS) and antibody for the corresponding non-phosphorylated proteins (t-eNOS). Results in (A) are presented as a ratio of phosphorylated and non-phosphorylated eNOS. Data are shown as mean ± SEM. *** $p < 0.001$ by unpaired Student's *t*-test. U46619-precontracted chorionic arterial rings were preincubated with (B) W146 (1 μmol/l) or (C) VPC23019 1 μmol/l followed by exposure to nHDL (200 μg/ml). Cumulative results are shown as mean ± SEM of at least 3 rings per condition from at least 3 placentae. *** $p < 0.001$ one-way ANOVA with Tukey's post hoc test.

endothelial cells [62,63]. Christoffersen et al. reported that upon stimulation with apoM-containing HDL and albumin-S1P, HUVEC show rearrangements of the F-actin network and adherens junctions' formation [18]. In line with these previous findings we observed that nHDL and S1P-HSA treatments on HPAEC stimulate cortical actin ring arrangement and improved adherens junction integrity as defined by VE-cadherin association with the cytoskeleton. These effects were abolished by pre-treatment with the S1PRs antagonists W146 and VPC23019, indicating that S1P drives these effects of nHDL on feto-placental barrier function.

Maintenance of vascular homeostasis is strongly dependent on the integrity of the endothelial barrier as well as on the regulation of the vascular tone. This concept is particularly relevant in the context of pregnancy, where the local vascular tone and fetal cardiac output regulates the vascular resistance of the human placenta. Moreover, the placental vasculature is mainly regulated by circulating, locally-produced hormones and vasoactive compounds such as prostaglandins, endothelium-derived hyperpolarizing factor (EDHF) and NO since unlike most other vascular beds, the feto-placental circulation is not innervated [64]. Given that the binding of S1P to its receptors on endothelial cells can activate eNOS signaling cascade, we hypothesized that the ability of nHDL to activate eNOS in HPAECs could be S1P dependent. Indeed, we observed that nHDL-induced eNOS activation relies on S1P-mediated signaling. It has been shown that S1P as well as HDL via SR-BI can modulate vascular tone in intact vessels [34,65,66]. In addition, Nofer and co-workers have been demonstrated that the vasodilatory effect of HDL in mice aortic rings is partly mediated by bioactive lysophospholipids of HDLs via S1PR3 [6]. Our results show an efficient vasorelaxation of precontracted placental chorionic arteries after nHDL stimulation. However, we couldn't prove the engagement of S1P signaling in this effect as pharmacological inhibition of S1PRs failed to significantly reduce the nHDL-induced vessel vasodilation. Interestingly, enzymatic S1P displacement abolished nHDL ability to induce vasorelaxation although the S1P depletion from the particle did not affect size of the hydrodynamic radius of nHDL (data not shown), suggesting that S1P plays a role in nHDL-induced regulation of vascular tone in placenta. However, further investigations are needed to effectively evaluate mechanism of S1P action. Taken together these data suggest that although the nHDL-S1P complex can activate eNOS signaling, the mechanism underlying the neonatal particle-induced vasodilatory effect may be more complex. Therefore, it requires differential analysis. The strength of our study is that we provide a novel insight into the human physiology of pregnancy, focused on placental vascular homeostasis, by investigating the biological function of neonatal HDL-S1P particles. However, given the limited access to the biological material obtained, results need further examination in larger cohorts. Moreover, it must be noted that conclusions drawn from this study apply only to the end of pregnancy. In conclusion, our studies define nHDL as major carrier of S1P in the feto-placental circulation and provide evidence that the delivery of S1P to its receptor can regulate placental vascular homeostasis by preserving stability and integrity of the endothelial barrier Fig. 6. These observations underline the crucial role of circulating S1P in placenta biology and fetal development, suggesting that an impairment of S1P signaling may predispose to placental vascular dysfunction and to a higher risk to develop long-term morbidities for the offspring.

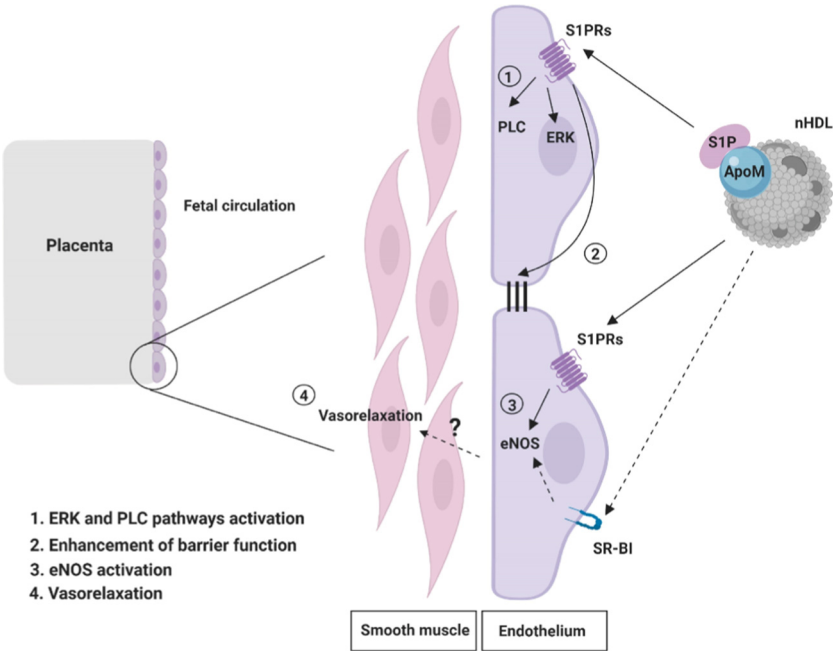

Fig. 6. Model of nHDL-S1P complex-mediated regulation of vascular function at the feto-placental barrier. nHDL activates 1) ERK an PLC signaling pathways, heightens 2) endothelial barrier function and promotes 3) eNOS activation by delivering S1P to the S1PR1 expressed on the placental endothelium. Additionally, nHDL can induce 4) placental rings vasorelaxation. The mechanisms underlying the nHDL-induced vasodilatory effect have yet to be determined. The figure has been created with BioRender.com.

Author contributions

I. Del Gaudio designed the research. I. Del Gaudio, I. Sreckovic, P. Zardoya-Laguardia and E. Bernhart conducted the experiments. I. Del Gaudio, S. Frank, G. Marsche, C. Christoffersen and S. E. Illanes contributed to data interpretation and manuscript editing. C. Wadsack supervised the entire study. I. Del Gaudio and C. Wadsack wrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Neonatal HDL Counteracts Placental Vascular Inflammation via S1P–S1PR1 Axis

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Abstract: Placental inflammation and dysfunction during pregnancy are associated with short- and long-term adverse outcomes for the offspring. However, the mechanisms of vascular protection at the feto-placental interface are still poorly investigated. The high-density lipoprotein (HDL) associated sphingosine-1-phosphate (S1P) has been described as a powerful anti-inflammatory complex. This study aimed to elucidate the role of cord blood-derived HDL (nHDL) in feto-placental endothelial dysfunction. Here, we report that the exposure of primary fetal placental arterial endothelial cell (fPAEC) to healthy nHDL-S1P attenuated the ability of TNFα to activate NF-κB signaling and increase the expression of pro-inflammatory markers. Moreover, the angiotensin II (AngII)-induced reactive oxygen species (ROS) production was blunted in the presence of nHDL, whereas it was preserved when the cells were preincubated with S1P receptor antagonists, suggesting that S1P accounts for the vascular protective function of nHDL at the feto-placental unit. These results highlight the importance of HDL and S1P metabolism and signaling in pregnancy pathophysiology.

Keywords: neonatal high-density lipoprotein; vascular inflammation; feto-placental dysfunction; sphingosine-1-phosphate

1. Introduction

Abnormal placentation and failure of the maternal vasculature to adapt to pregnancy hemodynamics can result in the development of pregnancy-associated diseases such as gestational hypertension and pre-eclampsia (PE) [1,2]. For instance, PE affects 6–10% of all pregnancies worldwide and remains a leading cause of maternal and fetal morbidity and mortality [3,4]. Vascular inflammation and endothelial dysfunction are the major and closely interconnected hallmarks underlying these pathological conditions. The feto-placental endothelium is part of a complex barrier which separates maternal and fetal circulation and regulates the gas and nutrients exchange between mother and fetus. Because of its localization at the interface of the two circulations, the proper function of the feto-placental barrier is of crucial importance for fetal health and development.

