Dissertation

INFLAMMATORY MEMORY OF FETAL ENDOTHELIAL CELLS: INTRAUTERINE PROGRAMMING BY MATERNAL METABOLISM AND INFLUENCE OF FETAL SEX

submitted by

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations 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'.

Elisa Weiss

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Disclosures

This cumulative dissertation is based on the following original publications:

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Simovic V[1] supported in performing qPCR and immunoblotting for analysis of MME expression

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Imran S[3,7] supported in data analysis to investigate inflammatory memory of fetal EC

Shepherd R[3] contributed to methods writing and data interpretation of inflammatory memory of fetal EC

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All co-authors have agreed to the inclusion of their published data in the dissertation.

I further contributed to the following publications during my PhD studies:

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I dedicate this work to my nephew **Aurel**. Your just completed intrauterine programming shall give you a golden life.

Table of contents

Abbreviations

BMI	body mass index
BOEC	blood outgrowth endothelial cells
CpG	cytosine-phosphate-guanine
CRP	C-reactive protein
CVD	cardiovascular disease
CVRF	cardiovascular risk factor
DOHaD	Developmental Origins of Health and Disease
EC	endothelial cell
ECFC	endothelial colony forming cell
EGF	epidermal growth factor
ELF	E74 like ETS transcription factor
eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cell
FAK	focal adhesion kinase
Flk	fetal liver kinase
fpAEC	feto-placental arterial endothelial cell
fpEC	feto-placental endothelial cell
FPG	fasting plasma glucose
GDM	gestational diabetes mellitus
GLP	glucagon-like peptide
H3K4me3	H3 histones trimethylated at lysine 4
hPSC	human pluripotent stem cell
HUVEC	human umbilical vein endothelial cell
ICAM	intercellular adhesion molecule
IFITM	interferon-inducible transmembrane
IL	interleukin
IRF	interferon regulatory factor
KDR	kinase insert domain receptor
KLR	killer cell lectin-like receptor
lincRNA	large/long intergenic non-coding RNA
lncRNA	long non-coding RNA

LPS	lipopolysaccharide
MHC	major histocompatibility complex
miRNA	microRNA
MME	membrane metalloendopepidase
NO	nitric oxide
NP	natriuretic peptide
oGTT	oral glucose tolerance test
oxLDL	oxidized low-density lipoprotein
Poly I:C	polyinosinic:polycytidylic acid
ROS	reactive oxygen species
TGF	transforming growth factor
TLR	toll-like receptor
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

Zusammenfassung

Die Plazenta ist ein fetales Organ und bildet die Verbindung von Mutter und Kind. Jegliche Störungen der Plazentafunktion können schwerwiegende Auswirkungen auf die fetale Entwicklung und die postnatale Gesundheit haben. Eine wichtige Rolle spielen dabei die Blutgefäße der Plazenta, die - wie alle Blutgefäße - mit einer Zellschicht, dem Endothel, ausgekleidet sind. Kardiovaskuläre Risikofaktoren wie Übergewicht oder Hyperglykämie beeinträchtigen die Endothelfunktion, und können während der Schwangerschaft von der Mutter auf den Fötus übertragen werden. Interessanterweise gibt es einen Geschlechtsdimorphismus bei der endothelialen Funktion und dem Risiko für Herz-Kreislauf-Erkrankungen im Erwachsenenalter. Daher stellten wir die Hypothese auf, dass der mütterliche Stoffwechsel die fetale Endothelfunktion durch eine geschlechtsspezifische intrauterine Programmierung beeinflusst. Dazu verwendeten wir fetale Endothelzellen aus der Plazenta (fpAEC; feto-placental arterial endothelial cells), der Nabelschnur (HUVEC; human umbilical vein endothelial cells) und dem Nabelschnurblut (ECFC; endothelial colony forming cells). Zuerst untersuchten wir die Auswirkungen des mütterlichen Stoffwechsels und des fetalen Geschlechts auf das Auswachsen der ECFC-Kolonien als wichtige Funktion dieser zirkulierenden endothelialen Vorläuferzellen. Männliche Zellen wuchsen schneller aus als weibliche, und waren anfälliger gegenüber Änderungen des Nüchternblutzuckerspiegels: Ein höherer mütterlicher Blutzucker, obwohl im gesunden Bereich, verlängerte die Zeit bis zum Auswachsen der ECFC-Kolonien. Außerdem untersuchten wir die Auswirkungen von mütterlichem Übergewicht auf das fetale Endothel. Dafür wurde die Expression der Peptidase MME (membrane metalloendopepidase) gemessen, welche Peptide spaltet, die den Gefäßtonus regulieren. Die MME-Expression war in fpAEC und im Nabelschnurblut von Kindern übergewichtiger Mütter verringert, was einen Effekt von mütterlichem Übergewicht auf kindliche endotheliale Funktion zeigt. Schließlich untersuchten wir die Rolle von inflammatorischen Prozessen bei fetaler Programmierung. Dazu wurde die Genexpression von ECFC und HUVEC als Reaktion auf eine inflammatorische Stimulation *in vitro* durch bakterielle und virale Reize analysiert. Neben einer ähnlichen transkriptionellen Veränderung dieser endothelialen Zellen fanden auch unterschiedliche Reaktionen statt. Zudem induzierte die Stimulation ein Immungedächtnis, das die Reaktion auf eine erneute Stimulation veränderte. Dieses ‚Gedächtnis' der fetalen Endothelzellen könnte auch dazu führen, dass sie sich an immuno-metabolische Veränderungen *in utero* erinnern.

Abstract

The placenta is a fetal organ that enables an exchange between the maternal and fetal circulations. Disturbances herein result in severe implications for fetal development and postnatal health. A major role is attributed to the placental vasculature with special emphasis on endothelial cells. While cardiovascular risk factors, such as overweight or elevated blood glucose, adversely affect endothelial function in adults, they can transmit from the mother to the fetus during pregnancy. Furthermore, a sexual dimorphism in adult risk for endothelial dysfunction and cardiovascular diseases exists. Thus, we hypothesized that maternal metabolism determines fetal endothelial function via intrauterine programming in a sex-specific manner. We employed fetal endothelial cells isolated from placenta (fpAEC; feto-placental arterial endothelial cells), umbilical cord (HUVEC; human umbilical vein endothelial cells) and umbilical cord blood (ECFC; endothelial colony forming cells). First, we analysed the effect of maternal metabolic parameters and of fetal sex on initial ECFC colony outgrowth, as a crucial determinant of endothelial progenitor function. Our data indeed revealed a sexual dimorphism in ECFC function already in the perinatal period. While male offspring cells exhibited faster outgrowth than female offspring cells, their outgrowth dynamics were susceptible towards maternal fasting plasma glucose levels. Higher glycemia within a healthy, non-diabetic range prolonged the time until ECFC outgrowth. Secondly, we investigated the effect of maternal overweight on fpAEC. To do so, we measured the expression of MME (membrane metalloendopepidase), an enzyme that cleaves several peptides involved in vascular tone regulation. Interestingly, we identified reduced MME levels in fpAEC and in cord blood of overweight mothers, highlighting the effect of maternal metabolism on fetal and neonatal endothelial function. Finally, we investigated the role of inflammatory processes in fetal programming. To analyse endothelial response to inflammation and memory thereof, we profiled the gene expression of ECFC and HUVEC in response to stimulation with bacterial and viral mimics. While similar transcriptional remodelling was induced in both, some cell type specific expression patterns were observed. These inflammatory responses were barely related to modifications in DNA methylation, suggesting other epigenetic mechanisms to be involved. We then showed that the initial stimulation induced immune memory, altering the cellular response to a microbial re-challenge. This inflammatory memory trait of fetal endothelial cells may explain their programming by immuno-metabolic changes *in utero*.

Introduction

The role of the human placenta

The placenta is a transient fetal organ that physically and functionally connects mother and fetus throughout pregnancy. It is essential for fetal growth and development via transporting nutrients and oxygen from the mother to the fetus paralleled by removing waste products and carbon dioxide from the fetal circulation. Moreover, the placenta acts as endocrine organ by releasing various hormones, thus regulating fetal development. In addition, it forms a barrier to protect the growing fetus from xenobiotic molecules. The highly vascularised human placenta is composed of a maternal and a fetal side, termed the basal and the chorionic plate. They border the intervillous space, which is a region surrounding placental villous tree-like structures that are highly packed with blood vessels and lined by the syncytiotrophoblast. This is a multinucleated syncytium forming the placental surface of exchange with the maternal blood. The microvascular vessels within the placental villi unite to large chorionic blood vessels that branch from umbilical cord vessels, which consist of one oxygenated blood carrying vein and two arteries transporting oxygen-deficient blood (8). The cellular components of umbilical cord blood are similar to those of adult peripheral blood, but the exact composition of cell subtypes differs (9). Additionally, human cord blood contains lots of stem cells from the hematopoietic, mesenchymal and unrestricted somatic lineage as well as endothelial progenitors (10).

Placental growth and development need to be highly regulated to ensure proper function at each stage of pregnancy (11). Availability of maternal nutrients and capacity of placental transport thereof can determine fetal growth (12). Disturbances in placental development and function may dramatically affect the fetus or even lead to miscarriage (8). Especially the formation of placental vasculature and regulation of angiogenesis is of great importance (13).

Feto-placental vasculogenesis and angiogenesis

The placenta and the growing fetus depend on adequate formation and development of their vasculature in order to be in proper connection with the maternal circulation. Endothelial progenitor cells (EPC) are particularly important for the growing embryo to establish a vascular network. While vasculogenesis describes the new formation of vessels from

precursors that differentiate and migrate, vessels are formed from already existing ones during angiogenesis. This includes sprouting and elongation via proliferation of endothelial cells (EC) on site combined with attachment of EPC (14). Disorders in both processes during pregnancy can have severe effects on feto-placental vasculature formation with disturbed fetal supply (15). That way, several pregnancy pathologies may lead to vascular alterations in the placenta with subsequent dysregulated nutrient supply to the fetus and impaired feto-maternal signalling (16).

Endothelial function and dysfunction

The endothelium is a multifunctional tissue forming the inner lining of blood vessels. Besides the above described formation of blood vessels via vasculogenesis and angiogenesis, it maintains a selectively permeable barrier between blood and surrounding tissue. Via paracrine and endocrine signalling, EC regulate vascular tone and blood coagulation, participate in inflammation and modulate immune response (17-19). One factor associated with proper endothelial function is the vasorelaxing nitric oxide (NO), which is produced by endothelial nitric oxide synthase (eNOS) (20, 21). Loss in endothelial function, i.e. endothelial dysfunction, is hallmark of vascular disorders such as cardiovascular disease (CVD) (22-25). This is the sequel of inflammation mediated 'endothelial activation' resulting in an imbalance between vasoconstrictive and -dilative factors (26). Consequently, the endothelium shifts to a pro-inflammatory and prothrombic stage (24).

Heterogeneity of (fetal) endothelial cells

Although common overall features, phenotype and function of EC is heterogeneous (27) depending on the site of the body they originate from (28), and the vascular bed they are present in, as seen when comparing arterial and venous derived EC (29, 30). This is also true for feto-placental EC (fpEC). While arterial fpEC are polygonal-shaped cells growing in cobblestone patterns, venous fpEC are characterised by spindle-shaped morphology, forming a swirling growing pattern. In addition to the morphological variance, arterial and venous derived fpEC also exhibit different proliferation rate and gene expression profile (31). Therefore, it is important to consider the heterogeneity of EC not only on inter- but also on

intraorgan level from physiological and molecular perspective (32). One reason for the heterogeneous phenotype of EC is epigenetic regulation via altered DNA methylation patterns (33-35). Although all mature EC originate from endothelial progenitors, their individual structure and function is determined by epigenetic modulation and microenvironmental signals depending on the site of the vascular bed they are embedding (36). For instance, despite deriving from the same organ, namely the placenta, fpEC differ from HUVEC of the large umbilical cord vessels regarding morphology, secretion of vascoactive substances and cytokine-mediated cell proliferation (37). Of note, also the developmental stage of the fetal endothelium during gestation determines its phenotype, as we have recently confirmed in umbilical artery derived EC from first trimester pregnancy (6).

In the present dissertation, three types of fetal EC were investigated:

1. Human feto-placental arterial endothelial cells (fpAEC) isolated from placental arteries of the chorionic plate
2. Human umbilical vein endothelial cells (HUVEC) isolated from the umbilical cord vein
3. Human endothelial colony forming cells (ECFC), which are circulating endothelial progenitor cells, isolated from venous umbilical cord blood

Their different origin in the vascular bed of the term placenta and cord blood is schematically explained in figure 1.

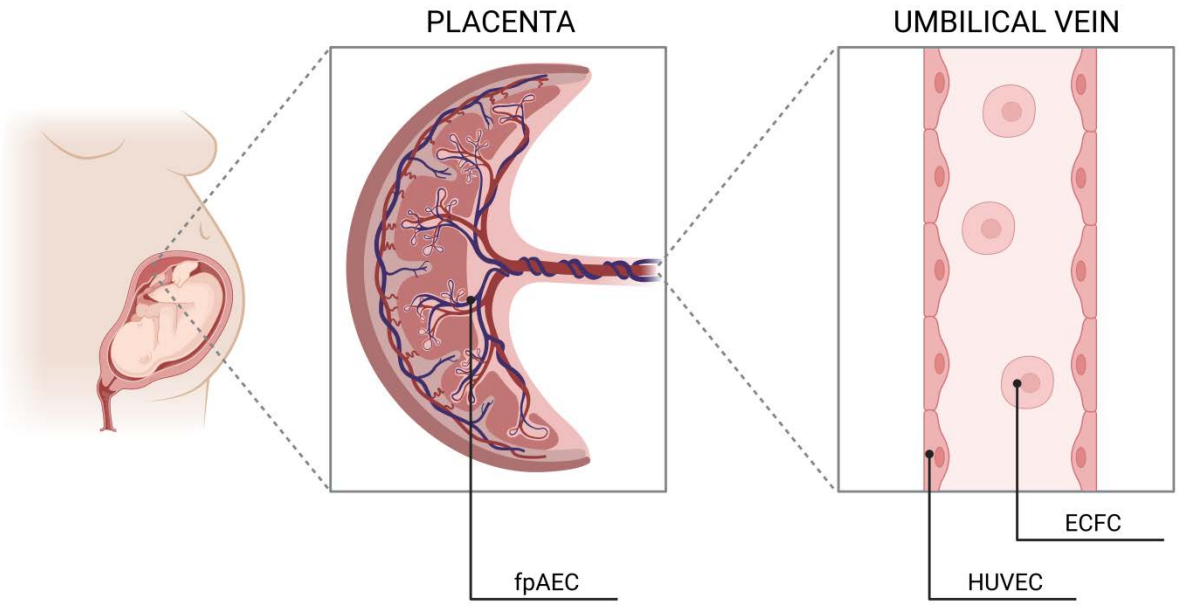

Figure 1 Origin of fetal endothelial cells. fpAEC line arteries located on the chorionic plate of the placenta. HUVEC line the umbilical cord vein. Fetal ECFC are isolated from circulating endothelial progenitors in the venous umbilical cord blood. Created with BioRender.com

Feto-placental arterial endothelial cells

fpAEC are detached from the endothelium of arteries on the placental chorionic plate via flushing of the vessel with an enzymatic solution (31). They have recently been used to investigate the effect of altered maternal metabolism as present in various pregnancy pathologies, or of *in vitro* emulated conditions on fetal development (7, 38-41).

