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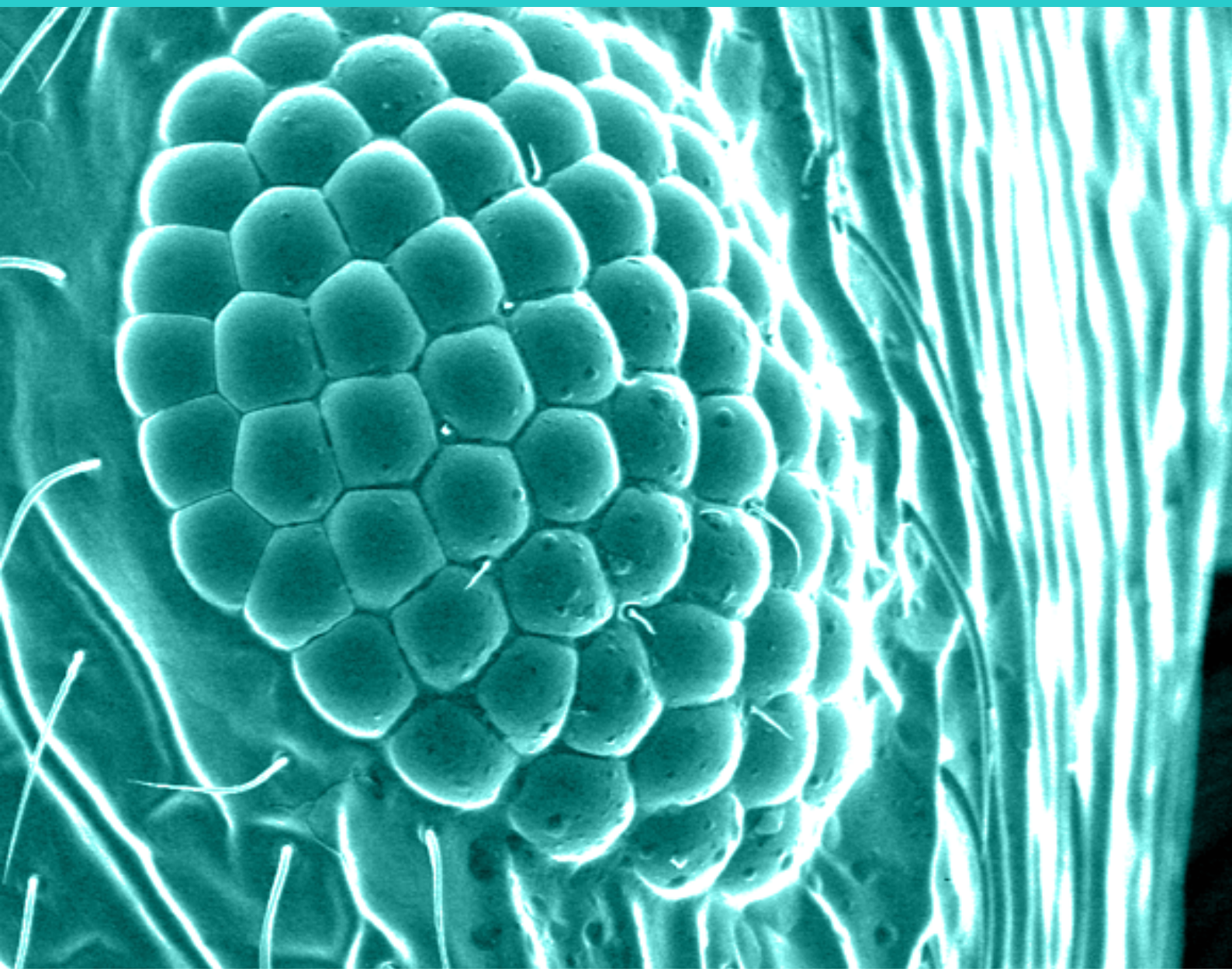

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FETOPLACENTAL ENDOTHELIAL DYSFUNCTION IN MATERNAL HYPERCHOLESTEROLEMIA AND OBESITY IN PREGNANCY

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ABSTRACT

Human fetoplacental vascular function is altered in several pathologies of pregnancy as a result of endothelial dysfunction. Pregnancy is a physiological condition coursing with increased circulating plasma levels of cholesterol in the mother, to respond to the higher demands from the growing fetus. An abnormal increase in maternal plasma cholesterol configures a pathological state referred as maternal supraphysiological hypercholesterolemia (MSPH). In MSPH, L-arginine transport and synthesis of nitric oxide (i.e., L-arginine/NO signalling pathway) as well as arginases/urea cycle in the fetoplacental endothelium are altered. Equally, an increase in the physiological gain of weight in pregnant women could end with obese women at the end of pregnancy leading to a condition referred as obesity in pregnancy (OP). OP seems to be also associated with alterations in the L-arginine/NO signalling pathway in endothelium in animal models; however, nothing is known regarding alterations of the human fetoplacental endothelium in OP. Insulin, adenosine and NO are vasodilators in the fetoplacental vascular bed, and a role for these molecules is proposed in MSPH and OP. Alternatively, involvement of intracellular pH modulation and the potential involvement of adenosine receptors is proposed as phenomena that could improve endothelial dysfunction associated with these diseases of pregnancy.