High-density lipoproteins (HDLs) are potent anti-atherogenic mediators in adult human circulation due to their capability to remove cholesterol from peripheral tissues [5,6]. However, basic research and clinical studies suggest that the anti-inflammatory and endothelial protective function of HDL may play a much more important role in vascular health than cholesterol removal [7]. HDL possesses several beneficial pleiotropic properties which have been partially attributed to the action of the bioactive lipid

sphingosine-1-phosphate (S1P), which is mainly carried through apolipoprotein M (ApoM) by the HDL particle in the circulation and, to a lesser extent, by serum albumin [8].

S1P is present at high concentrations in the lymphatic and circulatory systems (~1μM), whereas its tissue levels are relatively low (~75 pmol/mg) in physiological condition [9,10]. This vascular S1P gradient is crucial for the vascular homeostasis preservation. S1P can regulate endothelial function through specific G protein-coupled receptors (GPCRs) expressed on the vasculature. S1P receptor 1 (S1PR1) is highly abundant in endothelial cells and plays a pivotal role in vascular homeostasis [11]. Moreover, mice lacking the S1PR1 specifically in the endothelium exhibit a pro-inflammatory phenotype, suggesting the protective function of S1P-S1PR1 signaling in vascular pathology [12].

The role of free or HDL-associated S1P in vascular dysfunction has been predominantly studied in the context of atherosclerosis, diabetes or coronary artery disease (CAD) with controversial results [12–17]. However, nothing is known about the role of the HDL–S1P complex in placental vascular inflammation and dysfunction. In this study we aimed to investigate the role of cord blood-derived HDL (neonatal HDL, nHDL) in feto-placental dysfunction, focusing our attention on the fetal side of the circulation.

Our results clearly demonstrate that nHDL limits vascular inflammation by inhibiting NF-κB signaling and pro-inflammatory markers expression on the feto-placental vasculature. Moreover, the complex attenuates AngII-induced feto-placental dysfunction acting via S1P signaling. This is the first work which involves the use of human HDLs and isolated primary human placental endothelial cells to unravel the mechanisms of vascular dysfunction in placenta biology and pregnancy.

2. Results

nHDL-S1P Complex Attenuates Inflammation and Dysfunction of the Feto-Placental Endothelium

To study the role of nHDL-S1P or albumin-associated S1P (HSA-S1P) on placental vascular inflammation, we challenged primary human placental arterial endothelial cells (fPAEC) with TNF-α for 6 h and determined the changes in gene expression in the presence of the two complexes. We analyzed a panel of genes which are triggered by inflammation and are known to be associated with TNF-induced apoptosis and preeclampsia (Figure 1A). As expected, TNF-α strongly induced the expression of genes belonging to the TNF and TNF-receptor superfamily, such as TNFSF10, TNFSF18, TNFRSF1A and TNFRSF10. Genes involved in apoptosis (CASP3, CASP7, FAS and BID) were also upregulated. However, the presence of nHDL-S1P and HSA-S1P, although differentially effective, mitigated the expression of TNF-α-induced genes. Moreover, the mRNA of different effectors of the NF-κB signaling pathway, like TRADD, TRAF2, TRAF3, BRIC2, was upregulated upon TNF-α stimulation and downregulated in the presence of nHDL. Interestingly, the most pronounced increase in fold change was observed for the pro-inflammatory cytokines IL-1β, IL-8 and for TLR3, which are key mediators of inflammation in preeclampsia [18,19]. However, nHDL-S1P and HSA-S1P strongly abolished their transcription. By contrast, TNF-α markedly downregulated the expression of nitric oxide synthase 3 (NOS3), which is involved in the production of nitric oxide (NO) in the endothelium. Notably, nHDL was able to prevent this effect more efficiently than HSA-S1P, suggesting a carrier-dependent regulation of NOS3 expression.

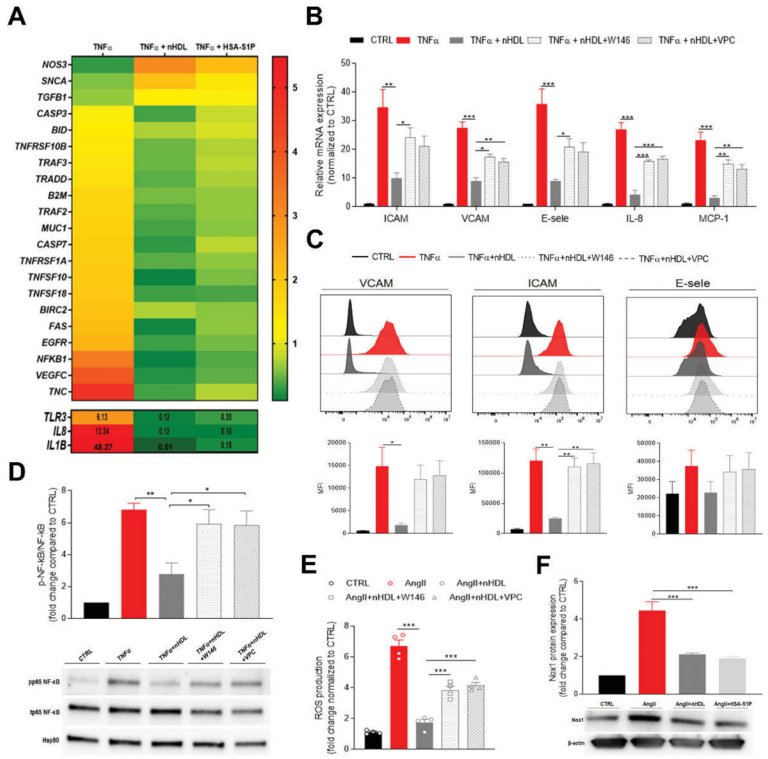

Figure 1. Cord blood derived high-density lipoprotein (nHDL)-bound S1P suppresses endothelial inflammatory markers and oxidative stress at the feto-placental vasculature. (**A**) Fetal placental arterial endothelial cells (fPAECs) were incubated with 10 ng/mL TNF-α in the presence of 800 μg/mL nHDL (~0.4 μmol/L S1P) or 1 μmol/L albumin-associated (has)-S1P for 6 h. Total RNA was analyzed by RT-PCR. Genes associated with preeclampsia and TNF-induced apoptosis, which were significantly regulated, as determined by multiple t-testing, are depicted in the heatmap as the average of $n = 3$ different isolations representing three biological replicates. Genes with values exceeding the scale shown in the upper panel are reported in the lower panel. (**B**) Changes in the expression of *VCAM*, *ICAM*, *E-selectin*, *IL-8* and *MCP-1* in fPAECs pre-treated with S1PRs inhibitors W146 and VPC23019 for 30 min before TNF-α and nHDL incubation for 6 h. Total RNA was analyzed by RT-PCR using TaqMan probes. Data were normalized to control and presented as mean ± SEM ($n = 4$). One-way ANOVA followed by Tukey's multiple comparison. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (**C**) fPAEC were treated as in (**B**) and analyzed by flow cytometry: VCAM, ICAM and E-selectin. Representative flow cytometry histograms (upper panel) and bar graphs showing the median fluorescence intensity (MFI) (lower panel). Data are presented as mean ± SEM ($n = 3$). One-way ANOVA followed by Tukey's multiple comparison. * $p<0.05$; ** $p<0.01$. (**D**) Cells were serum-starved treated as in (B) for 1 h. Phosphorylation (p) of the NF-κB subunit p65 was analyzed by Western blot of total cell lysates. Hsp90 was used as loading control. Data were normalized to control and expressed as fold change ratio of phospho p65/total p65 (mean ± SEM of $n = 4$ independent experiments). One-way ANOVA followed by Tukey's multiple comparison. * $p < 0.05$; ** $p < 0.01$. (**E**) fPAECs were pre-treatment with S1PRs inhibitors (W146 and VPC23019 1 μmol/L for 30 min) and incubated with dichlorofluorescin diacetate (DCFDA) for 45 min followed by exposure to 1 μmol/L AngII in the presence of 800 μg/mL nHDL. Intracellular oxidation of was detected by fluorescence spectroscopy (Ex/Em, 295/529). Tert-butyl hydrogen peroxide (200 μmol/L) was used as a positive control. The bar chart shows the reactive oxygen species (ROS) production as fold change (mean ± SEM; $n = 4$) compared to control. One-way ANOVA followed by Tukey's multiple comparison. *** $p < 0.001$. (**F**) fPAECs were serum starved and treated with 1 μmol/L AngII in the presence of 800 μg/mL nHDL or 1 μmol/L HSA-S1P for 6 h. Protein expression of Nox1 was analyzed by western blot in whole cell lysates. β-Actin served as loading control. Data are presented as mean ± SEM ($n = 3$). One-way ANOVA followed by Tukey's multiple comparison. *** $p < 0.001$.