Human umbilical vein endothelial cells

HUVEC serve as well-established cell model to investigate human endothelial function. Similar to fpEC, they are isolated by enzymatic digestion of the umbilical vein without detachment of cells from the surrounding connective tissue (42).

Endothelial colony forming cells

ECFC, also termed late outgrowth EPC or blood outgrowth endothelial cells (BOEC), are highly proliferative circulating EPC generating unequivocally cells of the endothelial lineage (43, 44). They were first isolated by Asahara et al. in 1997 based on magnetic bead selection

of CD34 and Flk-1 (fetal liver kinase 1; vascular endothelial growth factor receptor 2 (VEGFR-2); kinase insert domain receptor (KDR); CD309) positive cells that differentiated into EC *in vitro* and formed vascular structures *in vivo* (45). ECFC are recruited to perform angiogenesis of existing vessels, support vessel repair, but also possess the ability of neovascularisation (46, 47). They are highly abundant in umbilical cord blood as compared to adult peripheral blood (48, 49). We and others isolate fetal ECFC via density gradient centrifugation of cord blood and culturing of mononuclear cells (4).

All three fetal EC types used in this study show similar morphology in culture and grow in an endothelial-characteristic monolayer, as depicted in figure 2. Endothelial cell purity was confirmed for all individual donors as described previously (4). While HUVEC and ECFC show similar proliferation rate, fpAEC proliferate more slowly (6). All subjects used in this study derived from uncomplicated term pregnancies of mothers without any infections or pregnancy disorders.

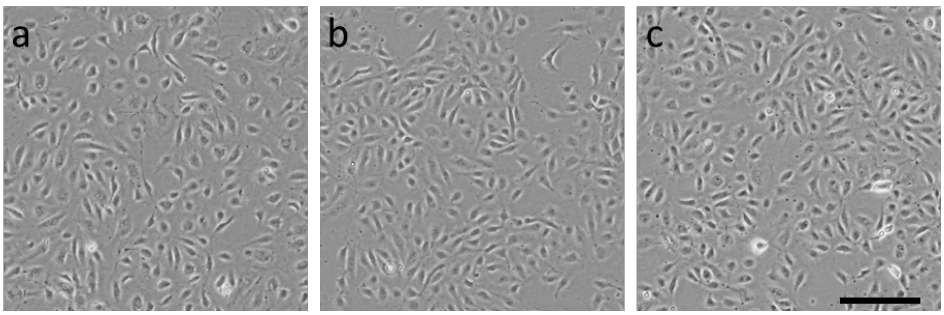

Figure 2 Morphology of fetal endothelial cells. The investigated fetal EC types grow in a monolayer and exhibit similar phenotype in culture. a = fpAEC, b = HUVEC, c = ECFC, scale bar represents 200 µm, modified from Gruber et al. (6) with permission of Histochem Cell Biol (Springer), http://creativecommons.org/licenses/by/4.0/

Overweight/Obesity and diabetes as cardiovascular risk factors

Several cardiovascular risk factors (CVRF), such as hyperglycemia, insulin resistance, overweight and obesity, can lead to endothelial dysfunction and associated vascular disease (50-53). Obesity is rapidly increasing worldwide and has reached pandemic dimensions (54). The commonly used method to define overweight and obesity is the body mass index (BMI), with 'normal' weight ranging from 18.5 to 24.9 kg/m^2. A BMI between 25 and 30 is classified

as overweight, while values above are further subdivided into three obesity classes (55). Increasing BMI directly correlates with endothelial impairment (56), since adipose tissue acts as inflammatory and endocrine tissue (57). It secretes pro-inflammatory cytokines resulting in decreased vascular availability of NO combined with increased production of oxidative stress (53). Leptin, whose plasma levels correlate with obesity (58), triggers endothelial dysfunction with subsequent vascular disease (59). Additionally, there is an indisputable link between overweight and obesity with type 2 diabetes (60), characterised as insulin resistance (61), systemic oxidative stress (62) and enhanced inflammation with upregulated levels of tumor necrosis factor alpha (TNF-α) (63). Insulin resistance in turn correlates with BMI, the inflammatory marker C-reactive protein (CRP) and leukocyte frequency (64). Diabetes impairs insulin secretion and can lead to vascular deficiency through the hyperglycemic milieu (65). Similar to overweight, hyperglycemia induces endothelial dysfunction by reducing NO bioavailability paralleled by enhancing the production of reactive oxygen species (ROS) and advanced glycation end-products (51, 66, 67). Even high glucose levels within a healthy, non-diabetic range may impair endothelial function (68) and enhance the risk for cardiovascular death (69). Likewise, already modest weight gain in healthy, normal-weight subjects can cause endothelial dysfunction (70).

Intrauterine programming by maternal cardiovascular risk factors in pregnancy

The DOHaD (Developmental Origins of Health and Disease) paradigm describes that the prevalence of adult diseases is affected by early life factors during intrauterine development (71), such as maternal smoking (72), nutrition (73-77), glucose levels and glucose tolerance (78-81). This fetal programming underlies epigenetic modifications as a result of environmental alterations *in utero* (82-84). Epigenetic mechanisms, which also occur in vascular EC, include DNA methylation, histone modifications and specific RNA-based mechanisms, thus determining genomic activity and disease susceptibility (85-87). A schematic overview of epigenetic regulation on different levels is depicted in figure 3.

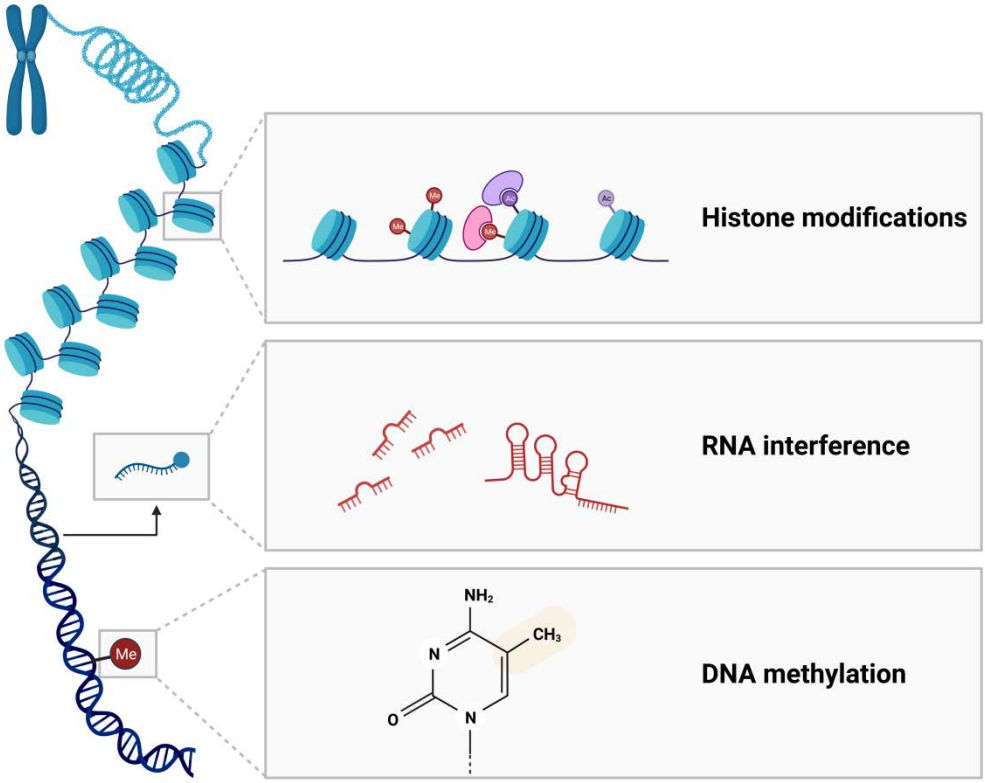

Figure 3 Epigenetic mechanisms. Gene transcription and protein translation can be modified on different levels via epigenetic regulation. Modifications can occur on histone tails or on the DNA strand directly. Also regulatory RNAs, such as microRNAs or long non-coding RNAs, are involved. Created with BioRender.com

All these modifications interact (88) and determine DNA compaction, resulting in open or closed chromatin regions, allowing or blocking gene transcription (89). The chromatin consists of nucleosomes, which are histone proteins wrapped by the DNA string. Several posttranslational modifications in histone tails have been described, mostly affecting the amino acids lysine, arginine, serine and threonine. Modifications like acetylation, methylation or ubiquitination regulate transcriptional activity (87). RNA-based mechanisms include short and long non-coding RNAs, which regulate again the chromatin state and determine gene expression (87). They mainly involve microRNAs (miRNA) (90, 91), long non-coding RNAs (lncRNA) (92), and large/long intergenic non-coding RNAs (lincRNA) (93). DNA methylation refers to the transfer of a methyl group (CH_3) on the nucleotide cytosine via methyltransferases and mostly occurs on a cytosine following guanine (cytosine-phosphate-guanine, CpG). Genomic regions with high CpG content are termed CpG islands and are often located within gene promotors. The DNA methylation state determines chromatin compaction

and consequently transcription factor binding or recruitment of proteins repressing gene expression. Depending on the DNA sequence, methylation differently affects gene expression in a tissue-specific way. For instance, DNA methylation is essential for genomic imprinting and inactivation of the X chromosome. It is a highly dynamic process including *de novo* methylation and demethylation, leading to a stable DNA methylation pattern in differentiated cells (88). Hence, epigenetic mechanisms regulate gene transcription and protein translation in the absence of changes in the DNA sequence (89). These modifications can be heritable and passed to daughter cells during cell division (94). Especially DNA methylation can persist in the long-term (95). In particular implantation and prenatal development is sensitive to environmental conditions, as cells are still plastic, but epigenetic alterations might persist (89). This high susceptibility towards transient environmental influences in early life was also demonstrated in investigations on the impact of the Dutch Hunger Winter. Intrauterine exposure to famine imprinted the offspring's DNA methylation pattern, which persisted throughout life (96). Therefore, it is not only the gene sequence alone that defines our phenotype, but an interplay of genetics and environmental exposures, which already start at conception or are acquired during pregnancy (89). The impact of maternal CVRF during pregnancy on the offspring is described in the following by the example of overweight/obesity and hyperglycemia.

Effect of maternal overweight/obesity in pregnancy

In parallel to the increase of obesity in the general population, the prevalence of obese pregnant women rises (97), which can affect mother and fetus from the very beginning of pregnancy (98). The risk for miscarriage (99) and stillbirth (100) increases, and so does the risk for developing other pregnancy pathologies, such as gestational diabetes mellitus (GDM) and preeclampsia (101), whose incidence increases with stage of obesity (102). Obesity in pregnancy is characterised by maternal chronic low-grade inflammation accompanied by disturbed endothelial function (103-105). For instance, interleukin 6 (IL-6) and CRP are increased in obese pregnant women (106, 107), and levels of TNF-α positively correlate with BMI (108). Altered cytokine levels in the maternal circulation also affect fetal and perinatal development with increased placental inflammation (107) and oxidative stress (109). Inflammatory markers in fetal blood are impacted, potentially determining the neonate's

ability to control inflammation (110). Furthermore, pre-pregnancy obesity lowers NO availability in HUVEC (111) and alters expression of genes involved in mitochondrial and lipid metabolism (112). Beyond pre- and perinatal period, maternal overweight programs the offspring in long-term with enhanced risk to develop obesity, type 2 diabetes, metabolic syndrome and CVD (113-117). For example, higher pre-pregnancy BMI reduces insulin sensitivity and increases blood pressure in the offspring at an elementary school age, which in turn can track on to adulthood and evoke CVD (118, 119). Furthermore, high maternal pre-pregnancy BMI alters the cardiac structure in the offspring at the age of six years, which is again associated with high childhood BMI (120). One mechanism that may be involved in the enhanced risk for metabolic diseases in later life after being exposed to intrauterine obesity can be the above described epigenetic programming via modifications in DNA methylation and histone tails, as well as the involvement of non-coding RNAs (121-125).

Effect of maternal hyperglycemia in pregnancy

GDM is one of the most frequent pregnancy pathologies. Similar to overweight, GDM has become a worldwide pregnancy complication with increasing prevalence within the last decades (126), now affecting around 14% of all pregnancies worldwide (127). It is defined as glucose intolerance firstly manifesting in pregnancy, characterised by insulin resistance combined with reduced insulin secretion (128). GDM triggers the development of hypertensive disorders, such as preeclampsia (128), but also has adverse effects on mother and fetus *per se* - even beyond pregnancy. The pro-inflammatory state and associated risk for vascular disease in GDM affected mothers continues after pregnancy (64). Affected women have a higher risk to develop subsequent type 2 diabetes (129), possibly linked via insulin resistance (128), and related CVD (130). Underlying mechanisms are persistently high levels of CRP and IL-6 in post-GDM women, as the boosted innate immune response associated with GDM progresses to a chronic condition (64). Maternal hyperglycemia can also determine fetal glycemic state (131), leading to fetal hyperglycemia and hyperinsulinemia (132). Moreover, maternal hyperglycemia does not only cause a pro-inflammatory environment associated with endothelial dysfunction in the mother (133, 134), but also induces inflammation in the placenta and fetal circulation (133). For instance, placental vessels of diabetic mothers show a pro-angiogenic phenotype (132) with increased branching

of capillaries (135). Even vascular changes in the neonate's iris have been observed (136). GDM upregulates inflammatory genes in the placenta (137) and affects barrier integrity of placental EC (35, 138). Similarly, GDM causes impaired function of HUVEC, such as proliferation, migration and tube formation (139, 140). In addition to perinatal complications, such as fetal macrosomia (141) and congenital malformations (128), GDM can program the offspring's metabolic stage in long-term. For instance, it increases the risk for metabolic syndrome in adulthood (142). Similar to the effect of maternal overweight, offspring of GDM complicated pregnancy have an enhanced risk for developing obesity, diabetes and CVD in child- and adulthood (141, 143-146). Again, this long-term effects result from intrauterine programming via epigenetic mechanisms (147), as exposure to GDM leaves epigenetic signatures in placenta and cord blood (148). GDM derived fpEC exhibit altered gene expression and associated cell function via changes in DNA methylation profile (35). Even in fpEC of non-diabetic mothers, transient hyperglycemic *in vitro* conditions are remembered by altered non-coding RNA expression (7).

Sex-specific differences in pregnancy

An increasing body of literature shows a sex-specific intrauterine development with varying impact on mother and fetus. Generally, women carrying a male baby are at higher risk to develop GDM and suffer from other adverse pregnancy outcomes than when having a female baby (149). Male fetal sex heightens the risk for stillbirth (150) and enhances the concentrations of pro-angiogenic factors and pro-inflammatory cytokines in the maternal circulation, while carrying a female baby increases regulatory cytokine concentrations in the mother (151). Also in placental gene expression analysis, male sex associated with immune genes and inflammatory pathways (152). However, different cell types within the placenta and cord blood show distinct sex-specific differences (152, 153). Resulting from sex-specific placental function, a difference in response to maternal metabolic derangements has been observed between male and female fetuses (154). Maternal nutrition during pregnancy has greater influence on male than on female offspring (155), with higher risk for insulin sensitivity in male offspring of obese women (156). These differences in susceptibility towards metabolic alterations may again underlie epigenetic programming, which is known to vary between the sexes (157).