Running title: Endothelial dysfunction in diseases of pregnancy

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MATERNAL HYPERCHOLESTEROLEMIA IN PREGNANCY

Maternal supraphysiological hypercholesterolemia

Pregnancy is a physiological condition characterized by a progressive, weeks of gestation-dependent increase (reaching 40-50%) of cholesterol and triglycerides in the maternal blood [1]. The normal increase of total cholesterol (TCh) during pregnancy is known as 'maternal physiological hypercholesterolemia in pregnancy' (MPH), and is considered to be an adaptive response of the mother to satisfy the high cholesterol demand by the growing fetus for cell membrane and hormone synthesis, among other functions [1, 2]. However, when pregnancy-associated hypercholesterolemia goes over this physiological adaptation a different condition is identified, i.e., maternal supraphysiological hypercholesterolemia (hereafter referred as 'MSPH') [1-3].
Maternal TCh is transported through the placenta to the fetal circulation [4]. A strong correlation between maternal cholesterolaemia during pregnancy and the size of atherosclerotic lesions in arteries of neonates and children has been established [5, 6]. Additionally, MSPH leads to human placental vascular dysfunction, a phenomenon associated with reduced synthesis of the vasodilator nitric oxide (NO) [2, 3]. The effects of MSPH in the fetal vasculature could affect the initiation and progression of fetal atherosclerosis and may influence the molecular memory of the vasculature in response to future risk factors, a phenomenon recognized as *in utero* programming, which occurs during a limited prenatal period and leads to permanent changes [7].

Cut-off point for MSPH in pregnancy

There are no clinical reference values established for total and lipoprotein cholesterol levels during pregnancy in the global pregnant population [1-3]. In a Chilean population of pregnant women the mean of TCh at term of pregnancy was estimated in 260 mg/dL [2, 3]. This value is similar to reports in the literature showing ~246 mg/dL for TCh at term [3, 8] and comparable with values for TCh in pregnant population from Argentina (~244 mg/dL), Brazil (~243 mg/dL) and Colombia (~269 md/dL) [3].
MSPH is a maternal condition defined by considering a *cut-off* point for TCh at term of pregnancy of ~280-300 mg/dL, which associates with vascular alterations at birth and even in the childhood [2]. Increased oxidative stress in the maternal and fetal blood and the placenta [9], as well as reduced expression of the placental low-density lipoprotein (LDL) receptor [10] in pregnancies coursing with maternal TCh levels over this value are described. Moreover, increased early atherosclerotic markers, such as fatty streaks and lipid peroxidation, were determined in human fetal aorta [5] and in 7- to 14-year-old children aortas [6] born from mothers coursing with MSPH. Furthermore, endothelial dysfunction in the human umbilical vein from pregnancies coursing with values of TCh over this *cut-off* point exhibit reduced NO synthesis and endothelium-dependent vascular relaxation [2, 3]. Interestingly, ~30% of the pregnant women that were included in the study developed MSPH during pregnancy associated with increased levels of LDL cholesterol (LDL-Ch) [2]. Thus, maternal LDLs could be the lipoproteins mainly involved in the vascular effects of MSPH in the fetal circulation. Furthermore, a significantly higher number of pregnant women will potentially present an adverse intrauterine condition leading to the development of vascular alterations as endothelial dysfunction and early atherosclerosis in the growing fetus.

NO and MSPH

MSPH modifies at least two cellular pathways related with the endothelial function of the fetoplacental vasculature, i.e., the L-arginine/NO signalling pathway and the arginases/urea cycle [2, 3]. Pregnant women with MSPH show altered umbilical vein reactivity and umbilical vein endothelial dysfunction at birth compared with MPH. Although the maternal levels of TCh and LDL-Ch were increased in the MSPH group, the newborns from this group of women exhibit normal levels of TCh, HDL-Ch and triglycerides [2]. This finding suggests that a regulation in the traffic of cholesterol (i.e., transport of cholesterol from the maternal circulation to the placenta and efflux of cholesterol from the placenta to the fetal circulation) is occurring in the placenta [4]. MSPH is associated with reduced calcitonin gene related peptide dilation of umbilical vein rings an endothelium-derived NO dilation of the placental vasculature [2]. The reduced NO synthesis detected in HUVECs from MSPH compared with MPH, was paralleled by reduced Serine^{1179} phosphorylation (phosphorylation associated with eNOS activation), but unaltered total eNOS protein abundance [2]. MSPH was also associated with increased activity of arginases and increased protein abundance of arginase II (ARG II), an enzyme that competes with eNOS for the substrate L-arginine [11]. Interestingly, arginases activity inhibition partially reverses MSPH effect on vein ring dilation. Thus, MSPH results in human umbilical vein endothelial dysfunction likely due to an imbalance between eNOS and ARGII activity and expression [2, 3] (**Figure 1**).