Vascular inflammation promotes phenotypic changes in the endothelium characterized by the over-expression of adhesion molecules and the release of pro-inflammatory cytokines, which initiate and contribute to endothelial dysfunction. To further validate the anti-inflammatory effect of nHDL and explore the role of S1P signaling within this pathway, we pre-incubated fPAEC with a selective antagonist for S1PR1 (W146) or an antagonist for S1PR1/3 (VPC23019) [20], which are the receptors subtypes widely distributed in the cardiovascular system, then challenged fPAECs with TNF-α and examined the mRNA expression of different inflammatory markers (Figure 1B). The addition of nHDL to the culture medium markedly reduced the TNF-α-induced expression of intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin, monocyte chemoattractant protein-1 (MCP-1) and interleukin 8 (IL-8). Strikingly, the inhibitors treatment restored inflammation, suggesting that the anti-inflammatory effect of the neonatal particle is partially mediated by S1P. These results were independently confirmed by flow cytometry analysis, which revealed that the surface expression of the adhesion molecules was decreased in the presence of nHDL (Figure 1C).

Among potential intracellular pathways involved in the regulation of endothelial inflammatory response, nuclear factor κB (NF-κB) represents a key player. Indeed, the NF-κB promoter controls the transcription of many pro-inflammatory genes, including adhesion molecules, chemokines and cytokines [21]. TNF-α stimulation of fPAEC increased NF-κB (p65) phosphorylation, which was significantly suppressed by co-incubation with nHDL (Figure 1D). Moreover, blockage of S1PR1 did not modify the endothelial inflammatory response. These observations corroborate the concept that nHDL exert its anti-inflammatory effect by delivering S1P to its receptors on the fetal endothelium.

To further explore the ability of nHDL to prevent the endothelial dysfunction, we exposed fPAEC to angiotensin II (AngII). It has been shown that AngII is a powerful stimulator of NADPH oxidase (Nox), which generates reactive oxygen species (ROS), thus triggering vascular inflammatory response [22]. AngII treatment remarkably increased ROS production, a response that was notably suppressed when nHDL was added to the cells (Figure 1E). Similarly to what we observed for the TNF-induced inflammatory response, the blocking of S1P signaling led to the reversal of nHDL's capability of lowering ROS production. Furthermore, we demonstrated that nHDL and HSA-S1P decreased the abundance of Nox isoform 1 (Nox1), which is expressed in the feto-placental vasculature. These data suggest that the nHDL–S1P complex plays a significant role in AngII-induced placental dysfunction.

HDL-S1P content and signaling has been shown to be impaired in cardiovascular diseases and diabetes [23]. Indeed, HDL-associated S1P levels are reportedly lower in subjects with CAD or T2D [14,15]. Our proteomic data showed that nHDL from PE pregnancies have a lower ApoM content, suggesting decreased S1P levels associated with the particle (Figure S1).

3. Discussion

Vascular inflammation represents an early event in several cardiovascular and metabolic diseases. On the other hand, low-grade inflammation is a physiological process for maternal adaptation to pregnancy. However, the line between a normal and pathological inflammatory response in pregnancy is blurred and still not fully understood. In recent years, much research has been done to clarify the role of placental inflammation and its association with adverse effects on fetal development. Nonetheless, there is a lack of knowledge of the mechanisms of placental vascular regulation and function. Maternal systemic inflammation can affect placental physiology.

As feto-placental endothelium is continuous with the fetal circulation, any dysregulation of its function may lead to impaired fetal growth. Pathologies such as obesity-associated gestational diabetes, fetal growth restriction and preeclampsia (PE) have been associated with exacerbated inflammatory response and changes in feto-placental endothelium phenotype and vascular tone [24,25].

Human pregnancy is accompanied by hyperlipidemia (particularly during the last trimester), with a rise in triglycerides and cholesterol levels in HDL and LDL particles, to meet the cholesterol demand of the fetus [26]. LDL particles represent the major cholesterol carrier in the maternal circulation, where they also serve as a substrate for placental progesterone synthesis [27,28]. Conversely, in cord blood

circulation the HDL particle is the cholesterol carrier [29], suggesting a specific and distinct role of the cord blood-derived particles compared to the adult ones.

The bioactive mediator S1P associated with the HDL particle is known to have a major impact on the physiology of the endothelium [30–33]. Several studies have shown that HDL and S1P are involved in the regulation of the endothelial inflammatory response in atherosclerosis [12,13]. However, the underlying mechanisms are still poorly understood in the context of pregnancy and placental pathophysiology.

Our study highlights the ability of neonatal HDL–S1P to suppress the inflammatory response and dysfunction of the feto-placental endothelium by reducing the expression of inflammatory markers and lowering the vascular oxidative stress. Increased levels of TNF-α in PE have been linked with endothelial activation and damage [34]. We could show that nHDLs, which represent a unique class of HDL circulating in the cord blood and in fetal circulation, as well as HSA-S1P, can affect gene regulation under inflammatory conditions. Although both complexes were able to suppress the expression of genes involved in cellular apoptosis and vascular dysfunction, nHDL were more efficient. The reason for this effect relies not only in the heterogeneity of the nHDL particle but presumably also in an S1P carrier-dependent regulation of inflammation [13]. However, we cannot draw any definitive conclusions in this context, since the concentration of S1P on the two carriers is different (~0.4 µmol/L S1P associated with nHDL versus 1 µmol/L S1P associated with HSA)

TNF-α triggers the expression of cytokines and cell adhesion molecules for leukocytes' recruitment during vascular inflammation. We demonstrated that fPAEC exposed to nHDL had decreased the mRNA expression of these inflammatory markers, confirming the anti-inflammatory properties of the particle. Fourth, the pre-incubation of fPAEC with W146 (selective inhibitor of S1PR1) and VPC23019 (inhibitor of S1PR1/3), pre-empted the inhibitory effects of nHDL on TNF-α-induced adhesion molecule expression. Furthermore, the pharmacological inhibition of both receptors did not show any further effects via receptor 3, supporting the concept that S1PR1 is the main actor in the anti-inflammatory signaling. Using defined preparations of human HDL (HDL+ApoM with S1P or HDL-ApoM lacking S1P), Ruiz et al. elegantly showed that the increased expression of VCAM, ICAM and E-selectin was suppressed only by the HDL fraction containing S1P. In agreement with their work, we showed that the surface expression of VCAM and ICAM was inhibited in presence of nHDL. However, E-selectin expression was only affected at the transcriptional level. Several factors might contribute to the observed discrepancy in previous results, such as different experimental incubation times (4 h vs. 6 h), type of HDL (adult vs neonatal) and type of endothelium (human aortic endothelial cells vs human feto-placental arterial endothelial cells). Furthermore, we used a different experimental approach involving the use of S1PRs antagonists in the presence of the native particles.

NF-κB signaling is activated with increased oxidative stress and inflammation. Indeed, placentae from PE-women have an exaggerated activation of the pathway compared to healthy ones [35]. Galvani et al. reported that HDLs isolated from WT mice can blunt the TNF-α- induced NF-κB activation, whereas HDLs from ApoM KO mice are not effective to the same extent, suggesting that S1P is mediating the HDL effect [12]. Moreover, they showed that human HDL in human umbilical vein endothelial cells (HUVECs) can achieve the same result in a dose-dependent manner. However, in the case of human HDL, they did not confirm the link between S1P signaling and HDL's capability of suppressing the activation of the NF-κB pathway. Our data corroborate the concept that human HDL can interfere with NF-κB signaling activation. In addition, we demonstrated a direct, S1P-dependent effect on the signaling cascade by using pharmacological inhibitors for S1PR1/3.

Disruption of the oxygen balance with concomitant increased ROS production plays an important role in inflammation-associated vascular cell damage [36]. Numerous studies have indeed reported an association between placental dysfunction, ROS production and the onset of hypertension in PE [37,38]. NAD(P)H oxidase is an important source of ROS within the vascular wall, and its activity is differentially regulated in pathophysiological processes. Tölle and co-workers demonstrated that HDL-associated lysosphingolipids inhibit the thrombin-induced NAD(P)H oxidase activity and ROS

production in vascular smooth muscle cells (VSMCs) [39]. In the present study, we show that nHDL negatively regulates AngII-induced ROS production in fPAEC via S1P signaling. Furthermore, we demonstrated that nHDL and HSA-S1P can decrease the protein expression of the NAD(P)H oxidase catalytic subunit Nox1. These data provide a partial insight into the mechanism by which nHDL and S1P protect the endothelium from oxidative stress in the placenta.

Another novel observation was that PE is associated with reduced ApoM levels on nHDL. Although further functional studies are needed to link the reduction in ApoM, as well as S1P content, to nHDL dysfunction, our findings corroborate the concept that the nHDL–S1P complex plays a pivotal role in the maintenance of placental vascular homeostasis.