Sex-specific differences in endothelial function and susceptibility towards cardiovascular risk factors

Endothelial function differs between the sexes, which may be one reason for the different prevalence of CVD between men and women (158). While males are more susceptible towards coronary artery disease, females have a higher risk to develop microvascular dysfunction (159). In adults, this difference in endothelial function and susceptibility towards CVD is mainly driven by different levels of steroid hormones (158). Besides a variation in hormones and their receptors, however, also genetic and epigenetic mechanisms cause sexual dimorphism in CVD. In fact, sex chromosomes encode cardiovascular genes (160) and regulate endothelial function (161). However, not only genes located on sex chromosomes differ between males and females, but also sex-specific differences in autosomal genes exist (159). For instance, gene regulation on autosomal regions differs depending on fetal sex in bovine blastocysts in the absence of differential hormonal environment (162). Similarly, sex differences in response to stressors have been confirmed before the onset of sex hormones in mice (163). A sexual dimorphism was also obtained in the human fetal endothelium: Sex-specific gene expression and cell function was observed in HUVEC from male and female babies with higher mRNA and protein levels of eNOS in female cells (164, 165). Barrier integrity and actin organization differs between the sexes in fpEC, which is associated with altered miRNA expression (5). These findings highlight intrinsic sexual dimorphism in fetal EC, suggesting that sex-specific cardiovascular function is already primed early in development. On top, a sex-specific response towards maternal CVRF exists, as fpEC exposed to a diabetic intrauterine environment differ between male and female fetuses (166).

Immune response and inflammatory memory of endothelial cells

Originally, the immune system has been divided into an innate and an adaptive part. While the innate immune system exhibits immediate but unspecific reaction upon exposure to a pathogen, the adaptive immune system leads to a more efficient response upon re-infection (167). Memory function was commonly associated with adaptive immunity only (168), but this dogma has been challenged by discovering immune memory in the innate immune system as well (169). Here, memory effects are subdivided into ‘trained immunity’ and ‘immune tolerance’. Trained immunity is characterized by resistant epigenetic alterations despite a

return in immune response to basal conditions after removal of the stimulus. This results in higher transcriptional and functional response upon re-stimulation as compared to the primary response. By contrast, tolerance transcribes the absent activation of immune response and associated gene expression after re-challenge with the same or similar pathogen (170). For instance, sepsis is described as a shift from a chronic pro-inflammatory to an immunosuppressive state via tolerance to endotoxins (171), resulting in an unresponsiveness with downregulated inflammatory cytokines (172). As opposed to this, trained immunity is characterised by an induction of pro-inflammatory cytokines, as seen in specific vaccines that also protect against other non-related microbial infections (173). Immune memory is generated via epigenetic programming of innate immune cells upon initial stimulation with altered accessibility of immune genes in case of a subsequent trigger (174). It has recently been discovered, that also non-hematopoietic cells, including EC, exhibit immune activity (175) and have important regulatory function in inflammation (176). EC were identified as immunoreactive cells involved in inflammation and host defence by producing and reacting to various immune modulators, such as cytokines, adhesion molecules, growth factors and vasoactive substances (19). During inflammation, activated EC release enhanced levels of pro-inflammatory cytokines, increase vascular permeability and leukocyte adhesion, and show pro-coagulant features, mediated by signal recognition via surface expressed toll-like receptors (TLR) (177-179). Furthermore, EC express major histocompatibility complex (MHC) class I and II molecules for antigen presentation (180). Recent body of evidence indicates that EC - similar to innate immune cells - are also capable of generating immune memory (177, 181). Besides the beneficial effect of a more powerful inflammatory response upon re-infection, endothelial trained immunity can lead to chronic inflammation associated with CVD (177). As an example, murine pathogen infected EC maintain their pro-inflammatory phenotype in culture, characterised by enhanced leukocyte adhesion associated with reduced NO production (182). Similarly, treatment of human aortic EC with oxidized low-density lipoprotein (oxLDL) primed the cells to release increased levels of pro-inflammatory cytokines upon re-challenge with an unrelated stimulus (181). Involved processes leading to trained immunity have been well studied in monocytes, and include epigenetic programming via alterations in the DNA methylation status at promotors of genes involved in inflammation (183, 184), and histone modifications (185, 186). As a hypothesis,

this may resemble mechanisms involved in programming of fetal endothelial function via maternal CVRF.

Hypothesis

We hypothesized that even small changes in maternal metabolism adversely affect fetal development via intrauterine programming of EC. The hereby established memory may determine the offspring's long-term health. Furthermore, we assumed that sex-specific differences in endothelial function as well as in their susceptibility towards metabolic changes already exist *in utero*. A schematic overview of this dissertation's hypothesis is represented in figure 4.

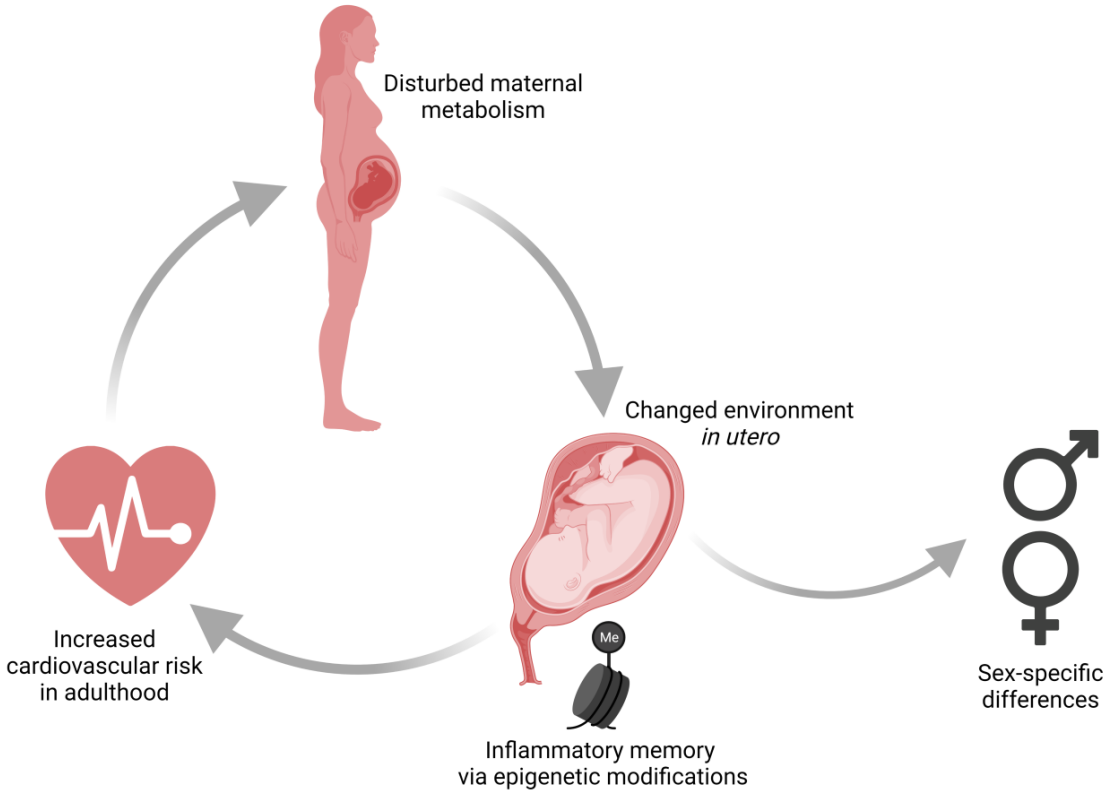

Figure 4 Dissertation overall hypothesis. Maternal metabolic derangements alter the intrauterine environment in a sex-specific manner. Via epigenetic programming, an inflammatory memory is generated, which in turn leads to an increased risk for cardiovascular diseases in adulthood. Created with BioRender.com

Dissertation objectives

In the present dissertation, I followed three overall aims to investigate fetal endothelial function in relation to a pro-inflammatory intrauterine environment considering fetal sex:

1. Determine influencing factors of fetal ECFC outgrowth as pivotal endothelial progenitor function
2. Investigate the effect of maternal overweight on fpAEC on the example of vascular tone regulating MME (membrane metalloendopepidase, also termed neprilysin and CD10) expression
3. Identify inflammatory response and memory of HUVEC and ECFC upon immunostimulation

Factors influencing fetal ECFC outgrowth

ECFC outgrowth reflects cell function and may determine future functional efficiency (49, 187). Initial clonogenicity represents an interplay of cell attachment, be it the culture dish or the *in vivo* vessel wall, differentiation from progenitor to mature cell, and finally proliferation to generate a colony. High clonogenic potential may be associated with fast vascular repair capacity (187).

While adult ECFC frequency and function is affected by CVRF, such as smoking (188), obesity (189) and diabetes (190-193), GDM during pregnancy can even transfer to fetal ECFC function (194, 195). The first objective of this dissertation was to investigate whether also maternal metabolic parameters within a healthy range affect fetal ECFC clonogenicity and whether there is an effect of fetal sex. To this end, I isolated ECFC from cord blood of male vs female neonates after healthy pregnancy and analysed initial outgrowth dynamics in respect to maternal metabolism during pregnancy.

Effect of maternal overweight on fpAEC on the example of MME expression

MME is a membrane-bound peptidase that degrades various peptides involved in vascular tone regulation (196). For instance, it cleaves peptides acting vasodilating, such as the natriuretic peptides A, B and C (ANP, BNP, CNP), bradykinin, and substance P, but also

peptides with vasoconstrictive features, like angiotensin II and endothelin-1. Therefore, the effect of MME on vascular tone is determined by the presence and composition of vasodilator or vasoconstrictor peptides (197).

Amongst other cell types, MME is expressed on EC (196, 197). Besides its membrane-bound form, the peptidase can be released from the cell surface as a soluble active enzyme. Thus, it can be released by EC into the culture medium (198), and soluble MME with catalytic activity has also been found in plasma (199). In adults aged above 45, circulating MME activity increases with weight and associates with metabolic syndrome (200, 201), which may underlie several factors of the pro-inflammatory environment. For instance, hyperglycemia and hyperlipidemia increase MME activity of cultured EC (202). Inflammatory mediators and growth factors, such as interleukin 1 beta (IL-1β), transforming growth factor beta (TGF-β) and epidermal growth factor (EGF) affect MME levels in different cell types (203, 204). Also the inflammatory markers CRP and IL-6 correlate with circulating MME concentration (205), revealing the susceptibility of MME expression and function to inflammatory status. Whether MME levels are also upregulated in the fetal circulation and endothelium by a pro-inflammatory intrauterine environment resulting from maternal overweight was the second objective of this dissertation. For that purpose, primary fpAEC deriving from pregnancies of lean vs overweight mothers were used as cell culture model to analyse MME mRNA and protein expression.

Inflammatory memory of HUVEC and fetal ECFC

In HUVEC, as model for fetal EC, immune response was recently analysed by stimulating cells with the viral mimic Poly I:C, which binds to endothelial TLR3, and with bacterial LPS, a ligand of TLR4 (206-209). Immune tolerance was observed after repeated doses of LPS, while initial stimulation with Poly I:C enhanced the immune response after a second hit of LPS or Poly I:C, showing increased cytokine production (207, 208). As explained previously, EC are structurally and functionally non-uniform, depending on their developmental stage, their location within the vascular tree and the size of the vessel. Hence, it is difficult to generalize their role in immune response. We assumed that the developmental stage of the endothelium plays a role in the cells' involvement in inflammatory process. In order to compare fetal EC from different source and differentiation status, we analysed the

transcriptomic immune response of the mature type of HUVEC and of fetal ECFC as endothelial progenitors. Combined with gaining information on the capacity and molecular basis of immune memory in different types of the fetal endothelium, it was the third objective of this dissertation. In order to pursue this research approach, analyses on the basal DNA methylation profile and transcriptomic alterations after initial stimulation with the viral mimic Poly I:C and re-challenge with bacterial LPS were performed.

Results

The results of this thesis are presented in the following publications:

Weiss E, Leopold-Posch B, Schrüfer A, Cvitic S, Hiden U. **Fetal sex and maternal fasting glucose affect neonatal cord blood-derived endothelial progenitor cells.** *Pediatr Res* (2022) Epub ahead of print; https://doi.org/10.1038/s41390-022-01966-4 (1)

Supplementary material is available at https://doi.org/10.1038/s41390-022-01966-4.

Weiss E, Berger HM, Brandl WT, Strutz J, Hirschmugl B, Simovic V, Tam-Ammersdorfer C, Cvitic S, Hiden U. **Maternal Overweight Downregulates MME (Neprilysin) in Feto-Placental Endothelial Cells and in Cord Blood.** *Int J Mol Sci* (2020) 21(3):834; https://doi.org/10.3390/ijms21030834 (2)

Supplementary material is available at http://www.mdpi.com/1422-0067/21/3/834/s1.

***Weiss E**, Vlahos A, Kim B, Wijegunasekara S, Shanmuganathan D, Aitken T, Joo JE, Imran S, Shepherd R, Craig JM, Green M, Hiden U, Novakovic B, Saffery R. **Transcriptomic Remodelling of Fetal Endothelial Cells During Establishment of Inflammatory Memory.** *Front Immunol* (2021) 12:757393; https://doi.org/10.3389/fimmu.2021.757393 (3)

Supplementary material is available at https://www.frontiersin.org/articles/10.3389/fimmu.2021.757393/full#supplementary-material.

*The experiments were performed in the laboratory of Prof. Saffery (Molecular Immunity, Infection and Immunity Theme, Murdoch Children's Research Institute, Parkville, VIC, Australia) during my research stay abroad.

In brief, maternal metabolism during pregnancy and fetal sex determined *in vitro* outgrowth of fetal ECFC. Mononuclear cells isolated from umbilical cord blood of male neonates required less days for initial ECFC colony outgrowth in culture than ECFC from female neonates, paralleled by faster cell confluency. In addition, the higher the levels of maternal fasting blood glucose measured during an oral glucose tolerance test (oGTT) at mid-pregnancy - within a non-diabetic range - the more days were required for fetal ECFC outgrowth. This effect remained only in the male subcohort after stratifying the analysis for fetal sex.

In addition, we have demonstrated that not only maternal glycemic state but also further subtle metabolic derangements transmit to the fetus. In order to determine the effect of maternal overweight on the fetal endothelium, we quantified the expression of the vascular tone modulating peptidase MME in arterial fpEC and in cord blood. Maternal overweight reduced fetal MME expression on mRNA and protein level, as well as the released MME amount. Similarly, cord blood MME concentration negatively correlated with maternal pre-pregnancy BMI.

To get further insights into the underlying mechanisms of intrauterine programming we investigated inflammatory memory of fetal EC. The basal DNA methylation profile of immune related probes clearly separated ECFC, HUVEC and fpEC (arterial vs venous). Also on RNA level, there were cell type specific signatures at baseline. Comparing ECFC and HUVEC, we identified common response patterns towards stimulation with the viral mimic Poly I:C on transcriptional level as well as ECFC-specific responses. However, these immune reactions were only little associated with the DNA methylation pattern at baseline, and thus might underlie other epigenetic regulation. Both cell types were further capable of establishing unspecific memory capacity, as seen after re-stimulation with a bacterial compound (LPS). Both employed fetal EC types showed trained (enhanced expression) and attenuated (decreased expression) genes. Our data suggest a progenitor-specific role in inflammation, highlighting the importance of separating data of endothelial function regarding developmental stage.

In summary, even subtle changes in maternal metabolism during pregnancy, such as higher glycemic state or overweight, affect fetal EC. Additionally, endothelial function and susceptibility towards maternal metabolism depend on fetal sex. These programming events

of fetal EC by a low-grade inflammatory intrauterine environment may underlie transcriptional remodelling, as observed in our inflammatory memory model after microbial stimulation.