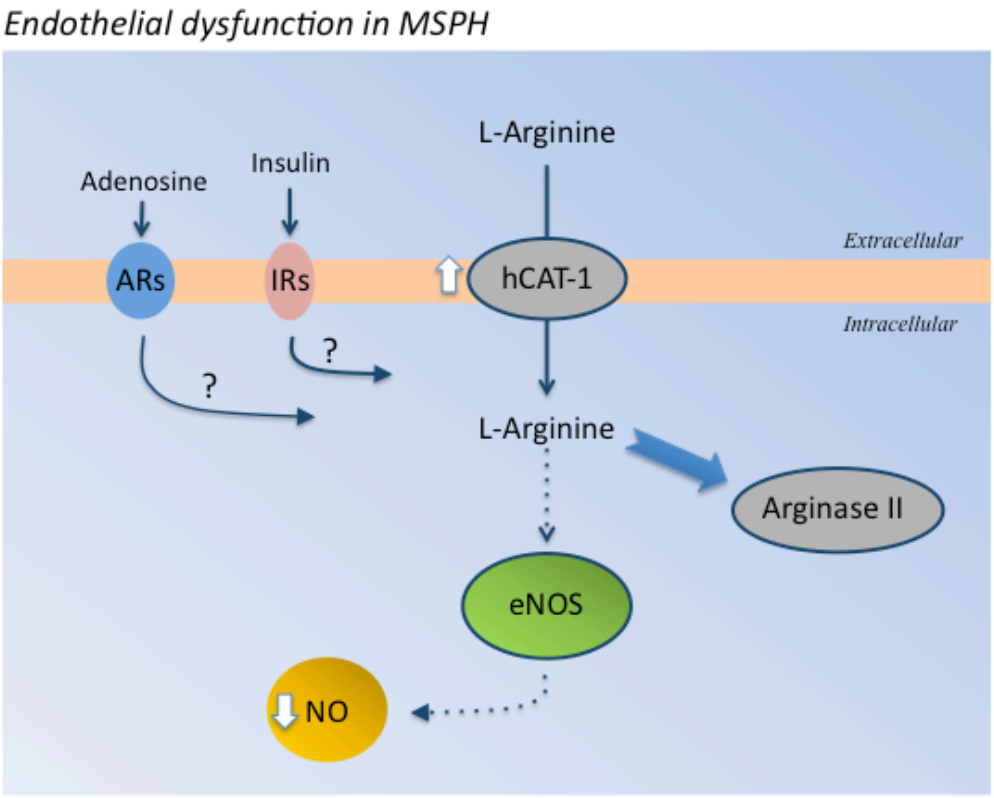

Figure 1. Role of insulin and adenosine on NO availability in MSPH. MSPH associates with increased (⇧) L-arginine transport in HUVECs compared with cells from MPH. This phenomenon is mainly due to higher transport capacity via the human cationic amino acid transporters 1 (hCAT-1) in this cell type. The increased L-arginine transport results in higher bioavailability of this amino acid in the intracellular space for its

catabolism via endothelial nitric oxide synthase (eNOS) and arginase II. L-Arginine is preferentially used by arginase II (solid blue arrow) compared with eNOS (dotted arrow), resulting in a reduced (⇩) synthesis of nitric oxide (NO), characteristic of endothelial dysfunction in MSPH. The potential contribution of insulin and adenosine signalling in this phenomenon in MSPH is unknown (?). However, since insulin via insulin receptors (IRs) activation does not alter L-arginine transport or NO synthesis in HUVECs from MSPH (A Leiva, L Sobrevia, *unpublished*), abnormal signalling following IRs activation probably due to altered level of cholesterol in the plasma membrane is proposed. Although the expression of adenosine receptors (ARs) may be altered in MSPH, the involvement of ARs in the genesis of MSPH is unknown as well as the contribution of their cell signalling mechanisms in NO metabolism. Considering that insulin and adenosine are potent vasodilators [2, 3, 12, 19], further studies are needed to characterize the potential cell mechanisms associated with NO synthesis in the fetoplacental vascular bed in MSPH.

Insulin and MSPH

Another molecule potentially modulated in MSPH is the hormone insulin, a key hormone for the placental metabolic regulation that is required in a normal pregnancy [12]. In the fetoplacental vasculature the insulin receptors (IRs) types A (IR-A) and B (IR-B) are expressed and are differentially regulated in the macrocirculation [13, 14] and in the microcirculation [15]. IRs are located in caveolae, a micro domain of the plasma membrane enriched in the protein caveolin [16], which is modified by plasma membrane cholesterol distribution [17]. Since high level of TCh increases the expression of caveolin and alters the caveolae structure modulating the activity of proteins located in caveolae such as eNOS [17], alterations in insulin signalling in pregnancies coursing with MSPH is feasible. Preliminary findings from our group show that MSPH associates with reduced vasodilatation of human umbilical vein rings in response to insulin (Leiva A, Sobrevia L, *unpublished*), a phenomenon that could be mediated by endothelial dysfunction since NO synthesis is also altered in HUVECs from these patients [3, 2].