Despite our study clearly demonstrating the vasculoptotective effects of the nHDL–S1P complex on the feto-placental vasculature, some limitations should be noted. As proof-of-concept, TNF-α and AngII were used as vascular disruptor molecules in our experimental design in order to evaluate the capability of nHDL to attenuate placental endothelial inflammation and dysfunction. However, we cannot directly translate these findings to the complex inflammatory and hypertensive environment which characterizes PE. Thus, studies assessing how and whether PE affects the function of the placental endothelium as well as the functionality of the nHDL particles are warranted.

4. Materials and Methods

4.1. Study Population

Clinical characteristics of the subjects enrolled in the study are summarized in Table S1. Each individual in this study gave written informed consent. All experiments were performed in accordance with the protocols approved by the ethics committee of the Medical University of Graz (Vote no: 29-319 ex 16/17, approval date: 29/03/2017).

4.2. Isolation of Cord Blood-Derived Neonatal HDL and S1P Quantification

Neonatal cord blood plasma (n = 10 male and n = 10 female) were centrifuged from collected mixed (arterial and venous) umbilical cord blood samples, which were obtained immediately after delivery of the placentas. nHDLs were isolated by ultracentrifugation [40,41]. The purity of total HDL was assessed by measuring apolipoprotein composition, total protein and cholesterol. Subsequently, S1P concentration was determined by HPLC, as described elsewhere [8].

4.3. Isolation and Treatment of Primary Fetal Placental Arterial Endothelial Cells (fPAEC)

Term placentae from caesarean section and vaginal delivery were used within 20 min of delivery (n = 4). fPAECs were isolated from arterial chorionic blood vessels as described by Lang et al. [42]. Cells were treated as follows: 10 ng/mL TNF-α or 1 µmol/L AngII in the presence of 800 µg/mL nHDLs (which were isolated according to Section 2 and contain ~0.4 µmol/L S1P) or 1 µmol/L HSA-S1P (Avanti Polar Lipids, Alabaster, AL, USA). Selective inhibitor of S1PR1–W146 (1 µmol/L) (857390, Avanti Polar Lipids) and S1PR1/3 inhibitor VPC23019 (1 µmol/L) (4195, Tocris Bioscience, Bristol, UK), were used in the cell culture experiments. When cells were treated with W146 or with VPC23019, Na_2CO_3, (2-hydroxypropyl)-β-cyclodextrin and DMSO respectively, were added as a vehicle.

4.4. Quantitative Real-Time PCR (qPCR) and PrimePCR of fPAEC

Cells were seeded in 12 well plates at a density of 100,000 cells/well. After two days, medium was changed to serum-free EBM and cells were treated for 6 h as described in Section 4.3. Treatment was performed in triplicates. After treatment, cells were washed twice and harvested in 350 µl RLT Lysis buffer (Quiagen, Hilden, Germany) supplemented with 1% β-mercaptoethanol (Sigma Aldrich, St. Louis, MO, USA). Next, total RNA content was isolated using the RNeasy® Mini Kit (Quiagen, Hilden, Germany) according to manufacturer's instructions. Quantitative real-time PCR was performed on the CFX384 cycler (BioRad Technologies, Vienna, Austria) using TaqMan® Gene Expression assays

(Applied Biosystems, Thermo Fisher Scientific, Carlsbad, CA, USA) and TaqMan® Universal PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, Carlsbad, CA, USA). Used TaqMan® Gene Expression assays are listed in Table S2. PrimePCR panels for pre-eclampsia (Pre-eclampsia Tier 1 H384; BioRad Technologies, Vienna, Austria) and apoptosis (Apoptotic TNF Family pathways H384; BioRad Technologies, Vienna, Austria) were used according to manufacturer's instructions. Relative quantification of gene expression was calculated by ΔΔCq method.

4.5. Western Blot

fPAEC were plated at a density of 200,000 cells/well in 6-well plates (Thermo Fisher Scientific). After two days in culture, cells were serum starved for 6 h and treated for 1 or 6 h as mentioned above. Thereafter, cells were collected in 50 µL RIPA lysis and extraction buffer (Sigma Aldrich) containing protease inhibitors (Roche, Basel, Switzerland). Total protein concentration was determined by bicinchoninic acid assay (BCA; Thermo Fisher Scientific). A total of 10 µg of total protein was loaded onto 4–20% SDS-PAGE gradient gels (BioRad Technologies) and resolved at 120 V for 1 h 10 min. Membranes were incubated with antibodies against phospho-p65 NF-κB (3033; Cell signaling, Danvers, MA, USA), total p65 NF-κB (8242; Cell signaling), Hsp90 (610418; BD Bioscience; New Jersey, NJ, USA), Nox1 (ab55831, Abcam, Cambridge, UK) and β-actin (ab6276, Abcam). All antibodies were diluted in 5% non-fat dry milk (BioRad Technologies, Vienna, Austria). Detection was carried out using SuperSignal® Chemiluminescent Substrate (Thermo Fisher Scientific). Immunolabeling was visualized with the Fusion FX imaging system (Vilber Lourmat, Marne-la-Vallée, France) and band densitometry was performed using the Fusion© Software (Vilber Lourmat). Hsp90 and β-actin were used as housekeeping genes.

4.6. FACS of fPAECs

Cells were carefully harvested by using 1 mL accutase (PAA, Pasching, Austria). Detached cells were collected in 10 mL HBSS, counted and centrifuged for 4 min at 800 rpm. The obtained cell pellet was resuspended ($1x10^6$ cells/mL) in staining buffer containing PBS (Thermo Fisher Scientific) supplemented with 0.1% bovine serum albumin (Sigma Aldrich) and 20 mmol/L EDTA (Thermo Fisher Scientific). Subsequently cells were incubated for 30 min at 4°C in the dark with the following antibodies: CD106-APC (305809, BioLegend, San Diego, CA, USA), CD54-Pacific Blue (322715, BioLegend) and CD62E-PE (322605, BioLegend). Unstained cells were used as a control. For each sample, at least 10,000 cells were counted. Cell sorting was performed on a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA) using the associated CytEXpert software (Beckman Coulter) for setting the gates and analysing data.

4.7. Reactive Oxygen Species (ROS) Assay

Cells were seeded in a dark-wall, clear-bottom, 96-well microplate (Costar®; Corning Inc., New York, NY, USA) at a density of 20,000 cells/well. 100 µL of DCFDA solution (10 µmol/L in HBSS) (Abcam) were added to each well and cells were stained for 45 min at 37 °C in the dark. Subsequently, cells were washed and treated with 1 µmol/L AngII (Sigma Aldrich) in the presence of 800 µg/mL nHDL with or without inhibitors for 6 h. A total of 200 µmol/L tert-butyl hydrogen peroxide (TBHP; Abcam) was used as positive control. All treatment compounds were diluted in phenol red free DMEM (Gibco, Thermo Fisher Scientific) without supplements. Fluorescent intensity of oxidized dichlorofluorescein (DCF) was measured at Ex/Em = 485/535 in end point mode (FLUOstar Optima; BMG Labtech, Offenburg, Germany).

4.8. Shotgun Proteomics by LC-MS/MS

Proteomic analysis were performed as previously described [43]. Relative protein abundance between samples was calculated based on the number of spectral counts (SpCs) of the total peptide.

4.9. Statistical Analysis

Graph Pad Prism 7 Software (GraphPad Software Inc., San Diego, USA) was used for all the statistical calculations and graph plotting. *t*-test or one-way ANOVA, including Tukey post-hoc, were performed if two or more groups were compared, respectively. For PrimePCR data, differences in experimental groups were evaluated by multiple Students' *t*-test using the Holm–Sidak method for multiple comparison correction. *p*-values below 0.05 were considered statistically significant.

5. Conclusions

In conclusion, our study defines S1P signaling as a key mediator of nHDL's anti-inflammatory effect on feto-placental endothelium. Furthermore, we provided evidence that the nHDL–S1P complex is a powerful regulator of ROS formation, thereby protecting the endothelium from oxidative stress and preserving placental vascular function. Thus, S1P and HDL metabolism and signaling might represent an attractive therapeutic target in pregnancy-associated diseases.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/21/3/789/s1.