Discussion

In this thesis, I investigated how maternal metabolism during pregnancy affects fetal endothelial function, and whether fetal sex has an impact herein as well. To this end, I first analysed initial fetal ECFC outgrowth in respect to maternal metabolic parameters within a healthy range and fetal sex. Secondly, I investigated the effect of maternal overweight on MME expression in fpAEC. Lastly, in order to get further insights into the underlying mechanisms of intrauterine programming, I examined immune response and inflammatory memory capacity of HUVEC and fetal ECFC.

Factors influencing fetal ECFC outgrowth: *Fetal sex and maternal fasting glucose affect neonatal cord blood-derived endothelial progenitor cells*

The first and indisputably pivotal function of endothelial progenitors is the initial outgrowth at the target site, which in turn relates to their frequency in the circulation. Due to their very low abundance, analysing EPC frequency and function is challenging. Therefore, *in vitro* grown ECFC are state of the art model to represent EPC (210). The number of outgrown ECFC colonies in culture is commonly used to report their frequency in the circulation (48, 210, 211).

Our first aim was to analyse the influence of maternal metabolism during pregnancy and of fetal sex on the outgrowth of neonatal endothelial progenitors. We identified that ECFC isolated from cord blood of female neonates required more days to grow out in culture compared to cells of male neonates. Literature on sexual dimorphism in adult ECFC is inconsistent. While some studies revealed lower ECFC numbers in females than in males when investigating middle-aged - mainly post-menopausal - women (212, 213), others reported higher ECFC colony numbers in female adults compared to age-matched men (214, 215). The authors suggested that this may underlie different levels of female sex hormones, as ECFC frequency in men is stable over time, but fluctuating in women (215). In females of reproductive age, estrogen plays a protective role on the cardiovascular system by enhanced NO mediated vasodilatation (216), which is mainly driven by estrogen receptors on EC and surrounding muscle cells (216, 217). However, a large cohort study did not detect any differences in cord blood estrogen levels of male and female newborns (218). This suggests

other, intrinsic factors to underlie the sex-specific difference in fetal ECFC function. Indeed, transcriptomic data of HUVEC showed a sexual dimorphism already in the perinatal period with around 10% of differentially expressed genes being located on sex chromosomes (219). Besides, intrauterine differences in growth factor and cytokine levels between male and female fetuses may prime their ECFC function. For instance, higher IGF-1 (insulin-like growth factor 1) and leptin concentration was measured in cord blood of female neonates (220-223). Moreover, the slower intrinsic ECFC outgrowth that we observed in females might be due to lower differentiation capacity, as reduced differentiation of human pluripotent stem cells (hPSCs) to EPC was observed in women (224). Overall, our data highlight the importance to distinguish between an intrinsic sexual dimorphism in endothelial function and acquired variations resulting from hormones and lifestyle. The present findings suggest that the intrinsic ECFC number favours males, then gets reversed by the beneficial effect of female hormones, which again diminishes after the menopausal age.

While we did not observe any effect of gestational weight gain or maternal pre-pregnancy BMI on fetal ECFC outgrowth dynamics, we further investigated the effect of maternal glycemic state therein. In diabetic patients, hyperglycemia is a main driver of endothelial dysfunction (67) and the effect of hyperglycemia, pre-manifested diabetes and GDM on fetal ECFC has been investigated in several studies. However, findings are inconsistent with reports about reduced (195, 225) or unchanged (194, 211) ECFC colony frequency in cord blood after diabetic pregnancy. Similarly, reduced (195) and enhanced (194) proliferation capacity after GDM pregnancy, but no effect on the time required until ECFC colony formation (195) was observed by others. Ingram et al. found a reduction in tube formation ability on Matrigel by more than 60% in fetal ECFC isolated after diabetic pregnancy (225). In our cohort of non-diabetic subjects, we did not observe a relation with maternal glycemic state in tube formation. However, we identified a correlation between maternal glycemia - within a normoglycemic range - at mid-pregnancy with the time required until fetal ECFC outgrowth. An increase in maternal fasting plasma glucose (FPG) by 10 mg/dL caused a 20% prolongation in fetal ECFC colony formation. The fact that higher maternal glucose impairs fetal ECFC outgrowth may underlie a hyperglycemia-mediated hypoxic intrauterine environment (226), as hypoxic *in vitro* conditions lower ECFC outgrowth, especially when deriving from cord blood (49). Hence, as a hypothesis, if glycemia is regarded as a continuum, high glucose levels - although in the normoglycemic range - may induce a subtle

hypoxic intrauterine environment. Similarly, even mild hyperglycemia during pregnancy can lead to endothelial dysfunction in the mothers (56), highlighting the susceptibility of mother and fetus already towards changes in glucose concentration within the non-diabetic range. The effect of maternal glycemia on fetal ECFC outgrowth did not apply to post-load glucose values. Also other studies support the importance of maternal basal glycemic levels on fetal development rather than values after sugar intake (227-229).

In parallel to basal sex differences in fetal ECFC function, our data also indicate that male and female cells respond differently to metabolic changes. The effect of maternal FPG on fetal ECFC outgrowth was only present in the male cohort after stratifying for fetal sex. Similarly, Liu et al. observed a higher correlation between maternal FPG and birth weight in male neonates (228). Accordingly, male sex generally associates with poorer outcome and with higher risk for morbidity after pregnancies complicated by maternal diabetes (230). GDM exposure increases the risk for developing childhood hypertension more in boys (231) and interestingly, also GDM treatment benefits are higher in male vs female offspring (232). Higher sensitivity towards metabolic risk factors in males can result from a higher tolerance threshold in placental adaption in females (233), and may represent a further reason for the enhanced susceptibility towards CVD in the male fertile population. Controversy, Krishnaveni et al. identified higher susceptibility of female babies to an intrauterine diabetic environment. However, authors added that the effect may result from an imbalance in the number of male and female subjects included in their analysis (145).

Limitations of our study include missing values for estrogen, IGF-1, leptin and insulin resistance, which might differ between the sexes (215, 220-223, 234) and thus contribute to the observed differences in ECFC outgrowth dynamics. In fact, these markers were identified to determine endothelial function in several studies (216, 217, 235-237). Alterations in insulin and IGF molecules in placental, maternal or fetal circulation also occur in diabetic pregnancies (238), and could thus evoke the differences in ECFC outgrowth depending on maternal glycemia. Besides, knowing C-peptide values would help to determine the actual metabolic profile present *in utero* (239).

In accordance with published literature, we confirmed that ECFC frequency and function is influenced by CVRF (188-190, 194, 195). It still remains unexplored whether impaired ECFC outgrowth is an indication of cardiovascular dysfunction that can be applied to the

endothelium in general, or whether the affected endothelial progenitors are directly contributing to CVD by reduced capacity to maintain a functional endothelium. The fact that intracoronary transplantation of circulating progenitor cells - almost entirely of endothelial phenotype - had beneficial effects on curing myocardial infarction (240), supports the latter hypothesis. The potential of ECFC to not only participate in angiogenesis, but also to contribute to *de novo* formation of blood vessels, highlights the importance of ECFC in embryonic development and the postnatal period (241). Also the rapid homing of $CD34^+$ hematopoietic stem cells from the fetal circulation directly after birth (242) suggests a key role of endothelial progenitors in perinatal vascular growth and postnatal angiogenesis. Investigating the effect of initial ECFC outgrowth dynamics and frequency on further neonatal vascular development remains topic of future studies. Although we only analysed the impact of gestational parameters on fetal endothelial function at the perinatal period, long-term effects are likely to occur. For instance, glycemic state during pregnancy determines offspring vascular health at the age of six years - again showing higher susceptibility in male children (243). Hence, we suggest that intrauterine programming of ECFC function determines the risk for CVD in adulthood, as the altered outgrowth dynamics of neonatal ECFC may transfer to postnatal angiogenesis and vascular repair.

Effect of maternal overweight on fpAEC on the example of MME expression: *Maternal overweight downregulates MME (neprilysin) in feto-placental endothelial cells and in cord blood*

Activity of the peptidase MME associates with obesity and metabolic CVRF in adults aged above 45 (200, 201), which may underlie the pro-inflammatory effect of hyperglycemia, hyperlipidemia, growth factors and cytokines (202, 204). Pregnancies characterised by maternal obesity do not only prime the offspring's risk to develop metabolic disturbances such as obesity, insulin sensitivity and type 2 diabetes, but can also lead to impaired blood pressure regulation in childhood, which further transmits to an enhanced risk for CVD in later life (115, 117-120, 244). In our study, we investigated whether overweight during pregnancy affects MME expression and release by the feto-placental endothelium and in the fetal circulation. In contrast to our expectations, maternal overweight did not increase but decrease

fetal MME levels. Additionally, fetal weight negatively correlated with circulating cord blood MME.

Overweight and obese pregnant women show elevated serum levels of pro-inflammatory markers, such as IL-6, TNF-α and CRP (245). Also in our study, overweight subjects showed higher CRP levels that correlated with pre-pregnancy BMI. The altered maternal metabolic and inflammatory environment transmits to the fetal compartment with increased fetal insulin resistance and higher cord blood levels of IL-6, TNF-α, CRP and leptin (246-249). Although inflammatory status in adults is associated with increased cell surface MME (203, 205), fetal MME seems to be regulated differently. A possible mechanism downregulating fetal MME may be an overweight-related hypoxic intrauterine environment. Rats exposed to hypoxia showed reduced lung and brain MME activity associated with decreased mRNA and protein expression (250, 251). Also in HUVEC, MME expression was inhibited by hypoxic environment *in vitro* (252). Indeed, subtle hypoxic intrauterine conditions are associated with pregnancies of overweight women (253, 254). However, culture of fpEC under hypoxic conditions did not affect MME expression in our study. Besides hypoxia, also NF-κB signalling may decrease fetal MME concentration. For instance, enhanced NF-κB expression in B-lymphoma cells reduces MME mRNA and protein (255). The inflammatory mediator TNF-α in turn activates NF-κB pathways in HUVEC (256), inducing ICAM-1 (intercellular adhesion molecule 1) expression (257). As mentioned above, cord blood TNF-α is increased after pregnancies of obese mothers, and fpEC respond to TNF-α treatment with increased ICAM-1 protein expression (38). Therefore, we further measured MME concentration *in vitro* after TNF-α stimulation. However, MME protein levels remained unchanged also in the presence of TNF-α, suggesting another mechanism to be involved in fetal MME regulation. For instance, shear stress downregulates MME expression in bovine vascular EC via ROS production (258). However, the fact that hypoxia did not affect MME expression in our study does not support the hypothesis of ROS-mediated MME reduction in the overweight-exposed fetal endothelium. Alternatively, as stated in our overall hypothesis, disturbed intrauterine environment can affect fetal gene expression via epigenetic mechanisms, including DNA methylation, histone modifications and regulation via non-coding RNAs. De facto, hypermethylation and histone deacetylation of the *MME* promoter region downregulate its transcription (259, 260), which was shown in various types of cancer (261-263). Additionally, MME is target of miRNAs, e.g. miR-155 regulates MME mRNA and protein expression

(255). According to the MicroRNA Target Prediction Database miRDB (www.mirdb.org), 156 miRNAs are involved in translational regulation of MME.

The physiological consequences of downregulated MME in the fetal circulation and endothelium are only speculative and may be versatile due to the multifunctional role of MME. For instance, inhibiting MME in HUVEC results in potentiation of the vasoactive factor bradykinin (264). However, as the effects of MME inhibition on vascular tone depend on the presence of vasoconstrictive and vasodilative factors (197), downregulation of MME may affect fetal vascular tone in both directions. In addition, MME plays a role in the insulin signalling cascade (265). On one hand, it cleaves and inactivates glucagon (266) and glucagon-like peptide 1 (GLP-1) (267), thus impairing insulin secretion (268). On the other hand, MME regulates the expression of insulin receptor subunits (269). Whilst inhibiting MME in mice leads to enhanced insulin sensitivity (269), obesity complicated pregnancies cause intrauterine insulin resistance (246). Therefore, the specific outcome of lower MME levels in the fetal circulation on insulin signalling is hard to predict. Furthermore, MME inhibits angiogenesis that is stimulated by FGF-2 (fibroblast growth factor) (270) or VEGF (vascular endothelial growth factor) (271). Besides enzymatic degradation of peptides (270), MME regulates vascularisation via signalling cascades by interfering with focal adhesion kinase (FAK) mediated pathways, thus decreasing cell migration (272). Indeed, placental vascularisation is affected by maternal obesity during pregnancy in humans and various animal models (273-278). These findings suggest that maternal overweight impacts placental and fetal vascularisation and development, which may contribute to programming events in the offspring's cardiovascular system.

A limitation of the study is that we do not have additional information on metabolic or inflammatory parameters from maternal and fetal circulation (e.g. glucose, insulin resistance, leptin, C-peptide, erythropoietin, cytokines) that would significantly contribute to the understanding of metabolically driven MME downregulation in the fetus. Instead, our group separation is based on maternal pre-pregnancy BMI, which may not fully reflect the intrauterine environment and the degree of inflammation. Besides, the concept of 'metabolically healthy obesity' describes the variation in individual risk for obesity-associated diseases (279), why follow-up analyses on the offspring are required. Moreover, stratified analyses for male and female offspring should be performed here as well, as maternal BMI

has sex-specific effects on placenta and fetus (280). However, the sample size needs to be increased for such subcohort analyses.

The finding about the effect of pre-pregnancy overweight on fetal MME levels again highlights the importance of considering even low-grade inflammation *in utero* as determinant of placental and fetal development.

Inflammatory memory of HUVEC and fetal ECFC: *Transcriptomic remodelling of fetal endothelial cells during establishment of inflammatory memory*

Finally, as metabolic derangements have a pro-inflammatory component as well, the question arose, whether also inflammatory processes can induce programming of fetal EC.

The principle of innate immune memory was established recently to play a role in both health and disease. Besides its beneficial effects such as the vaccination process, also immune-related diseases and chronic inflammation result from memory capacity (170, 174). This physiological and pathological function is an interplay of modification in metabolic pathways, the epigenome and transcriptome, leading to an altered response upon re-challenge (185, 186, 281-283). The capacity of immune memory is not limited to hematopoietic cells, but various other tissues do also remember inflammation after sensing foreign compounds or being subjected to damage (284). For instance, immune response and memory has already been identified in HUVEC analysing cytokine expression (207, 208). However, the underlying transcriptional responses have not been investigated. Therefore, we analysed transcriptomic remodelling during establishment of immune memory in HUVEC and ECFC to compare two fetal EC types. Although sharing a similar endothelial phenotype, ECFC show enhanced response towards pro-angiogenic stimuli along with higher proliferation rate compared to HUVEC. Thus, they exhibit greater repair potential, highlighting their progenitor stage (46). Their high frequency in the perinatal period (48, 49) underlines the key role of ECFC in vascular growth and remodelling soon after birth.

First, we showed that the DNA methylation pattern at baseline differs between fetal ECFC and HUVEC, further supporting the key function of epigenetic mechanisms in the heterogeneity of different EC types (33-35). Nevertheless, both cell types showed some

common transcriptional responses after being exposed to a microbial stimulus. Same as in monocytes, expression of genes involved in metabolic processes were highly dynamic upon stimulation (281, 283), suggesting consistent metabolic remodelling during development of immune memory between various tissues. Especially interferon-inducible antiviral genes such as *MX1*, *MX2*, *IFITM1* and *IFITM2*, were upregulated in response to Poly I:C challenge. Specifically, interferon-inducible transmembrane (IFITM) proteins protect against viral entry and early replication (285). This finding indicates that both ECFC and HUVEC contribute to vital protection against viral infection in the perinatal period. However, as explained above, a pro-inflammatory state is also associated with endothelial dysfunction and related CVD. This was also confirmed by the fact, that anti-inflammatory cytokines inhibit endothelial dysfunction (286). Therefore, a fine-tuned balance of avoiding inflammation versus defending pathogens is crucial for proper vascular function.