Adenosine and MSPH

Adenosine is an endogenous purine nucleoside that maintains the homeostatic equilibrium via activation of adenosine receptors (ARs) [18]. Adenosine biological effects include modulation of energy homeostasis (ATP metabolism) and regulation of vascular tone [12, 13, 18, 19]. Interestingly, ARs have a significant role in the regulation of the cholesterol levels and atherosclerosis. Endothelial cells of mice lacking the A_{2B} adenosine receptors (A_{2B}AR) have increased levels of adhesion molecules and pro-inflammatory cytokines, promoting endothelial dysfunction and a pro-atherogenic profile [20]. Indeed, deficiency of A_{2B}AR leads to increased atherosclerosis lesions in mice; a phenomenon associated with higher plasma TCh (predominantly very low-density lipoprotein (vLDL-Ch)) and triglycerides. Additionally, administration of A_{2B}AR specific agonist in control mice leads to a reduction in lesion formation, and lower plasma lipids level [21]. Null-mice for A_{2A}AR have elevated levels of plasma TCh (predominantly low-density lipoprotein (LDL-Ch)) and pro-inflammatory markers [22]. Pharmacological activation of A_{2A}AR in endothelial cells from human aorta associates with increased efflux of cholesterol from the cells, a phenomenon mainly due to up-regulation of the ATP-binding cassette (ABC), sub-family A, member 1 (ABCA1) and ABC, sub-family G, member 1 (ABCG1) transporters [23]. In addition, A_3AR deficiency does not alter plasma TCh or atherosclerosis development [24]. Altogether, this evidence strongly suggests that ARs are involved in the regulation of cholesterol levels and in the development of atherosclerosis. However, whether these receptors are involved in the genesis of MSPH or whether plasma levels of

adenosine are altered in MSPH is unknown. Interestingly, pregnancies coursing with gestational diabetes mellitus (GDM) associates with increased plasma adenosine concentration in the umbilical blood with subsequent alterations of endothelial cell function [13-15]. In some cases women coursing with GDM also develop maternal hypercholesterolemia [3]; however a functional link between adenosine metabolism and the plasma lipid level has been neglected. Additionally, expression of ARs is altered in HUVECs from in GDM (Guzmán-Gutiérrez E, Sobrevia L, *unpublished*), but the potential consequences of these alterations on the cholesterol metabolism are unknown.

MATERNAL OBESITY IN PREGNANCY

Maternal gain of weight in pregnancy

Obesity is a worldwide disease that presents a risk to health defined in a patient with a body mass index (BMI) ≥30 kg/m^2 [25]. The incidence of obesity is currently ~12% at worldwide and is certainly increasing. According with WHO reports ~14% of women in the world suffer obesity [25], of which ~29% are in their reproductive age [26]. Thus, obesity during pregnancy is a critical condition for pregnancy management since it is in most cases preventable. When women with normal BMI (range 18.5 – 24.9 kg/m^2) [25] at the beginning of pregnancy exhibit a supraphysiological gain of weight reaching BMI ≥30 result in a condition defined as obesity during pregnancy (OP). While obesity is a condition that courses with systemic metabolic misbalance leading to multiple complications including endothelial dysfunction, the impact of OP on fetal endothelial function remains unknown [19].

In pregnancies coursing with maternal obesity increased risk of fetal mortality and morbidity, congenital malformations, macrosomia and increased incidence of caesarean delivery is reported [27]. However, whether this condition is due to OP or to pregestational obesity is unfortunately unknown since most of these studies do not separate this group of patients from normal, overweight (BMI = 25.0 – 29.9) or obese patients. Additionally, an inflammatory profile in the placenta from obese women has been described [28]; however, the consequences of OP on fetoplacental vasculature function, including expression and function of the endothelial L-arginine/NO signalling pathway, remain unknown [19, 28].

Cut-off point for OP in pregnancy

The effect of OP on human fetal endothelium is unknown, with only some studies in the aorta in an obese murine model [29]. In addition to the need of knowing the mechanism(s) that are altered by OP leading to endothelial dysfunction, a *cut-off* point value for OP is required. During pregnancy, several guidelines propose a potential physiological (i.e., normal) gain of weight based on pre-gestational weight of the women. At least two different guidelines characterizing Chilean pregnant women have been proposed [30, 31]. Based on preliminary observations fetoplacental vascular dysfunction has been found in HUVECs from women exhibiting BMI >30 classified as obese at term under Mardones & Rosso's report [31] or overweight and obese under Atalah *et al*'s report [30] for Chilean population. Indeed, in pregnancies cursing with OP (i.e., BMI >30) the correlation between newborn weight and gestational age is lost (Pearson correlation, $r = 0.36$, $p<0.01$ for normal pregnancies compared with $r = 0.13$, $p = 0.5253$ for OP) (**Figure 2**), suggesting that this condition is critical and could result in altered fetal outcome. At present, the characteristics of foetal endothelial function in OP are not defined, even when there is

information regarding endothelium-dependent modulation of vascular homeostasis by NO, adenosine or insulin.