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

Abbreviations

ApoM	Apolipoprotein M
GPCR	G protein-coupled receptors
S1PR	Sphingosine-1-phosphate receptor
HSA	Human serum albumin
TNFα	Tumor necrosis factor alpha
AngI	Angiotensin II
fPAEC	Human primary fetal placental endothelial cell
ROS	Reactive oxygen species
Nox1	NADPH oxidase 1
ICAM	Intracellular adhesion molecule
VCAM	Vascular cell adhesion molecule
E-sele	Endothelial-leukocyte adhesion molecule
IL-8	Interleukin 8
MCP-1	Monocyte chemoattractant protein 1

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Sphingolipid Signature of Human Feto-Placental Vasculature in Preeclampsia

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Abstract: Bioactive sphingolipids are emerging as key regulators of vascular function and homeostasis. While most of the clinical studies have been devoted to profile circulating sphingolipids in maternal plasma, little is known about the role of the sphingolipid at the feto-placental vasculature, which is in direct contact with the offspring circulation. Our study aims to compare the sphingolipid profile of normal with preeclamptic (PE) placental chorionic arteries and isolated endothelial cells, with the goal of unveiling potential underlying pathomechanisms in the vasculature. Dihydrosphingosine and sphingomyelin (SM) concentrations (C16:0-, C18:0-, and C24:0- sphingomyelin) were significantly increased in chorionic arteries of preeclamptic placentas, whereas total ceramide, although showing a downward trend, were not statistically different. Moreover, RNA and immunofluorescence analysis showed impaired sphingosine-1-phosphate (S1P) synthesis and signaling in PE vessels. Our data reveal that the exposure to a deranged maternal intrauterine environment during PE alters the sphingolipid signature and gene expression on the fetal side of the placental vasculature. This pathological remodeling consists in increased serine palmitoyltransferase (SPT) activity and SM accrual in PE chorionic arteries, with concomitance impairment endothelial S1P signaling in the endothelium of these vessels. The increase of endothelial S1P phosphatase, lyase and S1PR2, and blunted S1PR1 expression support the onset of the pathological phenotype in chorionic arteries.

Keywords: sphingolipid metabolism; bioactive lipids; human placenta; placental vasculature; preeclampsia; feto-placental endothelium

1. Introduction

Preeclampsia (PE) is characterized by profound morphological and functional modifications in the arterial vessels of the uterus and the placenta. Together, poor vascular adaptions in the mother and an inflammatory intrauterine environment during pregnancy, affect the functionality of the feto-placental endothelium. This does not only lead to pregnancy-related maternal and fetal morbidities but also to adverse outcomes for the offspring later in life, e.g., increased risk to develop hypertension (3-fold) and cardiovascular disease (2-fold) later in life [1–3]. PE has an incidence of 3–5% of pregnancies in the United States and up to 10% of pregnancies worldwide [4], representing the leading cause of maternal and fetal morbidity and mortality. Clinically, PE is defined as the de novo onset of hypertension (systolic blood pressure > 140 mmHg or diastolic blood pressure > 90 mmHg) and proteinuria (>300 mg/24 h) after 20 to 24 weeks of gestation [5]. The symptoms of PE typically remain until delivery and are reversed after delivery, when blood pressure levels return to pre-pregnancy

levels. Although the specific mechanisms leading to the development of PE have to be elucidated yet, the indicators of the endothelial dysfunction in PE often synergize, exacerbating the condition. For instance, an association between impaired lipid metabolism and loss of endothelium functionality has been proposed in women who develop PE [6].

Sphingolipids, resulting from the de novo biosynthesis in the endoplasmatic reticulum (ER) or from the catabolism of complex sphingolipids and recycling pathway [7], have emerged as a class of bioactive lipids that play an important role in vascular homeostasis. Among the sphingolipids, ceramide (Cer) and sphingosine-1-phosphate (S1P) can differentially regulate endothelial functions. Once synthesized, mainly in red blood cells and vascular endothelium [8], S1P is rapidly exported out of the cells, where it can activate cell-surface receptors (S1PRs) in an autocrine fashion or can bind plasma chaperones. Approximately 65% of circulating S1P is bound to high density lipoprotein (HDL), while the remaining 35% associates to albumin [9]. S1P-mediated S1PR1 activation enhances cellular proliferation, survival, and nitric oxide (NO) production, resulting in blood vessel relaxation and atheroprotection [10,11]. Interestingly, plasma levels of S1P are significantly diminished in patients affected by myocardial infarction and coronary artery disease [12,13].

Multiple studies reported a correlation between Cer imbalance and cardiovascular diseases as well as metabolic disorders [14,15].

Sphingomyelin (SM), complex sphingolipid found in plasma and cellular membranes, plays a pivotal role in membrane stability and cell cholesterol homeostasis [16]. Several studies demonstrated an association between altered SM levels and cardiovascular diseases [17].

Whereas a substantial research effort investigated the impact of altered sphingolipids' metabolism and their role in the pathogenesis of cardiovascular diseases, their importance in pregnancy and the function of the placenta, a highly vascularized organ, remains poorly understood. It has been shown that there is a derangement in sphingolipid metabolism and levels in the human term umbilical cord artery (UCA) [18] and plasma of patients diagnosed with PE [19,20].

The aim of this study is to investigate whether PE affects the key players of the sphingolipid metabolism to impact their signature in the feto-placental vasculature, which plays a critical role in regulating angiogenesis, vasomotor tone, and placental perfusion, pivotal to fetal development.

2. Results

2.1. Sphingolipid Profile of Placental Chorionic Arteries from PE and Normotensive Subjects

It has been reported that sphingolipid signaling plays an important role in the vascular function and blood pressure homeostasis [21]. Considering that hypertension and the endothelial dysfunction are major hallmarks of PE, we reasoned that sphingolipid metabolism might play a role in the PE-induced placental vascular dysfunction. Thus, we determined the sphingolipid levels in chorionic placental arteries from normotensive (PN) and PE pregnancies. Interestingly, LC-MS/MS analysis showed a decreasing trend in total Cer levels in PE arteries compared to heathy subjects, although this was not statistically significant (Figure 1A(a)). The quantification of single ceramide species showed that, whereas an overall trend towards reduced levels could be observed in different species, only C20:0-cer was significantly decreased in PE compared to PN chorionic arteries (Figure 1A(b),(c)).