Besides common response patterns, expression of some genes differed between the two fetal EC types upon Poly I:C treatment. Genes solely induced in ECFC associate amongst others with 'antigen presentation'. This ability might be enabled via the expression of MHC class II molecules, as seen in EC of the microvasculature (287, 288). ECFC-specific genes were associated with enriched ELF promoter motifs (E74 Like ETS transcription factor; *ELF1*, *ELF4*), while equally-induced genes between ECFC and HUVEC were marked by IRF enrichment (interferon regulatory factors; *IRF1*, *IRF2*, *IRF3*). ELF1-mediated antiviral response separates from interferon signalling, supplying an additional layer of innate immune response (289), which seems to be absent in HUVEC. The top ranked ECFC-specific Poly I:C induced gene was *KLRD1* (killer cell lectin-like receptor D1, CD94). Of note, we observed a complete hypomethylation in one CpG site of the *KLRD1* promotor only in ECFC, resulting in an open chromatin state. In contrast, *KLRD1* was not expressed in HUVEC at any time, independent of Poly I:C challenge. KLRD1 is a natural killer cell-specific receptor binding MHC class I molecules. According to The Human Protein Atlas, a slight expression of KLRD1 in EC exists, but its function in fetal ECFC has not been studied yet. However, only the minority of differential immunological transcriptome between the two fetal EC types could be ascribed to DNA methylation differences at baseline. Therefore, other epigenetic regulatory mechanisms, such as histone modifications, might be the main trigger for the distinct behaviour upon microbial stimulation and need to be considered in future studies. While both processes are interconnected, histone modifications often have a short half-life

and are swiftly reversible, whereas DNA methylation is more stable and thus may affect gene expression in long-term (290). Furthermore, acute stimuli highly affect nucleosome remodelling (282). Particularly, H3K4me3 (H3 histones trimethylated at lysine 4) is enriched at promoters of genes encoding pro-inflammatory cytokines like TNF-α and IL-6 in macrophages that were trained with oxLDL (283).

Moreover, we demonstrated that HUVEC as well as fetal ECFC generate inflammatory memory on transcriptional level. After 24 h of Poly I:C treatment, cells were cultured in basal media for another 24 h, before being re-stimulated with LPS. ECFC and HUVEC showed some variation in the number of trained or tolerized genes upon re-challenge with LPS. Interestingly, attenuated genes in both cell types included cell adhesion genes (*SELE*, *ICAM1*, *VCAM1*) involved in leukocyte transendothelial migration. These genes get upregulated upon LPS stimulation in human pericytes, a muscle cell type interacting with EC in capillaries (291). Thus, the observed tolerization of leukocyte adhesion genes in EC might be necessary to avoid excessive leukocyte recruitment to the site of infection, which would result in hyperinflammation. A limitation of the study is that the small sample size prevents the statistical comparison of inflammatory memory in ECFC and HUVEC. However, the overall direction of either trained or tolerized response was the same for both fetal EC types, and expression levels of *TLR3* and *TLR4* did not differ. ECFC showed a higher response towards LPS re-exposure, pointing to their role in the microvasculature. Also human lung microvascular EC respond higher to LPS exposure than HUVEC (292), and only microvascular EC isolated from human hearts, but not their macrovascular counterpart, facilitate L-selectin mediated leukocyte adhesion (293).

We are aware of the fact, that *in vitro* culture of cells can affect their epigenetics and transcriptomics, and that the observed immune response may thus slightly differ from the *in vivo* condition. Besides, the used media contains growth factors, which can activate both EC types. However, a difference in response to the initial Poly I:C stimulation demonstrates that intrinsic reactions are preserved. Besides, to avoid any bias from upregulated cytokine presence in the supernatant still after removal of the stimulus, as observed by Koch et al. (208), we only included genes with an expression similar to the media control at 48 h for defining trained and attenuated genes, but not genes showing a persistent expression after the initial stimulation.

Future studies should also compare immune response of fetal EC from male vs female neonates, as sex-specific differences in inflammatory response exist (159). Women show lower incidence of viral or bacterial infection, but higher rates of autoimmune diseases, indicating their enhanced immune activity. These sex differences underlie genetic as well as epigenetic regulation (294). Also, female HUVEC show enhanced expression of genes involved in stress and immune response compared to their male counterpart (165).

Due to inexperienced pathogen exposure and thus not yet well-established adaptive immunity, the neonate relies on proper function of the innate immune system. Fetal ECFC show lower expression of pro-inflammatory genes than adult ECFC (295), suggesting trained immune memory in adults due to various pathogen exposures over lifetime. However, excessive immune response is associated to CVD. Severe infections during early childhood adversely influence the cardiovascular risk in adulthood (296), further suggesting that the neonate remembers its intrauterine infection and inflammation. The evidence that fetal endothelial immune response is primed after pathogen exposure indicates that memory capacity of fetal EC is involved in intrauterine programming mechanisms. The fact that a pro-inflammatory intrauterine environment as present during maternal pregnancy disorders can determine the long-term offspring's disease risk, highlights the susceptibility towards programming effects in fetal development (89). Environmental conditions can affect the epigenome and thus induce memory function. The phenomenon called 'glycemic/metabolic memory' or 'metabolic legacy' describes the effects of primary exposure to hyperglycemia that persist even after resetting to normoglycemic condition (297, 298). Transient hyperglycemia induces permanent epigenetic signatures in EC (299), including altered non-coding RNA expression (7) and persistent alterations in DNA methylation on gene loci involved in inflammation (300). These epigenetic modifications by a pro-inflammatory hyperglycemic milieu during pregnancy are linked to altered cell function (35). Metabolic memory does not only occur in fully differentiated EC, but especially endothelial progenitors were also identified as 'carriers' thereof (301). For instance, disturbances of the intrauterine environment induce epigenetic changes in genes associated with cardiovascular function (302). Similar to our findings that ECFC and HUVEC show distinct immune response, fetal EC from macrovascular vs microvascular origin respond differently to maternal metabolic derangements during pregnancy (303). Besides, it has been demonstrated that TLR4, the receptor for bacterial LPS, also regulates insulin sensitivity and is thus enhanced in placentas of GDM pregnancy. TLR4

expression correlates positively with maternal fasting glucose and also with glucose levels after glucose intake (304). These findings further confirm an important connection between immune response upon pathogen exposure and inflammatory signalling in response to an altered intrauterine environment as present in metabolically dysregulated pregnancies.

Conclusion

We showed that i*n vitro* cultured fetal EC remember their *in vivo* environment during pregnancy as well as short-term inflammatory stimulation in culture. Although the capacity-load model of placental adaption suggests that the placenta protects fetal environment in case of metabolic disturbances up to a certain threshold (233), we observed that the feto-placental endothelium and endothelial progenitors already respond to subtle changes in maternal metabolism. We identified that moderate changes in maternal metabolism, i.e. maternal overweight, influence fpAEC by altering the expression of the vascular tone modulating peptidase MME. The fact that inflammation indicating CRP levels in the maternal circulation correlate more with BMI than with glucose or insulin levels (64), emphasizes the importance of considering maternal weight as relevant metabolic parameter. In addition, fetal development was identified to be affected the most during the periconceptional stage (96, 305), why maternal pre-pregnancy metabolism may significantly influence the offspring. Maternal hyperglycemia and overweight are highly connected to each other and intensify the risk for adverse pregnancy outcomes when present in combination (306). However, they both affect the placenta differently (307), and should thus be monitored both. Not only hyperglycemia affects endothelial function, but so does maternal glycemic state in a healthy, non-diabetic range, as we observed in fetal ECFC outgrowth. These findings highlight the importance of strict screenings even for 'healthy' pregnant women, as the placenta and fetus can already be sensitive to subtle changes in maternal metabolism. Thus, not only severe maternal metabolic disturbances but also small dysregulations should be considered as influencing factors for fetal health. Indisputably, genetics affect future generations, however, siblings before and after maternal diabetes emergence were compared and clearly showed a relation of intrauterine diabetic exposure with childhood obesity and type 2 diabetes (308). This memory effect may underlie transcriptional remodelling, as we observed when exposing HUVEC and ECFC to inflammatory stimulation. Although we do not have any follow-up data

on mother or fetus, which would help in understanding the long-term effects of altered intrauterine environment, we confirmed that the fetal endothelium 'remembers' inflammation. Together our findings support the DOHaD paradigm: environmental influences in pre- and perinatal development define the susceptibility for diseases in later life via an interplay between genetics and epigenetics (309). Informing mothers-to-be and even women before conception about the impact of their metabolism on the future generation is needed. While screening and diagnosis of pregnancy pathologies, e.g. GDM, is inconsistent, early treatment can improve the outcome (310). Exhibiting diet and physical activity, as well as medication intake can enhance insulin sensitivity in high risk subjects (128). Beneficial effects of treating maternal diabetes during pregnancy also apply to the fetus, e.g. insulin therapy enhances fetal EPC levels (311). Nevertheless, such adverse effects of disturbed maternal metabolism on the fetus are not fully reversible, as explained above in the long-term effect of prior disturbed glycemic conditions. Moreover, treatment of obesity should not only be commenced at the beginning of pregnancy, but already before conception to improve maternal and fetal outcome (312).

Furthermore, we confirmed that sex-specific differences already exist *in utero*, i.e. faster initial colony outgrowth of male ECFC. Different outgrowth dynamics between male and female cells were further determined by maternal fasting plasma glucose level. Hence, our data reveal that intrinsic sex-specific differences in basal fetal EC function exist, as well as differences in programming by intrauterine environment. Together they might contribute to varying disease susceptibility in adulthood. These results indicate further that sex should be considered in all biomedical research fields. It is important to avoid the bias of overlooked differential gene expression when showing data of one sex only (313).

In addition, our data highlight the importance of distinguishing between various fetal EC types and of assessing the results separately based on the cell source - even within the same organ. Important to remember is the heterogeneity of EC depending on developmental stage, vessel type and tissue location (314). This results in structural and functional differences, which need to be considered when developing therapeutic strategies to target specific sites of the vasculature (36). In our study, besides different immune response of ECFC and HUVEC, also maternal metabolism had different effects dependent on fetal EC type: While maternal

glycemia influenced ECFC outgrowth, overweight determined the expression of vascular tone regulating peptidase MME in fpAEC.

A general limitation in our investigations of maternal metabolic effects on fetal EC is missing detailed information on maternal and fetal metabolic and inflammatory markers that should be determined and considered in future studies. Indisputably, metabolic changes are tightly linked to inflammation as metabolic derangements such as overweight, obesity and diabetes are associated with an altered pro-inflammatory environment. The herein discussed publications highlight the importance of a healthy maternal metabolism in regards of the offspring's future health. As various metabolic and inflammatory signals may be underlying and prime the offspring, detailed information on maternal metabolic and inflammatory state should be collected. However, maternal inflammation may not directly resemble fetal inflammation (103), why measuring cytokines in cord blood is an important future step. Further investigations should also include analyses on DNA methylation, histone modifications and non-coding RNA regulation in relation to the intrauterine environment, in order to get further insights into epigenetic programming *in utero*. Besides, additional functional assays need to be performed to better understand the consequence of initial progenitor outgrowth dynamics and differential gene expression upon inflammation.

Future perspectives include follow-up studies investigating endothelial function and vascular health of the offspring in later life. Only in this way, the long-term effects of altered endothelial function on cardiovascular health can be understood. If it turns out that the effects of maternal metabolic changes and of fetal sex on fetal endothelial cells indeed lead to altered endothelial function in long-term, lifestyle interventions in the sense of sustainable health management could be recommended.

Finally, fpEC, HUVEC and cord blood derived ECFC are an easily accessible and available source to study endothelial function in general, but especially to investigate the programming effects of intrauterine environment, affecting each and every one of us. We must not forget that already the short-term exposure to low-grade inflammation during pregnancy can program our endothelial function for a lifetime, why early prevention of gestational disorders is necessary.

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BASIC SCIENCE ARTICLE OPEN

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Fetal sex and maternal fasting glucose affect neonatal cord blood-derived endothelial progenitor cells

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BACKGROUND: Maternal cardiovascular risk factors (CVRF) in pregnancy, i.e., obesity and hyperglycemia, transmit to the fetus and affect placental and fetal endothelial function. Moreover, a sex dimorphism in endothelial function and susceptibility towards CVRF exists already *in utero*. Endothelial colony-forming cells (ECFC) are circulating endothelial progenitors highly present in neonatal cord blood and sensitive to CVRF. This study investigated whether fetal sex or subtle maternal metabolic changes within healthy range alter fetal ECFC outgrowth.
METHODS: Outgrowth of ECFC from cord blood of male ($n = 31$) and female ($n = 26$) neonates was analyzed after healthy pregnancies and related to fetal sex and maternal metabolic parameters.
RESULTS: Male ECFC grew out earlier (−20.57% days; $p = 0.031$) than female. Although all women were non-diabetic, higher levels of fasting plasma glucose (FPG) at midpregnancy increased the time required for colony outgrowth (OR: 1.019; $p = 0.030$), which, after stratifying for fetal sex, was significant only in the males. Gestational weight gain and BMI did not affect outgrowth. Colony number was unchanged by all parameters.
CONCLUSIONS: Fetal sex and maternal FPG within normal range alter ECFC function *in utero*. A role of ECFC in postnatal angiogenesis and vasculogenesis has been suggested, which may be affected by altered outgrowth dynamics.

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IMPACT:

- This study is the first to report that a sexual dimorphism exists in ECFC function, as cells of female progeny require a longer period of time until colony outgrowth than ECFC of male progeny.
- Our data show that ECFC function is highly sensitive and affected by maternal glucose levels even in a normal, non-diabetic range.
- Our data raise the question of whether maternal plasma glucose in pregnancy should be considered to play a critical role even in the non-diabetic setting.

INTRODUCTION

Endothelial cells are multifunctional cells and regulate vascular tone, angiogenesis, formation of a barrier, blood clotting, and modulate the inflammatory response.[1] Disturbance of these functions is regarded as endothelial dysfunction (ED), which is closely associated with cardiovascular health and ED is a precursor to cardiovascular disease (CVD).[2]

Increased blood glucose, as well as overweight and adiposity, are well-established risk factors for ED and CVD.[3–7] Hyperglycemia increases the production of reactive oxygen species and induces the formation of advanced glycation endproducts which affect endothelial function.[4,8] Adipose tissue causes a pro-inflammatory environment through the production of cytokines, which contributes to ED.[3] Notably, not only do pathological metabolic derangements lead to ED, even subtle changes within normal, healthy range alter the function of endothelial cells in adults. For instance, in non-diabetic, normoglycemic subjects, higher fasting plasma glucose (FPG) associates with altered endothelial function and markers for ED,[9,10] and modest weight gain around 4 kg impairs endothelial function in women.[11]

During pregnancy, maternal cardiovascular risk factors (CVRF) transmit to the fetal compartment: Maternal obesity in pregnancy is associated with a pro-inflammatory fetal environment[12,13] and maternal hyperglycemia directly transfers to the fetus,[14] and both may affect the fetal and placental endothelium. In fact, gestational diabetes (GDM) alters microvascular architecture in the placenta[15] and in the neonatal iris,[16] and maternal GDM and obesity alter gene expression and function of endothelial cells from the placenta and umbilical cord vein.[17–21] A recent study has revealed that not only the metabolic derangement of GDM, but also moderate metabolic alterations of maternal overweight affect placental endothelial cells and alter protease expression, suggesting that the placental and

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Table 1. Maternal and infant main characteristics of ECFC donors.