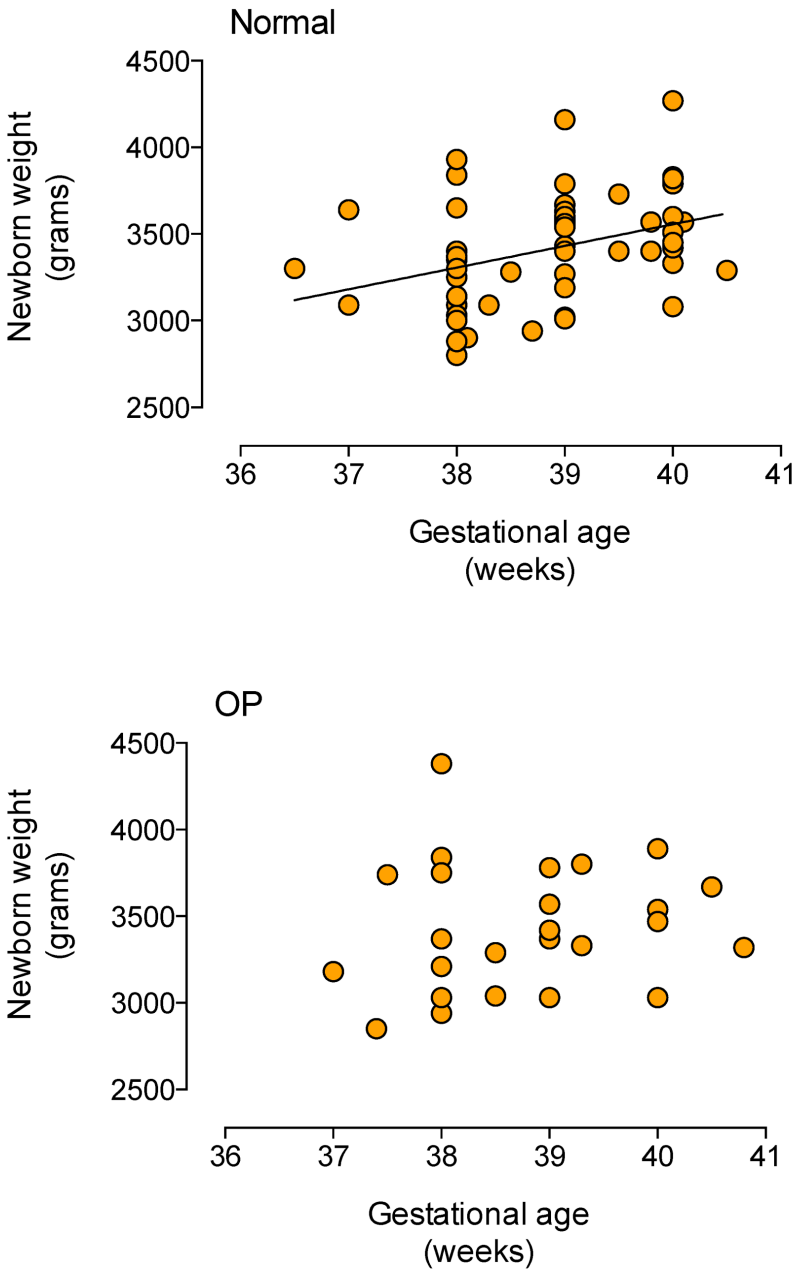

Figure 2. Correlations between newborn weight and gestational age. Pearson correlation for newborn weight and gestational age at term in pregnancies where maternal gain of weight ended within a normal (Normal) body mass index (BMI = 18.5 – 24.9 kg/m^2) or in obesity (OP) (BMI >30 kg/m^2).

NO and OP

Control mechanisms of NO synthesis in endothelial cells, such as eNOS activation or deactivation by phosphorylation, protein association and cellular localization, among others, are well known [32]. eNOS associates to caveolin 1 leading to reduce NO synthesis, which is even more attenuated when caveolin-1 is overexpressed [32, 33].

However, even knowing that HUVECs express caveolin, nothing is reported regarding OP and NO synthesis. As expected, an increase in caveolin-1/eNOS association would lead to lower NO synthesis in the human fetoplacental endothelial cells from normal pregnancies [19]. The latter findings and eNOS expression in OP is not documented. However, lower eNOS protein abundance an NO synthesis is reported in human subcutaneous fat arterioles endothelium from obese patients [34]. Furthermore, increased caveolin-1 expression while eNOS expression remains unaltered in saphenous arteries is reported [35]. Thus, both increase in caveolin-1 and/or decrease in eNOS could be potential mechanisms leading to lower NO bioavailability in the fetoplacental vasculature in OP in humans [19].