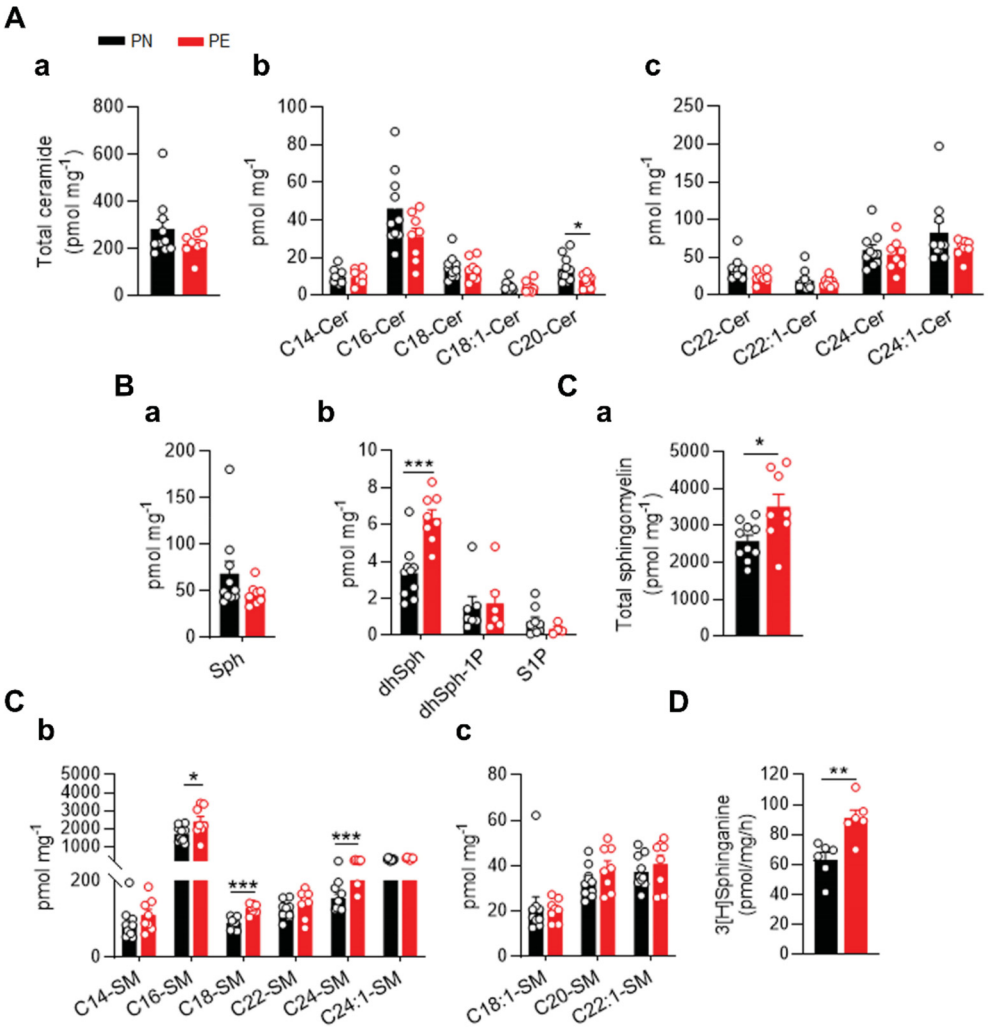

Figure 1. Sphingolipids content and serine palmitoyltransferase (SPT) activity of normotensive (PN) and preeclamptic (PE) chorionic arteries. LC-MS quantification of ceramide (**A**), sphingosine intermediates (**B**), and sphingomyelin (**C**) of isolated placental chorionic arteries from normotensive (n = 10) and preeclamptic women (n = 8). Total (**A**, **a**) and individual (**A**, **b**; **A**, **c**) ceramide species. (**B**) Dihydrosphingosine (dhSph), sphingosine (Sph), dihydrosphingosine -1-phosphate (dhSph-1P), and S1P. Total (**C**, **a**) and individual (**C**, **b**; **C**, **c**) sphingomyelin species. (**D**) Chorionic arteries lysates were assessed for SPT activity. [3H]-serine and palmitoyl-CoA were used as substrates by SPT to generate 3-ketosphinganine, subsequently reduced to sphinganine, followed by TLC separation. Sphinganine was used as the marker (n = 6 per group). Data are expressed as mean ± SEM * $P < 0.05$; ** $P < 0.01$ *** $P < 0.001$ compared to PN. Statistical significance was determined by unpaired t-test.

Interestingly, sphingosine and S1P, downstream products of ceramide, also presented a decreasing trend in PE arteries versus control patients, although this was not significant (Figure 1B(a),(b)). Notably, the level of dihydrosphingosine (dhSph), a downstream product of serine palmitoyltransferase (SPT), the first and rate limiting enzyme of the de novo biosynthesis, was significantly augmented in chorionic arteries of preeclamptic women (Figure 1B(b)), suggesting an upregulation of this pathway. The dhSph levels have been reported to be associated with cardiovascular disease [22]. Accordingly, PE was accompanied by an accrual of total SM levels (Figure 1C(a)). The analysis of the single SM species revealed a significantly higher content of C16:0-, C18:0-, and C24:0-cer in PE arteries compared to PN (Figure 1C(b)). These data collectively suggest that during PE, the sphingolipid metabolism of the feto-placental vasculature is shifted towards SM, by altered production/catabolism.

The de novo sphingolipid synthesis takes place at the ER through the action of SPT, encoded by the genes SPTLC1 and SPTLC2) [23]. To corroborate an upregulation of SL biosynthesis, the SPT enzymatic assay was performed as previously reported [21]. As shown in Figure 1D, SPT activity was significantly increased in PE versus PN chorionic arteries, in agreement with the sphingolipid profile.

2.2. PE Impairs S1P Signaling at the Feto-Placental Vasculature

The endothelium is a major source of S1P. This bioactive lipid is a potent regulator of vascular integrity, due to its ability to enhance the endothelial barrier function, induce the production of NO and exert anti-inflammatory and anti-atherogenic effects [9,10,24,25]. Disruption of S1P metabolism has been implicated in many cardiovascular diseases, in which the endothelial dysfunction represents a common denominator [26].

Within the vascular bed, endothelial cells can produce and release (via spinster 2 transporter, SPNS2) S1P [8]. Its intracellular levels are tightly regulated by sphingosine kinases (*SPHK1* and *SPHK2*) and degrading enzymes such as S1P phosphatase (*SGPP1*) and sphingosine-1-phosphate lyase (*SGPL1*) [27]. Once S1P is secreted out of the cell it may signal via G protein-coupled receptors (GPCRs) via S1PR1, S1PR2, and S1PR3, which are all expressed in the vasculature [28]. We have previously reported that Nogo-B (encoded by the gene *RTN4*) is a negative regulator of SPT activity, highly expressed in the endothelium of blood vessels [21]. Mice lacking endothelial Nogo-B are protected by hypertension and heart failure [29] via upregulation of S1P-S1PR1-NO signaling [21], suggesting the Nogo-B-mediated inhibition of SPT plays a pathological role in the onset of cardiovascular diseases.

To assess the effect of PE on S1P signaling in the human placenta, we evaluated the expression of key genes involved in the sphingolipid pathway in placental chorionic arteries (Figure 2A) and fetal placental arterial endothelial cells (fPAECs) (Figure 2B). RNA analysis revealed that PE caused an increased expression of SPTLC1 and SPTLC2, suggesting an increased sphingolipid biosynthesis in the arteries of PE subjects (Figure 2A). The expression of the sphingosine kinases was not different suggesting that the formation of S1P is not impaired in the whole vessel. However, the amount of Sph in PE arteries is reduced, although not statistically different, indicating that the limited substrate of SPHK1/2 might contribute to decreased S1P production. PE chorionic arteries showed also a significant increase in *SGPL1* expression, a degrading enzyme of S1P [30]. These data suggest that, despite the increased sphingolipid biosynthesis and SM, S1P production might be impaired.

Notably, the expression of *S1PR1* was significantly downregulated, whereas *S1PR2* levels were increased in isolated chorionic arteries from PE subjects compared to controls. These data corroborate the concept that S1P can induce opposing effects according to the expression levels of the respective receptors involved in the signaling cascade. S1PR1 activation is often associated with vascular protection [31–33]. Conversely, induction of S1PR2 has been related to diabetes [34] whereas expression of S1PR3 was unchanged.

Considering that the endothelium is one of the major cellular sources of S1P, and an important regulator of vascular tone and barrier, we next examined the gene expression in fPAECs from PE and PN patients (Figure 2B). Consistent with the findings obtained from the chorionic arteries, fPAEC of PE showed increased expression of SGPP1 and SGPL1, although only the latter was not statistically significant. These findings suggest an impaired endothelial-derived S1P. Interestingly, Nogo-B was upregulated at mRNA levels in fPAEC and at the protein level in the endothelium of PE chorionic arteries versus controls (Figure 2B,C(a),(b)).

Furthermore, mRNA expression of S1PR1 and S1PR2 in the endothelium mirrored the same pattern described in the vessels, with the decrease of the former and increase of the latter (Figure 2B). Accordingly, immunofluorescence staining showed significant diminished levels of S1PR1 in the endothelium of PE chorionic arteries (Figure 2C(a),(b)). These findings suggest that PE impairs S1P production and/or degradation, hence S1PR1 signaling.

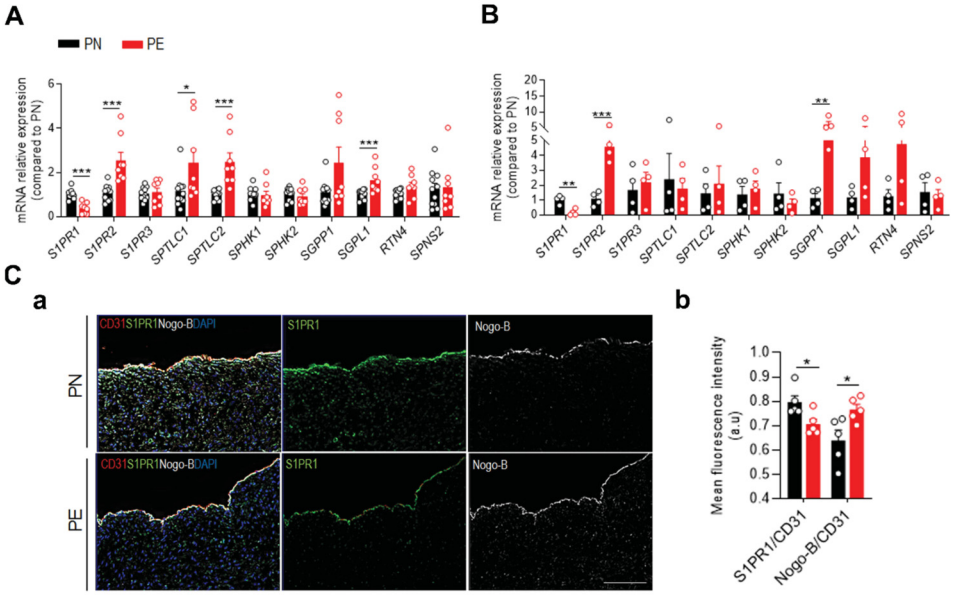

Figure 2. PE alters S1P signaling. RT-PCR of PN (n = 10) and PE (n = 8) homogenates of chorionic arteries (**A**) and isolated fPAEs (PN n = 4; PE n = 4) (**B**). Representative immunofluorescence staining of CD31, S1PR1 and Nogo-B in placental chorionic arteries of PN (n = 5) and PE (n = 5) subjects. Scale bar: 100 µm (**C**, **a**). Scatter plot of fluorescence intensity quantified by using ImageJ. Mean fluorescence intensity was calculated as the ratio of S1PR1/CD31 and Nogo-B/CD31. CD31 was used as the reference marker for the endothelium (**C**, **b**). Data are expressed as mean ± SEM * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared to PN. Statistical significance was determined by unpaired t-test.