Characteristics	Entire cohort	Male	Female
Number of subjects	57	31	26
Maternal age (year)	31.1 ± 4.9	31.6 ± 5.4	30.5 ± 4.3
Pre-pregnancy BMI (kg/m^2)	24.7 ± 4.3	24.4 ± 4.5	24.9 ± 4.0
BMI at delivery (kg/m^2)	29.5 ± 4.3	29.7 ± 4.4	29.2 ± 4.1
Gestational weight gain (kg)	13.8 ± 6.0	14.5 ± 6.9	12.8 ± 4.3
oGTT 0 h (mg/dL)	80.3 ± 6.5	80.1 ± 6.7	80.5 ± 6.4
oGTT 1 h (mg/dL)	118.4 ± 28.3	110.9 ± 29.6	127.3 ± 24.4*
oGTT 2 h (mg/dL)	97.1 ± 22.8	93.1 ± 22.9	102.0 ± 22.0
Gestational age at delivery (week)	39.5 ± 1.1	39.4 ± 1.0	39.7 ± 1.2
Mode of delivery (vaginal/C-section)	22/35	10/21	12/14
Neonatal weight (g)	3513.8 ± 401.5	3535.7 ± 394.8	3487.7 ± 415.6
Neonatal height (cm)	51.2 ± 2.2	51.5 ± 2.1	50.8 ± 2.3
Placental weight (g)	649.5 ± 130.2	662.7 ± 127.7	634.2 ± 133.9

Data are presented as mean ± SD. Statistical differences are calculated by unpaired Student's *t*-test comparing male vs female.
oGTT oral glucose tolerance test, *C-section* cesarean section.
*$p < 0.05$.

fetal endothelium may be responsive to subtle maternal metabolic changes.[13]

Endothelial function and susceptibility towards CVRF differ between the sexes.[22] This sexual dimorphism contributes to a higher incidence of CVD reported in males[23] and a more frequent occurrence of microvascular dysfunction in females.[24] In adults, estrogen is regarded as the main cause underlying this difference. However, an increasing body of evidence demonstrates that a sexual dimorphism in endothelial function exists already *in utero*. In fact, in previous studies, we have revealed a sex dimorphism in miRNA expression and actin organization of feto-placental endothelial cells.[25] Moreover, HUVEC isolated from male vs female donors reveal a sex dimorphism in endothelial nitric oxide synthase (eNOS) protein levels.[26] In early development, genetic and epigenetic reasons are likely the main drivers of sexual dimorphism. These may involve transcripts located on sex chromosomes that encode genes associated with cardiovascular function[27] or which may regulate the expression of autosomal genes participating in endothelial function.[28] Notably, the fact that changes in miRNA profiles of placental endothelial cells after exposure to GDM differ in male vs female fetuses indicates that the response of endothelial cells to maternal CVRF differs depending on fetal sex.[29]

Endothelial colony-forming cells (ECFC) are circulating endothelial progenitor cells which are recruited for endothelial repair, vascular growth, and angiogenesis[30] and give rise to cells of mature endothelial phenotype.[31] Fetal and neonatal period is characterized by a particularly high number of circulating ECFC, probably due to massive angiogenesis and vascular remodeling.[32] Colony outgrowth is a critical parameter reflecting ECFC function[33,34] and requires attachment of progenitor cells to the surface, i.e., the vessel wall in vivo or the cell culture dish in vitro, differentiation into mature endothelial cells, and proliferation to form colonies. ECFC number and function are sensitive to CVRF[35–37] and also maternal diabetes in pregnancy affects cord blood-derived ECFC.[38,39] Whether ECFC are sensitive to metabolic characteristics within the normal, non-diabetic range is still elusive.

In this study, we addressed the question whether, within healthy, non-diabetic pregnancy, fetal sex, and maternal metabolic hallmarks, i.e., FPG and post-load glycemia at midpregnancy, gestational weight gain, and pre-pregnancy BMI, affect neonatal ECFC function. Therefore, we isolated ECFC from umbilical cord blood of male and female neonates after a healthy pregnancy and related ECFC outgrowth, i.e., the days required for colony outgrowth, the number of colonies, and the days required for confluency, to these maternal metabolic characteristics.

METHODS

Study cohort

The present study was conducted at the Department of Obstetrics and Gynaecology at the Medical University of Graz, Austria, in accordance with the Declaration of Helsinki. Isolation of ECFC from umbilical cord blood was approved by the local ethics committee (29-319 ex 16/17) and written informed consent was obtained from all participants. A total of 57 participants were enrolled after delivery of singleton pregnancy (37–42 weeks of gestation) and cord blood samples of male ($n = 31$) and female ($n = 26$) neonates were obtained. Maternal and infant characteristics are shown in Table 1. Exclusion criteria included GDM (diagnosed by a 75 g oral glucose tolerance test (oGTT) at 24–28 weeks of gestation[40] and defined by at least one glucose value being above the cut-off levels (92, 180, and 153 mg/dL at 0, 60, and 120 min after glucose intake, respectively)), smoking (self-reported), medical disorders or pregnancy complications (except thyroid dysfunction), use of any medication (except thyroid hormones), or adverse medical history. Two included subjects were diagnosed with polyhydramnios and in one case pregnancy was initiated by in vitro fertilization.

Isolation and culture of ECFC

ECFC were isolated as described previously.[41] In brief, 8–16 mL of venous cord blood was collected in lithium heparin-coated tubes (Greiner Bio-One, Kremsmünster, Austria) immediately after delivery of the placenta. Mononuclear cells (MNC) were separated via density gradient centrifugation using Lymphoprep™ Density Gradient Medium (Axis Shild, Alere Technologies AS, Oslo, Norway). The emerged buffy coat was washed and resuspended in culture medium (Endothelial Cell Growth Medium MV Kit (PromoCell, Heidelberg, Germany) supplemented with 0.1% Gentamycin (ThermoFisher Scientific, Waltham, MA)). 2×10^7 cells were seeded per well of six-well culture plates (ThermoFisher Scientific) pre-coated with rat tail collagen type 1 (Corning, Corning, NY) and cultured at 37 °C, 21% O_2, 5% CO_2 in a humidified incubator. After overnight incubation, the culture medium was changed and then twice a week.

ECFC outgrowth

Colony outgrowth of ECFC was monitored daily using an inverted microscope (Olympus CKX53, Tokyo, Japan). Following the seeding of MNC, a major part of ECFC isolations formed colonies after 4–6 days. Cells revealed the typical cobblestone morphology of endothelial cells. Figure 1 illustrates the outgrowth of a representative ECFC colony followed over 4 days. The time until the first colonies formed, the number of colonies as well as the time until cells reached confluency for passaging, was recorded.

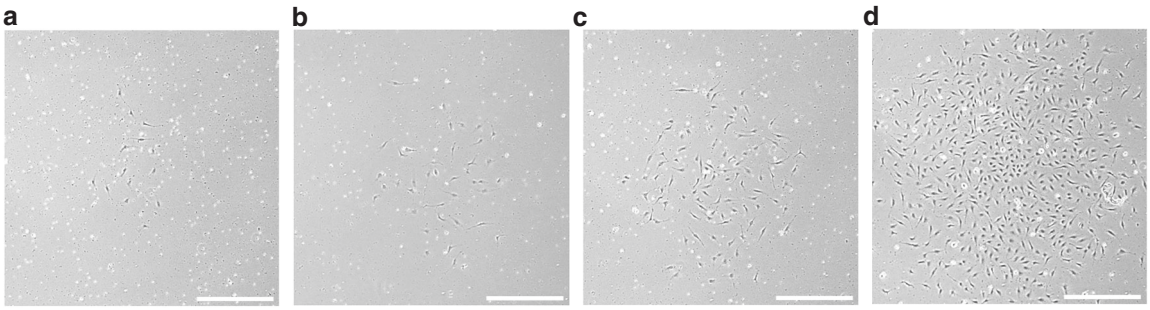

Fig. 1 Colony outgrowth of cord blood ECFC. Microscopic photography of a representative colony using an inverted microscope from day 4 (**a**), day 5 (**b**), day 6 (**c**) until day 7 (**d**) after plating of MNC. Scale bar = 500 µm.

For passaging, the culture medium was aspirated, cells washed with 1 × Hank's Balanced Salt Solution (HBSS, ThermoFisher Scientific), and detached using TrypLE™ (recombinant cell-dissociation enzyme, ThermoFisher Scientific). Cells were resuspended in culture medium and transferred to gelatine pre-coated (1% porcine skin gelatine (Sigma-Aldrich, St. Louis, MO) + 1% Gentamycin (ThermoFisher Scientific)) culture flasks (ThermoFisher Scientific). Colony number was related to the amount of cord blood used for isolation. Supplementary Fig. 1 represents the schematic study design.

Characterization of ECFC via flow cytometric analysis

Phenotype of ECFC was evaluated by flow cytometric analysis (FACS) as described[41] in 6 representative ECFC isolations. In brief, ECFC were detached, washed and stained with pre-titrated volumes of fluorochrome-conjugated antibodies (Supplementary Table 1) for 20 min at 4 °C. Measurements were performed on a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA) using the CytExpert software (Beckman Coulter). For controls, unspecific antibodies of the same isotype and concentration were used. Data were analyzed using the Kaluza Analysis software (Beckman Coulter).

Characterization of ECFC via immunocytochemistry

For quality and purity control, each ECFC isolation was subjected to immunocytochemical characterization using antibodies against endothelial cell markers (CD31, von Willebrand factor (VWF)), fibroblast markers (CD90, TE-7) and muscle cell markers (smooth muscle actin (SMA), Desmin) as described previously.[41] In brief, cells were seeded on glass chamber slides (ThermoFisher Scientific) and fixed with acetone (Merck). TBE pH 8.0 (Gatt-Koller, Absam, Austria) with 0.1% Tween (Sigma-Aldrich) was used as rehydration and washing buffer. Primary antibodies (Supplementary Table 2) were applied for 30 min and visualization was performed with the Ultra Vision horseradish peroxidase Polymer Kit (ThermoFisher Scientific). Images were generated using a light microscope (Olympus BX53) with the UC90 camera (Olympus) and the corresponding cellSens Standard software (Olympus).

Network formation assay on Matrigel

2D network formation ability of ECFC was analyzed in a subcohort of 23 ECFC donors (12 male, 11 female). ECFC were resuspended in culture medium supplemented with 5% FCS but without additional growth supplements, and seeded in a density of 10^4 cells per well on 96-well culture plates (Costar, Corning), pre-coated with growth factor reduced Matrigel (Corning). Plates were incubated at 21% O_2 at 37 °C and monitored for 24 h using an inverted phase-contrast microscope (Cell Observer; Zeiss, Oberkochen, Germany) with a digital camera and the AxioVision software V 4.8. Images at 3, 6, 12, and 24 h were quantified using the AngioJ-Matrigel assay plugin in ImageJ, V 1.43l (https://imagej.nih.gov/ij/index.html, RRID:SCR_003070) as described previously.[42] The assay was performed in triplicates per individual donor.

Statistics

Statistical analyses were performed using the IBM SPSS Statistics software, V25 (https://www.ibm.com/products/spss-statistics, RRID:SCR_019096). Maternal and infant characteristics, as well as FACS results, are presented as mean ± standard deviation (SD). Statistical differences in subjects' characteristics were calculated by unpaired Student's *t*-test. Data of Matrigel assay are presented as mean ± SD and were analyzed by unpaired Student's *t*-test and Pearson correlation, respectively. Visual assessment with *QQ*-plots revealed that data describing ECFC outgrowth were not normally distributed. Potential confounding factors were identified via Mann–Whitney *U* test. Data are presented as median ± interquartile range (IQR). For other analyses, skewed data of the outcome/dependent variable were log-transformed and re-transformed by exponentiation for the presentation of results. Differences between groups were tested by ANCOVA (analysis of covariance), and correlation with specific parameters by linear regression analysis, in both adjusting for delivery mode. Results of ANCOVA are presented as estimated marginal means (EMM) and 95% confidence intervals (CI). Linear regression analysis revealed the unstandardized regression coefficient *B*, which requires interpretation as odds ratio (OR), i.e., percentage of increase/decrease of the dependent variable per unit increase of the independent variable, due to re-transformation of log-transformed data. Results are given by OR and 95% CI. A p-value < 0.05 assumed statistical significance. Graphs were generated with the GraphPad Prism software, V9 (http://www.graphpad.com, RRID:SCR_002798) using untransformed and unadjusted data, unless otherwise specified.

RESULTS

ECFC phenotype

ECFC phenotype was assessed by FACS analysis and immunocytochemistry. FACS analysis was performed with a subset of 6 ECFC isolations. Analysis revealed that cells were positive for the endothelial cell markers CD31 (PECAM-1), CD144 (VE-Cadherin), CD146 (MCAM), and Tie-2, and did not express markers for myeloid cells (CD14), leukocytes (CD45), fibroblasts (CD90) and stem cells (CD133). The majority of cells (90.42 ± 1.44%) were positive for the endothelial cell marker CD309 (KDR). A small population (8.38 ± 7.42%) was positive for the endothelial and hematopoietic progenitor marker CD34 (Fig. 2). Immunohistochemistry confirmed the expression of endothelial cell markers (CD31 and VWF), and the absence of fibroblast markers (CD90 and TE-7) and muscle cell markers (SMA and Desmin) in all ECFC isolations ($n = 57$) (Supplementary Fig. 2).

Identification of confounding factors

To identify confounding factors, the influence of maternal and neonatal characteristics was analyzed. Delivery mode strongly affected the number of outgrown ECFC colonies (vaginal delivery: 5.35 ± 14.52, cesarean section (C-section): 1.31 ± 5.93, $p = 0.001$, Supplementary Fig. 3A). Although the effect of delivery mode on the number of days until initial colony outgrowth (vaginal delivery: 5.00 ± 3.00, C-section: 5.00 ± 3.00, $p = 0.136$, Supplementary Fig. 3B) did not reach significance, the days until reaching confluency was affected (vaginal delivery: 10.00 ± 2.00, C-section: 12.00 ± 3.00, $p = 0.020$, Supplementary Fig. 3C). Thus, for all further analyses, data were corrected for delivery mode.