Insulin and OP

It is known that pregnancy is a condition that courses with a physiological state addressed as low sensitivity to insulin [13-15, 19]. In other metabolic alterations during pregnancy, such as in GDM, insulin restores macrovascular and microvascular endothelial dysfunction [13-15, 19] via a mechanism including differential activation of IR-A and IR-B, as well as ARs [13, 14]. However, the involvement of these IRs and the potential requirements of ARs for its biological effects in OP are not available. Pregnant obese women exhibit increased fasting insulin compared to pregnant non-obese women [36]; however, the effect of this hyperinsulinemia in the placental endothelial function in normal pregnancies as well as in OP remains unknown [19].

Adenosine and OP

Obesity associates with altered human fetoplacental vascular function, and a relationship between ARs and adipogenesis in adipocytes is reported [37], there are not studies addressing the potential role of ARs and/or activity and expression of the human equilibrative nucleoside transporters (hENTs) in the fetoplacental endothelial cell function in OP [19]. hENTs are expressed in HUVECs [13, 14] and human placental microvascular endothelial cells (hPMECs) [15] and play crucial roles in the uptake of adenosine maintaining physiological extracellular levels of this vasodilator [13, 19]. In addition, a potential direct or indirect effect of ARs activation on NO level, as proposed to occur in pathologies of pregnancy such as GDM [13-15] or PE (Salsoso R, Sobrevia L, *unpublished*), has not been investigated in OP [13, 19]. Interestingly, the transcription factors complex CCAAT/enhancer binding protein (C/EBP) homologous protein 10 (hCHOP)-C/EBPα (hCHOP-C/EBPα), which down-regulates *SLC29A1* (encoding for hENT isoform 1 (hENT1)) expression in HUVECs [13], is also expressed in human adipocytes where causes down-regulation of *SCL2A4* (encoding for glucose transporter isoform 4 (GLUT4)) expression [38]. In addition, obesity is also associated with altered insulin signalling in several tissues, and activates mitogen-activated protein kinases (MAPK) signalling cascades enhancing insulin resistance [13], but nothing is known regarding the effect of OP in these signalling mechanisms in the fetoplacental vascular endothelium.

INTRACELLULAR pH IN PREGNANCY

Intracellular pH regulation and endothelial function

As a normal consequence of cellular metabolism mammalian cells generate H^+. Different membrane transport systems maintain a stable intracellular pH (pHi) value of ~7.2 with an

extracellular pH (pHo) of ~7.4 [39]. If the expression and/or activity of the membrane transport systems maintaining these pH values are altered, cellular processes are deeply affected compromising cell viability. Thus, the control of pHi and pHo is critical for an adequate cellular function. Extrusion of H^+ occurs via Na^+/HCO_3^- co-transporter (NBCn1) and Na^+/H^+ exchanger isoform 1 (NHE1), a member of NHEs family, in arterial vascular smooth muscle and endothelium [39]. Normal function of these membrane transport systems is critical for controlling pHi, with NHE1 mainly accounting for H^+ extrusion in low pHi conditions (i.e., acidic) compared with non-acidic pHi [39]. The syncytiotrophoblast, which is in contact with both maternal and fetal vasculature, is responsible of H^+ transfer from the fetal to the maternal circulation. In this cell type, the recovery of pHi after an acid-pulse depends on NHEs activity [40]. In addition, *NHE1* and *NHE2* mRNA are detected during the pregnancy, of which *NHE2* mRNA level is higher in the last weeks of gestation [41]. Importantly, NHEs expression in the syncytiotrophoblast is reduced in preterm and in growth restricted placentas, suggesting that modulation of NHE expression may play a role in diseases of pregnancy [42].

Inhibition of NHEs function is regarded as a condition leading to vasodilation via eNOS [43]. The relaxation caused by cariporide (NHEs inhibitor) in rat aorta is reduced in eNOS deficient-mice and after treatment with the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME). Mechanistically, cariporide reduces pHi leading to release of intracellular Ca^{2+} from the endoplasmic-reticulum stores in bovine aortic endothelial cells. This increase in Ca^{2+} level allows association between eNOS and calmodulin increasing NO synthesis [43]. Additionally, hyperglycaemia, a condition exhibited in patients with diabetes mellitus or obesity, associates with endothelial dysfunction [12, 19, 44]. It is reported that this adverse environmental condition decreases NO production by reducing eNOS activity. Under these conditions, NHE1 and the Ca^{2+}-dependent protease calpain are activated, resulting in the dissociation of eNOS from the heat-shock protein 90 (Hsp90) and its subsequent inactivation. Inhibition of NHE1 by cariporide or in HUVECs knockdown for NHE1 results in lower pHi value and restoration of eNOS activity [44].