3. Discussion

Altered sphingolipid metabolism has been associated with the pathogenesis of a repertoire of cardiovascular and metabolic diseases [26,35]. However, the role of sphingolipid metabolism and signaling in pregnancy and pregnancy-related disorders is understudied. Several reports highlighted the importance of sphingolipids in different aspects of the female reproductive system such as uterine decidualization [36], placental trophoblast differentiation [37], and uterine and placental angiogenesis [38]. In the context of PE, most of the studies focused on identifying potential biomarkers in plasma with conflicting results [20,39]. However, current knowledge of the role of sphingolipids in the pathophysiology of placental blood vessels remains elusive.

It is well established that the feto-placental vasculature plays an important role during fetal development. Indeed, fetal growth anomalies can occur despite a normal maternal uteroplacental perfusion, suggesting that a proper function of the feto-placental vasculature unit is mandatory independently of the maternal environment [40]. Our study reveals, for the first time, a pathological sphingolipid remodeling associated with gene profile changes of the placental chorionic plate in PE patients. Similarly, to what Romanowicz et al. found in UCA, we observed that PE was associated with a slight decrease in total ceramide levels [41], with only the reduction of C20:0-Cer levels being statistically significant. On the contrary, the analysis by Melland-Smith et al. revealed a significant increase in the concentration of different ceramide species including C18:0, C20:0, and C24:0 in preeclamptic placentae tissue compared to control [19]. This discrepancy could be explained by the different type of tissues used for the analysis of ceramides. Whereas they used the whole placenta tissues, in our study we focused on the contribution of the feto-placental vasculature, specifically we dissected chorionic arteries. It is also well accepted that sphingolipid metabolism is regulated in a cell type and/or context dependent fashion [7]. Additionally, different pathological mechanisms originated at the maternal side of the placenta might be accountable for the mild decrease in ceramide levels in PE. It is generally accepted that different acyl chains and/or double bonds confer specific biological

properties to ceramide species [42]. For instance, augmented levels of C16:0, C18:0, and C20:0 have been associated with anti-proliferative processes and apoptosis [43], whereas very long chain ceramides, such as C24:0, play an anti-apoptotic role [44]. Recently, multiple clinical studies demonstrated a robust correlation between specific plasma ceramide ratios and the occurrence of major cardiovascular events. Peterson et al. reported that higher plasma C24:0/C16:0 ratio negatively correlate with the increased risk of adverse cardiovascular events in patients affected by CAD [45], suggesting that specific changes in the plasma ceramide profile might be indicative of distinct pathological processes. However, measurements of sphingolipids in tissue and plasma samples from preeclamptic donors performed by our group, and others [20,39,46], did not correlate with PE conditions. On the contrary, we found a marked increase in SPT activity, dhSPh and SM, suggesting that a different remodeling of sphingolipid profile occurs during PE compared to CAD. The significant increase in dhSPh measured by LC/MS was strongly supported by the heightened SPT activity of chorionic arteries of PE patients. Increased dhSph levels have been associated with cellular lipotoxicity, in the context of diabetes and neurodegenerative and cardiovascular diseases [47–49], and most likely also contributes to the onset of the endothelial dysfunction of the chorionic arteries.

Disruption of SM homeostasis has been also linked to an adverse cardiovascular outcome [17]. Recent studies have demonstrated a positive correlation between serum SM levels and insulin resistance and inflammation [50]. Higher content of SM has been found in UCA as well as in syncytiotrophoblast-derived microvesicles of placentae of preeclamptic women [18,51]. In line with these previous reports, our lipidomic analysis showed a significant upregulation of C16:0-, C18:0-, and C24:0-SM in PE chorionic arteries compared to PN subjects. Notably, the increase in C18:0-SM was also reported in the apical membrane of lipid rafts from placentae as well as in plasma of preeclamptic patients during late gestation [20,52,53]. Considering the increased SM levels in tissue and plasma during preeclampsia [20,52,53], together with our findings of SM increase in PE chorionic arteries, it is tempting to speculate that altered SM content might play a role in the pathogenesis of this syndrome, although further studies need to be performed to validate this conclusion.

Sphingolipids have been implicated in processes regulating the endothelial barrier function and vascular tone [54–56]. S1P can enhance the endothelial barrier function [57] and reduce vascular tone by stimulating eNOS-derived NO production [56,58] These effects are mainly mediated by the endothelial S1PR1. Indeed, mice lacking endothelial S1PR1 are hypertensive and present blunted blood flow regulation [31]. Recent studies investigating the role of S1P signaling in pregnancy have demonstrated that the SPHK1/S1P/S1PR1 axis is crucial in early gestation to stimulate placental angiogenesis and the endothelial barrier function during pregnancy [59,60].

Notably, a study conducted by Dobierzewska et al. showed that PE induced a downregulation SPHK1 and S1PR1/3 expression in term placentae [46]. Our data corroborate in part these findings, with an increased expression of SGPL1, SGPP1, and S1PR2 in endothelial cells derived from chorionic arteries, and a concomitant reduction of S1PR1, both at mRNA and protein levels in the endothelium of preeclamptic chorionic arteries compared to controls. These results suggest a decreased endothelial-derived S1P, with a shift of the S1P signaling from S1PR1 to S1PR2-mediated functions. The upregulation of S1PR2 expression occurs in both culture endothelial cells and chorionic artery from PE patients compared to controls. S1PR2, which is described as a pro-inflammatory receptor, promotes the endothelial dysfunction in several pathological conditions [61,62]. Moreover, it mediates the contraction of diverse types of smooth muscle cells. Its activation has been associated with increased pulmonary vascular resistance [63]. Thus, the inverse regulation of S1PR1 and S1PR2 expression in chorionic arteries might explain, at least in part, the vascular dysfunction reported in preeclampsia, including a heightened placental vascular resistance.

Furthermore, the expression of key enzymes involved in the sphingolipid de novo biosynthesis, SPTLC1 and SPTLC2, were significantly increased, as reported in inflammatory and hypertensive [29,64,65]. Accordingly, we found a significant upregulation of SPT activity in chorionic arteries of PE compared to healthy subjects. Interestingly, the increased activity of SPT did not result in

the increase of all the sphingolipid subclasses. For instance, total ceramide levels were only slightly reduced, whereas SM significantly accumulated in PE arteries. This is not surprising because multiple enzymes of this metabolic pathway, and their expression levels and post-translational modification, can dictate the sphingolipid landscape in specific cell types, and hence tissues and organs.

We discovered that Nogo-B negatively regulates SPT activity, thereby controlling the de novo sphingolipid biosynthesis [21]. Mice lacking Nogo-B specifically in the endothelium are protected from hypertension and heart failure [21,29], mainly via the upregulation of endothelial-derived S1P S1PR1 signaling. Interestingly, Nogo-B expression was upregulated in the endothelium of preeclamptic chorionic arteries, as well as in the fPAECs. While it is difficult to correlate the SPT activity in the whole chorionic arteries with endothelial Nogo-B, since the expression of Nogo-B is very low in smooth muscle cells compared to the endothelium, our data suggest that Nogo-B upregulation in the endothelium of the feto-placental vasculature might play a role in the pathogenesis PE.

To our knowledge, this is the first study describing how PE affects sphingolipid metabolism at the feto-placental vasculature. Our study reveals a distinct alteration of the sphingolipid profile during preeclampsia, which includes (Figure 3): increased de novo biosynthesis, accumulation of dhSph, and augmented production of SM in chorionic arteries. In PE endothelial cells, the increased expression of Nogo-B, SPPase, and S1P lyase, together with the decreased S1PR1 expression and concomitant S1PR2 upregulation, indicate the onset of an endothelial dysfunction, typical of this disorder. In the present study, all the experiments were carried out exclusively on human tissues, which is the strength of our work. One limitation of our study is the small cohort. However, we believe that our study paves the way for future research on the role of sphingolipids in the pathogenesis of preeclampsia.