Effect of fetal sex and maternal metabolism on ECFC outgrowth and network formation

First, we investigated the effect of fetal sex on ECFC outgrowth comparing isolations from 31 male vs 26 female neonates. Indeed,

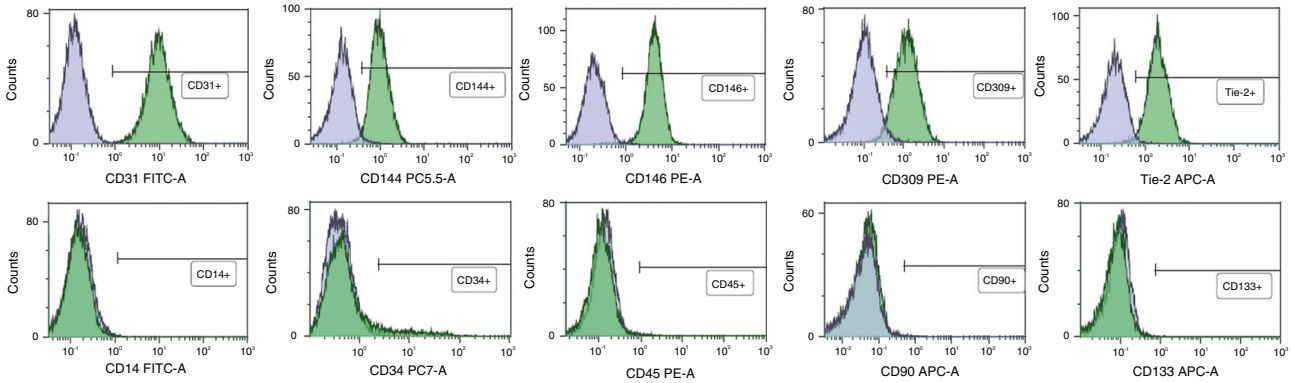

Fig. 2 Phenotype of cord blood ECFC as determined by flow cytometry. Cells were analyzed for surface expression of the endothelial cell markers CD31 (PECAM-1), CD144 (VE-cadherin), CD146 (MCAM), CD309 (KDR), and Tie-2, and for markers for myeloid cells (CD14), hematopoietic stem cells (CD34), leukocytes (CD45), fibroblasts (CD90) and stem cells (CD133). Depicted plots represent typical expression patterns and show specific antibody staining (green peak) vs corresponding isotype control (blue peak).

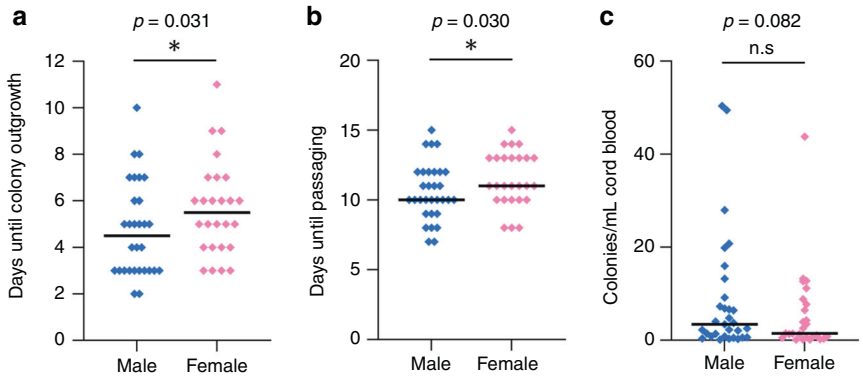

Fig. 3 Effect of neonatal sex on ECFC colony outgrowth. a Days required for colony outgrowth in ECFC of male and female neonates. **b** Days required for reaching confluency in ECFC of male and female neonates. **c** ECFC colony numbers of male and female neonates. Data were analyzed via ANCOVA adjusted for delivery mode after log-transformation of the dependent variable. Graphs represent untransformed and unadjusted data with median. *n* (male) = 31, *n* (female) = 26.

ECFC of female neonates required a longer period of time until colony outgrowth (male: 4.29 d (3.72; 4.93), female: 5.40 d (4.63; 6.28), $p = 0.031$, Fig. 3a) and a longer time period until reaching confluency (male: 10.30 d (9.66; 10.99), female: 11.46 d (10.69; 12.27), $p = 0.030$, Fig. 3b) than ECFC of male neonates. Similarly, the number of colonies per mL cord blood used for isolation was by trend greater in male ECFC (male: 3.30 colonies/mL (1.96; 5.56) vs. female: 1.67 colonies/mL (0.94; 2.94), $p = 0.082$, Fig. 3c).

In order to investigate whether not only outgrowth, but also the ability of ECFCs to form networks differs between the sexes, we performed a 2D Matrigel assay using a subset of 23 ECFC isolations (12 from male, 11 from female neonates). However, within this subset, there was no difference at any of the time points examined, i.e., 3, 6, 12, and 24 h, regarding the number of branching points and tube length (Supplementary Fig. 4 and Supplementary Table 3).

In the second analysis of outgrowth, we determined the effect of maternal metabolic parameters, i.e., FPG and glycemia after glucose challenge as well as gestational weight gain and pre-pregnancy BMI, on ECFC outgrowth. Analysis of the relation between glucose levels determined by an oGTT at midpregnancy (gestational week 24–28) and outgrowth of ECFC isolated after birth revealed a positive correlation between the number of days required for colony outgrowth with maternal FPG (OR: 1.019 (1.002; 1.035), $p = 0.030$, Fig. 4a). For instance, a 10 mg/dL increase in FPG prolongs colony outgrowth by 20%. Maternal FPG was neither associated with ECFC colony number (OR: 0.971 (0.912; 1.033), $p = 0.353$) nor with days until passaging (OR: 1.005 (0.998; 1.014), $p = 0.189$). There was no effect of plasma glucose levels after the glucose challenge on ECFC outgrowth (not shown). Moreover, neither gestational weight gain (range 0–26 kg) nor maternal pre-pregnancy BMI (range 19.0–35.6 kg/m^2) had an effect on the days required for ECFC outgrowth (OR: 0.998 (0.977; 1.016), $p = 0.734$, Fig. 4b and OR: 0.995 (0.971; 1.023), $p = 0.765$, Fig. 4c, respectively).

Since we found an effect of FPG on ECFC outgrowth, we further analyzed whether network formation on Matrigel was also affected by maternal glycemia. However, in the subset of 23 ECFC isolations, there was no correlation of network formation with FPG (Supplementary Table 3).

As we identified a sexual dimorphism in colony outgrowth in our first analysis, we further investigated whether the impact of FPG or any other of the investigated parameters (post-load glycemia, pre-pregnancy BMI, gestational weight gain) also depended on fetal sex. After stratifying for fetal sex, the correlation between FPG and ECFC outgrowth, i.e., days until colony outgrowth, remained significant only in ECFC of male (OR: 1.026 (1.002; 1.050), $p = 0.029$, Fig. 5a), but not in ECFC of female neonates (OR: 1.012 (0.984; 1.038), $p = 0.405$, Fig. 5b). This difference is illustrated by plotting the respective confidence intervals in Fig. 5c. The other parameters showed no dependence on neonatal sex (Supplementary Table 4).

DISCUSSION

We here investigated the effect of fetal sex and influence of maternal metabolic parameters within healthy pregnancy on the

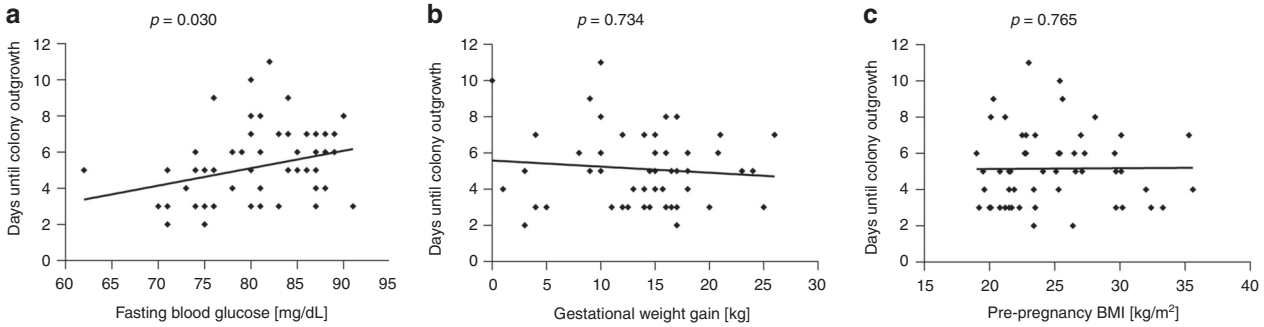

Fig. 4 Effect of maternal metabolic parameters on ECFC colony outgrowth. a Positive correlation of days required for ECFC outgrowth with maternal fasting plasma glucose. **b** Absent correlation of ECFC outgrowth with maternal gestational weight gain and **c** pre-pregnancy BMI. Data were analyzed via linear regression analysis adjusted for delivery mode after log-transformation of the dependent variable. Fasting plasma glucose was determined during oGTT at gestational weeks 24–28. Graphs represent untransformed and unadjusted data. $n = 57$.

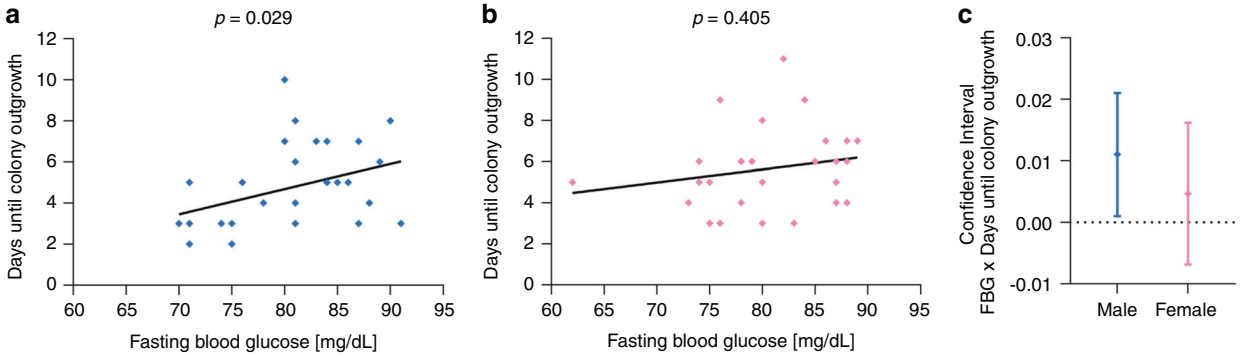

Fig. 5 Effect of maternal FPG on ECFC colony outgrowth depends on fetal sex. a Positive correlation between days required for ECFC outgrowth and maternal FPG in male subjects. **b** Absent correlation between ECFC outgrowth and maternal FPG in female subjects. **c** Confidence intervals from the linear regression analysis between maternal FPG level during pregnancy and required days until neonatal ECFC colony outgrowth revealed that the correlation is only significant in the male group. Fasting plasma glucose was determined during oGTT at gestational weeks 24–28. Data were analyzed via linear regression analysis adjusted for delivery mode after log-transformation of the dependent variable. Graphs in **a** and **b** represent untransformed and unadjusted data. Graph in **c** represents log-transformed and adjusted data. n (male) = 31, n (female) = 26.

outgrowth of neonatal ECFC. Our key findings were that ECFC of female progeny required a longer period of time until colony outgrowth than ECFC of male progeny. Moreover, we identified that, even within the healthy, non-diabetic range, higher maternal FPG present at midpregnancy prolongs the number of days required for colony outgrowth in ECFC of male neonates.

Due to the fact that ECFC represent a tiny cell population within the mass of circulating MNC, clear quantification by FACS analysis requires a previous enrichment step, which could reduce the accuracy. Also, the use of polychromatic flow cytometry to quantify ECFC was reported[43] although data of ECFC quantification were not shown. Therefore, the number of colonies formed in vitro is widely used as a measure for ECFC frequency. As an approximation for EPC number, Fadini et al. counted circulating $CD34^+/KDR^+$ cells in male and female individuals of different ages, including umbilical cord blood ECFC.[44] The $CD34^+/KDR^+$ phenotype is not exclusive for EPC and may include also cells that are not of endothelial progeny and thus, do not form endothelial colonies,[45] but results revealed that female neonates, as well as pre-menopausal women, possess higher levels of $CD34^+/KDR^+$ cells.[44]

Several studies investigated sex differences in ECFC colony formation in adults. Comparing colony outgrowth of adult ECFC in postmenopausal women and men of similar age, Hoetzer et al. reported 150% higher colony-forming capacity in women[46] and Fadini et al. observed a higher number of ECFC colonies in female pre-menopausal adults compared to age-matched males.[44] By contrast, Shaw et al. observed a reduced number of ECFC colonies in adult females.[47] In parallel, also Xiao et al. obtained a lower number of EPC in females, which was determined by counting the single endothelial-like cells attached after 5 days in culture, but no sex-difference in the number of colonies formed.[48] Interestingly, Randolph et al. identified lower differentiation capacity of female human pluripotent stem cells (hPSC) into endothelial progenitors[49] pointing at a possible role of differentiation capacity underlying the slower outgrowth of female cells. We did not find studies investigating ECFC outgrowth during the perinatal period or in children.

We also investigated the effect of neonatal sex on in vitro network formation of ECFC. In a subcohort of 23 cases, we observed no difference between ECFC from female vs male neonates. Comparing isolated ECFC from 25 women and 25 men around the age of 60, Hoetzer et al. employed a Boyden chamber to investigate migratory activity and observed a 40% increased migration in the female group.[46] However, we did not find any reports on the effect of sex on network formation of ECFC or of other endothelial cells.

Estrogen is a major protector against ED in adult, pre-menopausal females[50,51], and a central role of sex hormones in the regulation of EPC is suggested.[44] The fetal gonads start to produce steroid hormones already in early pregnancy,[52] but a large cohort study observed no difference in intrauterine estrogen levels between boys and girls.[53] Nonetheless, also other factors may underlie the sex differences in ECFC function. Intrinsic sex differences are based on genetic mechanisms that are directly or indirectly associated with sex chromosomal gene expression, or on epigenetic mechanisms. Such intrinsic sex differences become increasingly investigated and acknowledged.[54] In addition,

systemic sex dimorphism in growth factors, hormones, and cytokines may prime EPC and contribute to sex differences in ECFC outgrowth as a sexual dimorphism exists for several bioactive molecules present in the fetal circulation: For instance, cord blood levels of insulin-like growth factor 1 (IGF-1) and of leptin are lower in male than in female neonates.[55,56]

Besides the effect of fetal sex, we investigated the impact of maternal metabolism on neonatal ECFC. Hyperglycemia is a major cause of ED in diabetes[57] and the effect of GDM on neonatal ECFC has been investigated in several settings: Studies observed fewer[38] or unchanged[39,43] ECFC colonies after GDM pregnancies with no difference in days until colony appearance,[38] and revealed both, reduced[38] and increased proliferation[39] of ECFC after GDM.

In the subcohort used for Matrigel assays, we did not observe an effect of maternal glycemia on ECFC network formation. Ingram et al. has shown a strong negative effect of maternal diabetes on network formation of cord blood ECFC, although the sample size investigated was quite small with 5 controls and 4 diabetic subjects.[58] The fact that we observed no effect of maternal glucose levels on ECFC network formation may thus be to the fact that all ECFC donors are non-diabetic and potential subtle changes are too small to be identified within a cohort of this size.

When we analyzed samples of our healthy, non-diabetic cohort according to maternal FPG at midpregnancy, we discovered that increasing glucose levels prolonged the period of time required for outgrowth. FPG represents the value to which the body can lower blood glucose before meals, and pathologically elevated FPG in pregnancy represents a much stronger predictor for adverse pregnancy outcomes than elevated post-load glucose.[59] Our data indicate that even within the normal range, higher levels of FPG affect the fetus, which parallels a study from Liu et al., who demonstrated an association between maternal FPG at gestational weeks 10–24 with birth weight.[60] Authors concluded that maternal fasting glycemic state affects fetal development. Of note, this correlation was more pronounced in male than in female neonates. Also in our study, the effect of maternal FPG on fetal cells was sex-dependent, since the correlation with colony outgrowth was only significant in ECFC from male neonates. Besides, Liu et al. demonstrated, that neonatal birth weight was only associated with fasting glucose level measured during oGTT, but not with glycemia after the glucose challenge, highlighting the role of maternal basal blood glucose on fetal development.[60] Again, this result is in accordance with ours, as we also did not detect a correlation between ECFC outgrowth and glycemia after the glucose challenge. In our study, we did not identify an association between FPG and birth weight, which may be due to a much smaller sample size of our study. The study by Liu et al. investigated a cohort of 2284 cases, whereas our cohort included 57 subjects, making such correlations more difficult to detect.