NHE1 activation appears to be also involved in several steps of atherosclerosis, including NHE1-mediated adherence of leukocytes to endothelial cells [45]. NHE1 activity is decreased by LDL-Ch, but increased by HDL-Ch in human platelets. Thus, the use of NHE1-inhibitors is proposed as a potential therapy for atherogenesis [45]. Rabbits subjected to an atherogenic diet exhibit cholesterol-dependent endothelial dysfunction, and treatment with cariporide recovers these alterations improving cholesterol level [46]. These findings indicate that targeting NHE1 could be a promising alternative to recovers endothelial function in diseases of pregnancy **(Figure 3)**. However, whether the endothelial dysfunction observed in pathologies of pregnancy regards NHE1 activity and/or expression, particularly in OP or MSPH, is not reported.

Concluding Remarks

MSPH and OP are diseases of pregnancy, which could course with detrimental effects on the fetoplacental vascular and endothelial function. In the absence of a *cut-off* value for the MSPH it was recently suggested that maternal total cholesterol over 280 mg/dL associates with altered fetoplacental endothelial L-arginine/NO signalling pathway and vascular reactivity [2, 3]. A *cut-off* value for OP is not reported, including for Chilean population. However, it is suggested that a BMI >29 kg/m^2 (Atalah's chart) [30] or >30 kg/m^2 (Mardones & Rosso's chart) [31] could be considered as a *cut-off* value for OP leading to altered human fetoplacental dysfunction **(Figure 4).** These pathological conditions could result from altered endothelial function due to abnormal regulation of the pHi with a

critical role played by NHE1 for H^+ extrusion. Insulin, adenosine and NO vascular effects could associates with NHE1 expression and activity. In addition, adenosine receptors in concordance with insulin receptors could play a role in MSPH and OP associated endothelial dysfunction.