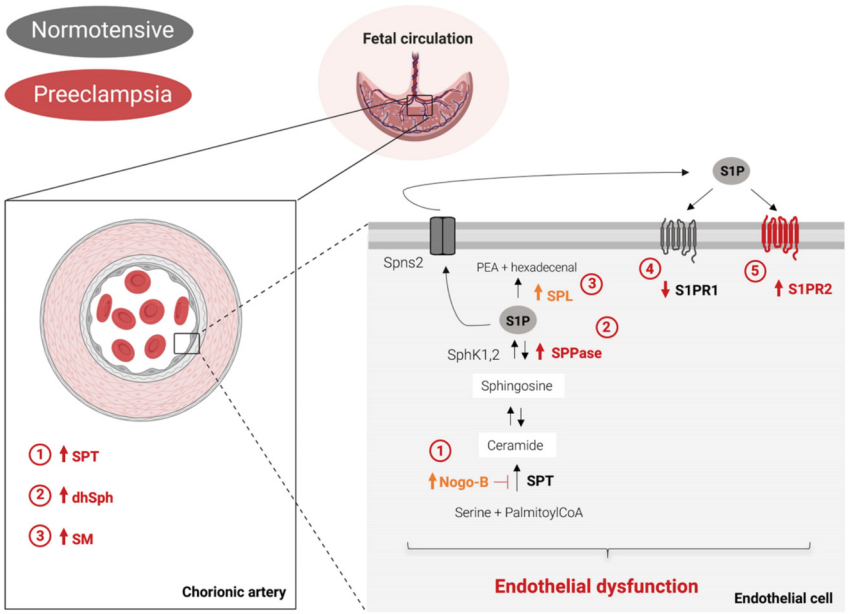

Figure 3. Summary scheme of PE-associated alterations of sphingolipid metabolism and signaling at the feto-placental vasculature. Left, PE is accompanied by increased SPT activity, accumulation of dhSph and increased SM production in chorionic arteries. Right, PE impairs the endothelial function by upregulating the expression of Nogo-B, SPPase, SPL and S1PR2, whereas it reduces the level of S1PR1 (statistically significant changes are depicted in red, whereas not statistically significant alterations are outlined in orange). SPT: serine palmitoyl transferase. KSR: 3-keto-dihydrosphingosine reductase. CerS: dihydroceramide synthase. DES: dihydroceramide desaturase. CDase: ceramidase. SM: sphingomyelin. SMS: sphingomyelin synthase. SMase: sphinglomyelinase. SphK1,2: sphingosine kinase. SPPase: sphingosine-1-phosphate phosphatase. S1P: sphingosine-1-phosphate. SPL: sphingosine-1-phosphate lyase. Spns2: spinster 2. S1PR1: sphingosine-1-phosphate receptor 1. The figure has been created with BioRender.com.

4. Materials and Methods

4.1. Study Population

In this study, preeclampsia was defined according to the guidelines of the American College of Obstetricians and Gynecologists (ACOG, 2019). Clinical characteristics for the PE study are summarized in the Supplementary Table S1. All subjects gave written informed consent. All experiments were performed in accordance with the protocols approved by the ethical committee of the Medical University of Graz (Vote no: 29-319 ex 16/17).

4.2. Isolation of Arterial Chorionic Vessels and Primary Human Placental Arterial Endothelial Cells (fPAEC)

Placentae from cesarean section and vaginal delivery were used within 20 min after delivery (n = 10/Control group, n = 8/PE). The amnion was removed and the arterial chorionic vessels with a length of ~3 cm were resected and washed in Hank's balanced salt solution (HBSS, Gibco, Thermo Fisher Scientific, Carlsbad, CA, USA). Next, isolated arteries were snap frozen or fixed in PFA for further processing. fPAECs were isolated from arterial chorionic blood vessels, as firstly described by Lang et al. [66].

4.3. Sphingolipid Analysis by LC-MS/MS

Chorionic placental arteries homogenates from normotensive and preeclamptic donors were used for quantification of sphingolipids by LC-MS/MS. The levels of ceramide (Cer) species, sphingosine (Sph), and S1P were analyzed by the Lipidomics Analytical Core at the Medical University of South Carolina, as previously described [67]. Lipid extraction was performed according to Bligh and Dyer [68]. For quantitative analysis of sphingolipid, eight-point calibration curves were generated for each target analyte. Synthetic as well as internal standards were spiked into an artificial matrix, and then subjected to an identical extraction procedure as the biological samples. These extracted standards were subsequently analyzed by the LC-MS/MS system operating in positive multiple reaction-monitoring (MRM) mode employing a gradient elution. Results were then calculated by plotting the sample area ratios against their corresponding standard. The MS analysis represents the mass level of particular sphingolipid (in pmols) per total sample used for lipid extract preparation. For the final data presentation, MS results were normalized to total protein (mg).

4.4. SPT Activity Assay

SPT activity in placenta arteries was measured as previously described [69]. Briefly, placenta arteries were homogenized in SPT reaction buffer (0.1 M HEPES (pH 8.3 at 25 °C), 5 mM DTT, 2.5 mM EDTA (pH 7.4), 50 µM pyridoxal 5'-phosphate (PLP; Sigma)). The assay was conducted in a volume of 0.1 mL composed by 200 µg of protein lysates, 0.45 µM [3H] serine (PerkinElmer), 0.2 mM palmitoyl-CoA (Sigma). After 15 min at 37 °C, the reaction was stopped with NH4OH and the product 3-ketosphinganine converted into sphinganine with NaBH4 (5 mg/mL). Radiolabeled lipids were extracted by using a modified Bligh and Dyer's method [68], dissolved in CHCl3, and analyzed by thin-layer chromatography.

4.5. Quantitative Real-Time PCR (qPCR)

fPAECs were washed twice in pre-warmed HBSS and harvested in 350 µL RLT Lysis buffer (Quiagen, Hilden, Germany) supplemented with 1% β-mercaptoethanol (Sigma Aldrich, St. Louis, MO, USA), whereas chorionic arteries were snap frozen in liquid nitrogen and homogenized in 400 µL TRIzol. Next, total RNA content from cells and tissue lysates was isolated using the RNeasy®®Mini Kit (Quiagen, Hilden, Germany). Reverse transcription was performed using 100 ng of RNA and Maxima Reverse Transcriptase (200 U/µL; Thermo Scientific, USA). For the real-time PCR analysis, SYBR green PCR Master Mix (Qiagen, Hilden, Germany) and iCycler Applied Biosystems 7700 were

used. 18S and HPRT1 were used as housekeeping genes. Primers sequences used for the real time PCR are listed in Supplementary Table S2.

4.6. Immunostaining

Isolated arterial chorionic placental vessels were incubated in calcium-free Krebs for at least 30 min to allow vessel vasodilation. Subsequently, the arteries were fixed with 4% PFA and left overnight at 4 °C. PFA-fixed arteries were OCT-embedded. For immunofluorescence, frozen placental artery sections were stained for Nogo-B (1:200, R&D), S1PR1 (1:200, R&D), and CD31 (1:200, Invitrogen) overnight at 4 °C and were then stained with Cy5-labeled anti-goat antibody (#A21436, Invitrogen, 1:500) Alexa 488 anti-rabbit (#016-540-084, Jackson ImmunoResearch, 1:200) and Alexa 568 anti-mouse in PBS for 1 h. Nuclei were stained with DAPI. Confocal immunofluorescence images of the tissues were captured on an Olympus Fluoview confocal microscope and quantified with ImageJ.

4.7. Statistical Analysis

Data are expressed as mean ± SEM. Statistical analysis were run with unpaired Student's *t*-test. Differences were considered statistically significant when $p < 0.05$. GraphPad Prism software (version 8.0, GraphPad Software, San Diego, CA, USA) was used for all statistical analysis.

5. Conclusions

In conclusion, to our knowledge, this is the first study to show the impact of PE on sphingolipid metabolism in the feto-placental vasculature. We demonstrated that in blood vessels of PE placentae, the sphingolipid biosynthesis is shifted towards sphingomyelin production rather than ceramide and, concomitantly, an impairment of the vasculoprotective S1P signaling was observed. Taken together, these results indicate a shift of the feto-placental vasculature towards a pathological state.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/21/3/1019/s1.

Author Contributions: I.D.G. conceived the study, designed most of the experiments and performed data analysis; L.S. performed the SPT activity assay and its data analysis; reviewing and editing was done by A.D.L.; I.D.G. and C.W. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

S1P	Sphingosisne-1-phosphate
S1PR	Sphingosisne-1-phosphate receptor
Cer	Ceramide
SM	Sphingomyelin
UCA	Umbilical cord artery
fPAEC	Fetal placental arterial endothelial cells
SPT	Serine palmitoyltranferase
SPTLC1	Serine palmitoyltranferase long chain base subunit 1
SPTLC2	Serine palmitoyltranferase long chain base subunit 2
SPNS2	Spinster transporter 2
SPHK1	Sphingosine kinase 1
SPHK2	Sphingosine kinase 2
SGPP1	Sphingosisne-1-phosphate phosphatase 1
SGPL1	Sphingosisne-1-phosphate lyase 1
Nogo-B	Reticulon-4B

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