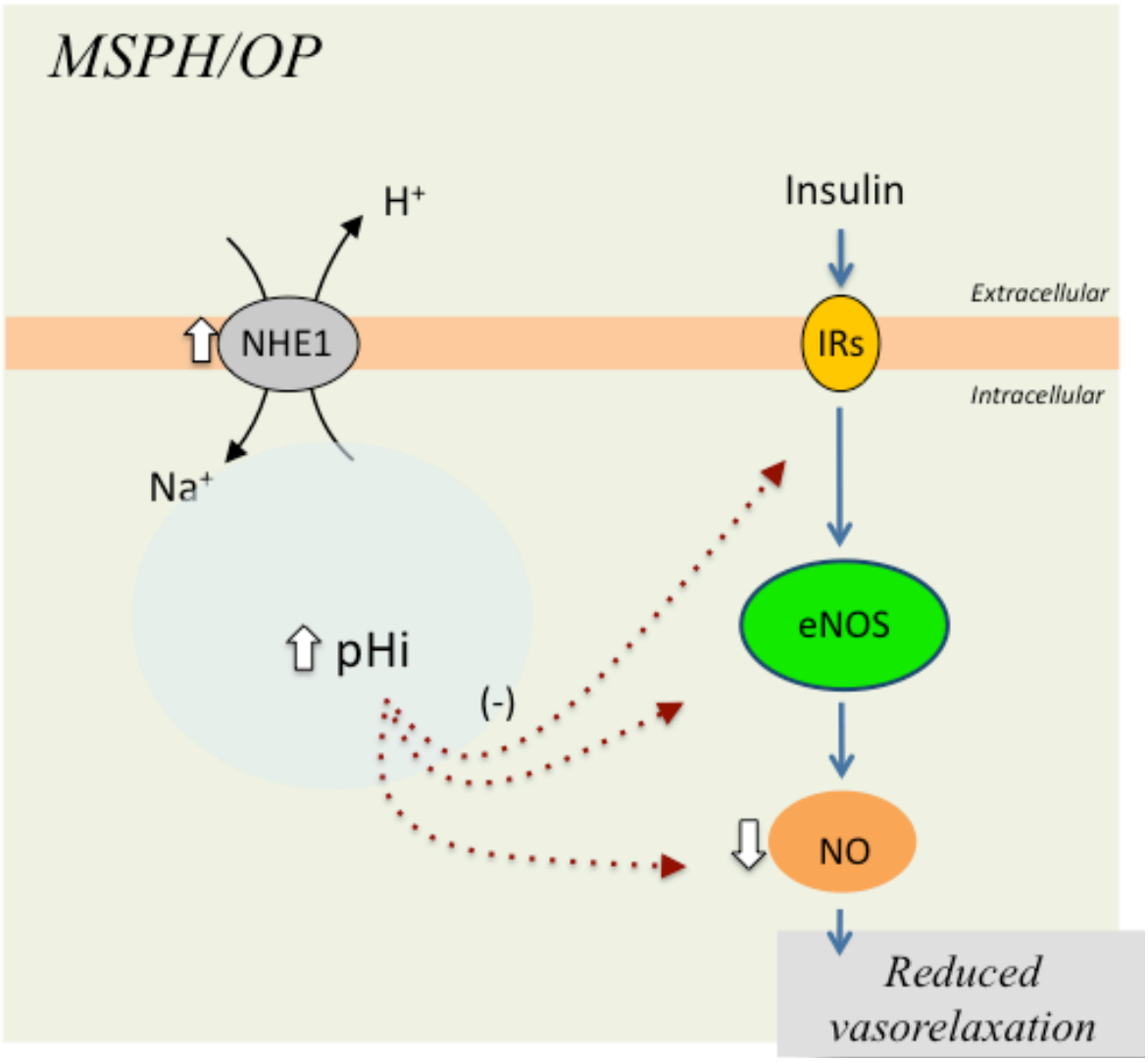

Figure 3. Proposed model for a potential role of NHE1 in human fetoplacental endothelial dysfunction. In non-pathological conditions the Na^+/H^+ exchanger 1 (NHE1) expression and/or activity remain in a physiological level, thus, maintaining a normal intracellular pH (pHi). Under pathological conditions such as maternal supraphysiological hypercholesterolemia (MSPH) or maternal gain of weight ending in obesity in pregnancy (OP), NHE1 expression and/or activity may increase (⇧), leading intracellular alkalization (increased (⇧) pHi value) involving transcriptional and/or post-translational mechanisms. Increased NHE1 expression and/or activity will result in inhibition of endothelial nitric oxide synthase (eNOS) activity, hence reducing nitric oxide (NO) synthesis leading to reduced vasorelaxation. In addition, NHE1 and increase in the pHi could result in deleterous effects decreasing insulin activation (+) of eNOS, thus contributing to MSPH and OP-mediated endothelial dysfunction in the fetoplancental vasculature. We envisage the possibility of future studies aiming to explore whether NHE1 inhibitors could result in improving fetal endothelial function in this diseases in human pregnancy.

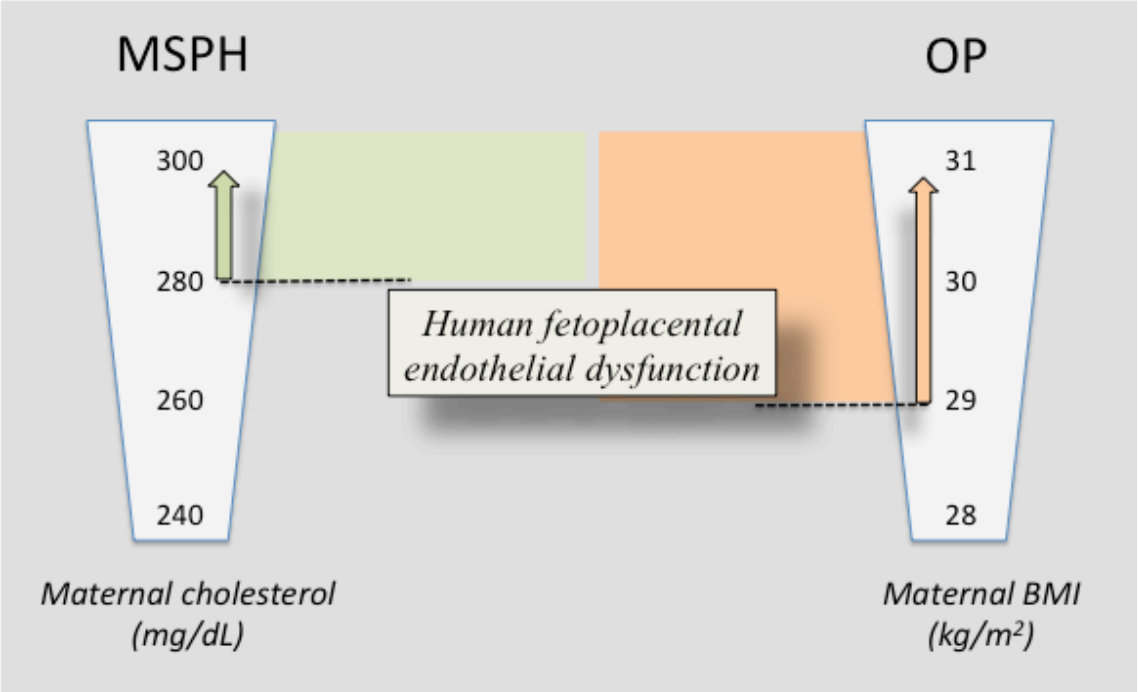

Figure 4. Proposed cut-off values for human fetoplacental endothelial dysfunction. In pregnant women coursing with maternal supraphysiological hypercholesterolemia (MSPH) the level of maternal plasma total cholesterol from and over 280 mg/dL associates with manifestation of human fetoplacental endothelial dysfunction (green area) [2, 3]. In pregnancies where the maternal gain of weight result in obesity in pregnancy (OP) a maternal body mass index (BMI) at term from and over 29 kg/m^2 associates with human fetoplacental endothelial dysfunction (orange area) (Pardo F, Sobrevia L, *unpublished*).

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Conflict of interest
The authors have declared that no conflict of interests exists.

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