Our data highlight the fact that endothelial function is subject to sex dimorphism. Whilst in adults and due to differences in sex hormones, females possess a better endothelial function and are at lower risk to develop cardiovascular disease, neonatal ECFC reveal a better outgrowth in males. This highlights the fact that intrinsic, genetic, and epigenetic sex differences differ from acquired sex differences as a result of hormones and lifestyle. Our data, however, further revealed that male neonatal ECFC are more sensitive to metabolic influences as outgrowth correlated with maternal FPG within the normal range. A higher susceptibility towards environmental risk factors, even when subtle, may contribute to a higher risk for CVD in male adults. ECFC outgrowth is sensitive towards CVRF in adults and neonates.[35–39,61] Whether slower ECFC outgrowth is assigned of adverse effects of CVRF on any exposed endothelial cell type, or whether ECFC dysfunction in fact contributes to CVD is difficult to distinguish observationally. However, the fact that treatment of acute myocardial infarction with blood derived progenitor cells by intracoronary infusion increased myocardial viability in the infarct zone[62] supports the hypothesis that disturbed ECFC function is not only indication of ED, but contributes to CVD by an impaired capacity to repair and maintain the endothelial layer.

We did not identify an effect of maternal weight gain or BMI, suggesting that maternal adipose tissue-derived paracrine factors do not affect neonatal ECFC outgrowth. Analyzing outgrowth of neonatal cord blood ECFC from 27 donors with maternal pre-pregnancy BMI ranging from 18 to 30 kg/m^2, Moreno-Luna et al. observed a positive correlation between maternal BMI and the number of ECFC colonies, without differences in cell phenotype and function.[63] The absence of such correlation in our study despite of a similar BMI range, may underlie differences in the isolation protocol: Freezing of MNC prior to ECFC isolation as performed by Moreno-Luna et al. may represent an additional selection step that promotes the growth of cells with a higher survival capacity. Thus, as a hypothesis, ECFC from donors with high BMI may be more robust and have a higher survival rate. In adults, however, BMI is negatively correlated with the number of circulating EPC,[35,64] which also show reduced proliferation following initial outgrowth.[64]

We identified delivery mode as an important confounder. In fact, soluble endothelial markers are more frequent in cord blood after spontaneous vaginal delivery compared to elective C-section.[65] Also Baker et al. reported reduced cord blood ECFC numbers after birth by C-section vs vaginal birth, with no difference between planned and emergent C-section in a study cohort of 62 preterm babies with and without pregnancy pathologies and further medical complications.[66] The authors explained this difference by perinatal stress induced by vaginal birth leading to an increased release of angiogenic progenitor cells. The fact that the same finding is present in our full-term cohort highlights the physiological role of ECFC release at delivery. We did not detect a correlation between gestational age and ECFC number (not shown), which was identified in preterm infants[67] and in a cohort with a gestational age range from 27 weeks to full term,[68] respectively. The absent correlation in our study may be due to the fact that our cohort included only full-term pregnancies with deliveries between gestational weeks 37.4 and 41.7.

We see it as a limitation of our study that we do not have data on fetal insulin resistance, estrogen, IGF-1, and leptin. All these parameters are known to differ between male and female neonates[44,69] and affect endothelial function.[50,51,70–72] Thus, they may contribute to the observed sexual dimorphism in ECFC outgrowth.

This study highlights that not only do severe metabolic derangements affect fetal development, but parameters within the normal range also play a role. Whilst a wide array of studies investigated the effect of maternal hyperglycemia on the offspring's short and long-term health, only very few studies investigated continuous glucose levels within the normal range. Yuan et al. demonstrated an impact of maternal FPG in pregnancy on endothelial function and cardiovascular risk markers in the offspring at age 6 years.[73] Interestingly, this association existed only in the male offspring, which highlights the importance of sex-specific analysis in programming events.

The role of pre-and perinatal ECFC is not fully understood, however, a function in postnatal vasculogenesis and angiogenesis has been suggested.[74] Additionally, the fast and active homing of hematopoietic progenitor cells from the neonatal circulation after birth[75] suggests a significant role of ECFC in the neonatal vasculature, such as promotion of the rapid vascular growth, or support of major vascular remodeling events happening immediately after birth. A relation between number and function of neonatal ECFC and further development has not yet been investigated and we did not find any studies investigating the neonatal vascular system in regards to sex or maternal glycemia in pregnancy. However, a link between neonatal ECFC dysfunction in preterm delivery and adult risk for CVD has been hypothesized.[76]

In the light of the DOHaD (Developmental Origins of Health and Disease) paradigm, which describes the role of intrauterine influences in the development of diseases in later life,[77] such long-term effects of adaptive and mal-programming events may be suggested and should be investigated in future studies.

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AUTHOR CONTRIBUTIONS

E.W. and U.H. designed the study and wrote the paper. E.W., B.L.-P., and A.S. performed experiments. S.C. contributed with analytic tools. E.W analyzed data. All authors revised the manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

CONSENT STATEMENT

The study was approved by the ethics committee of the Medical University of Graz (29-319 ex 16/17) and written informed consent was obtained from all participants.

ADDITIONAL INFORMATION

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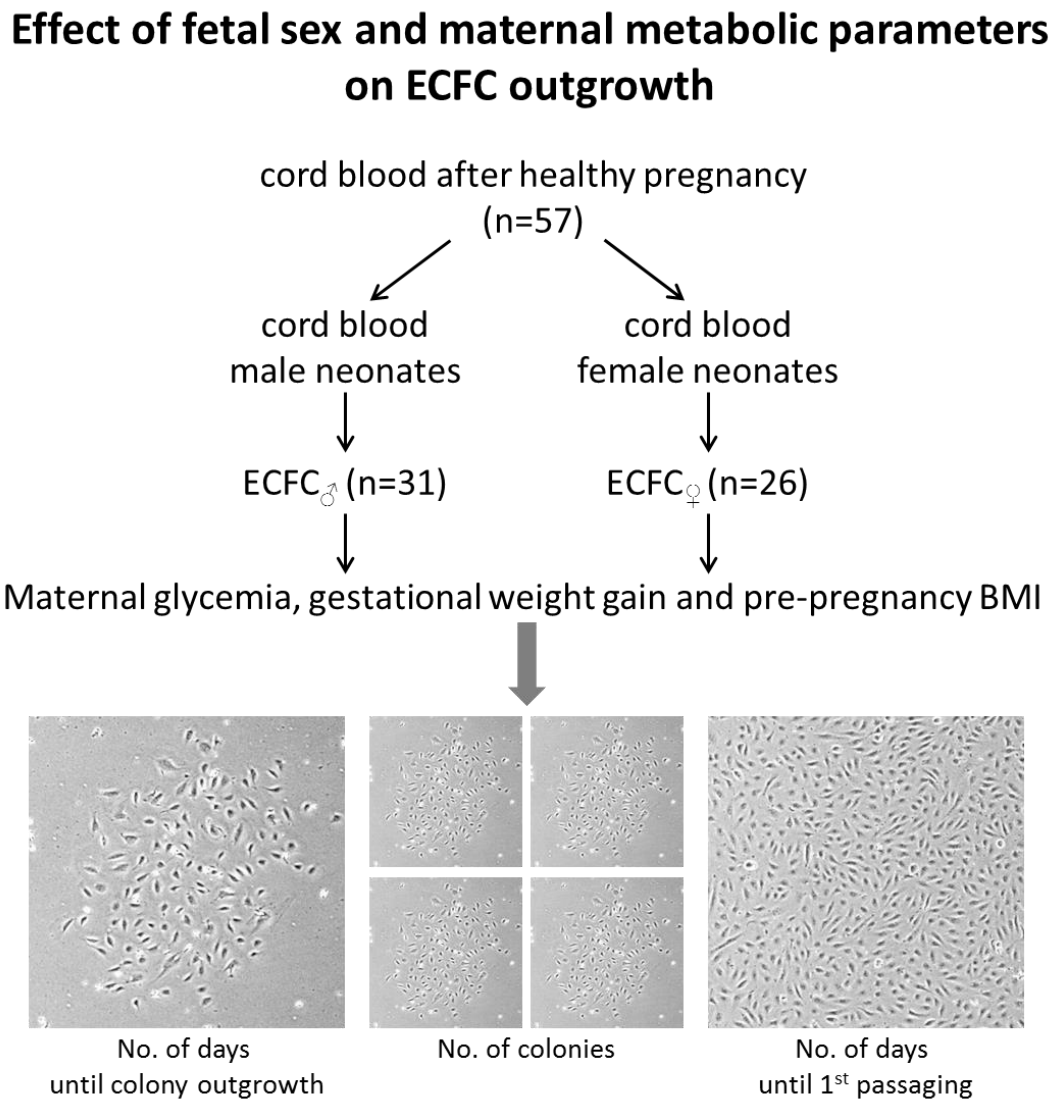
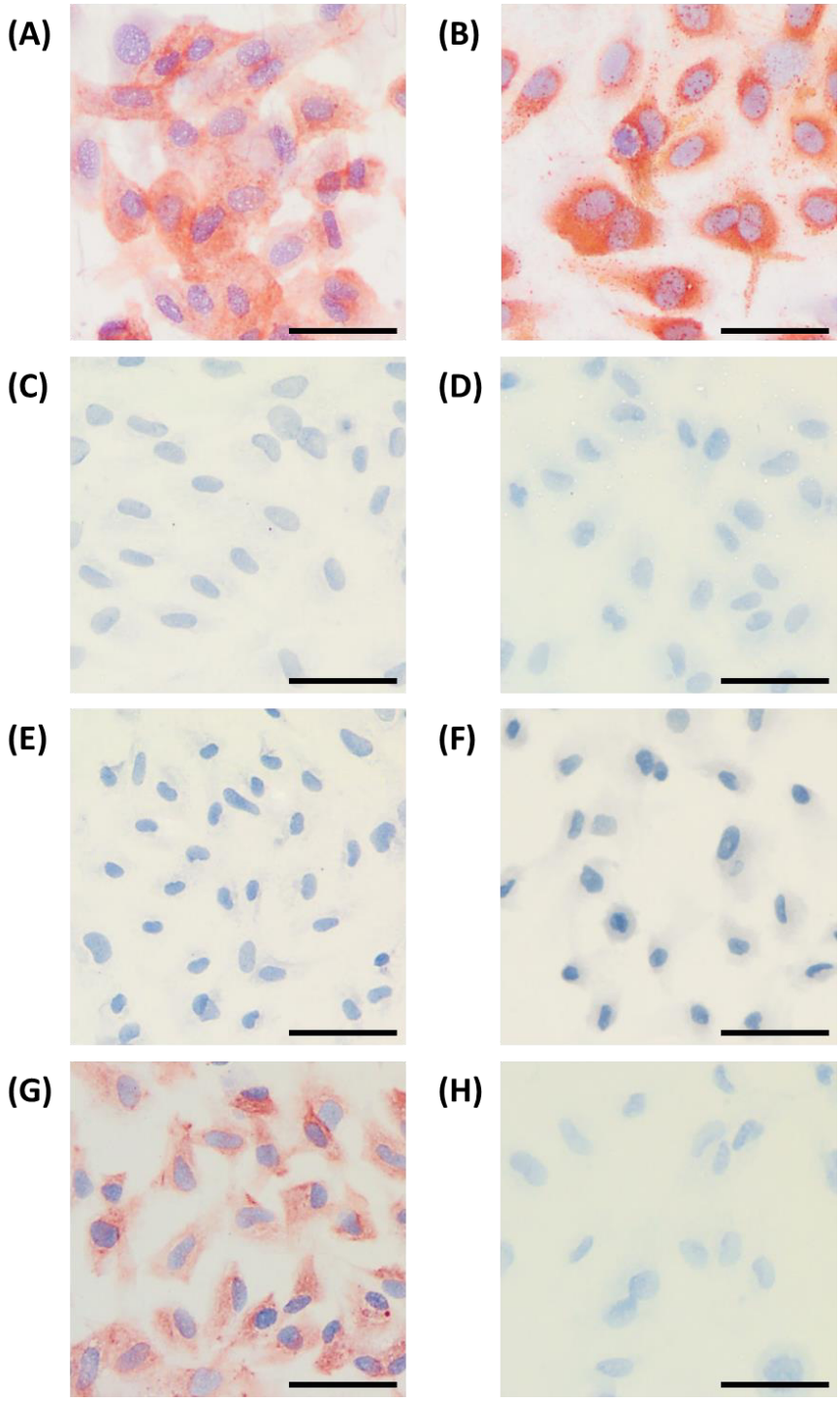
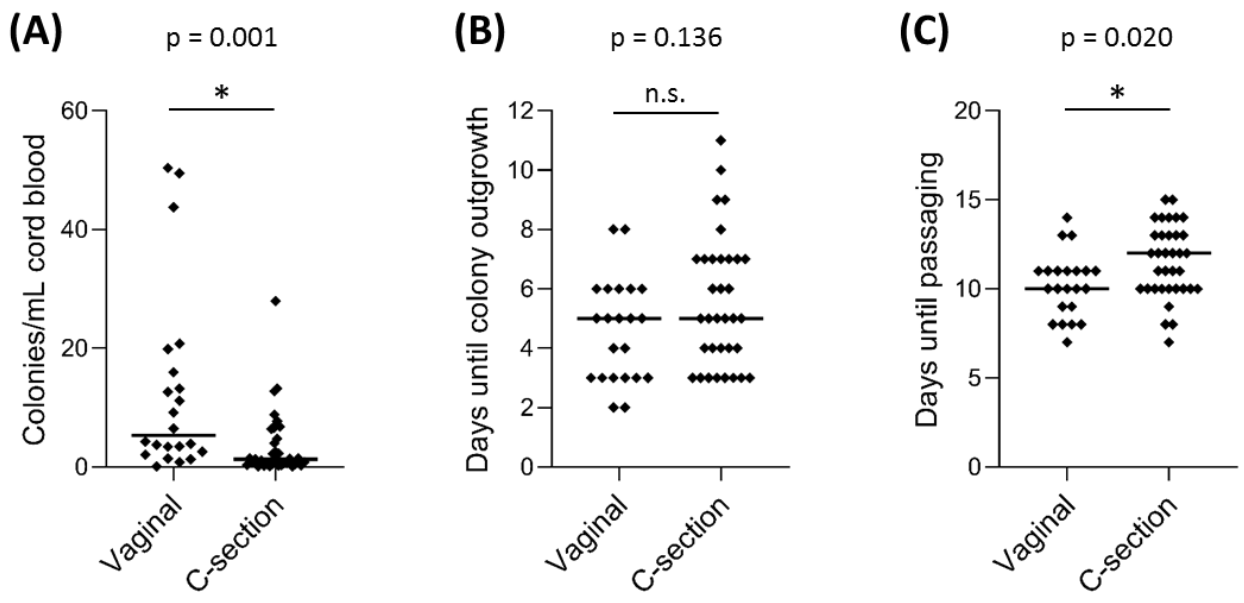


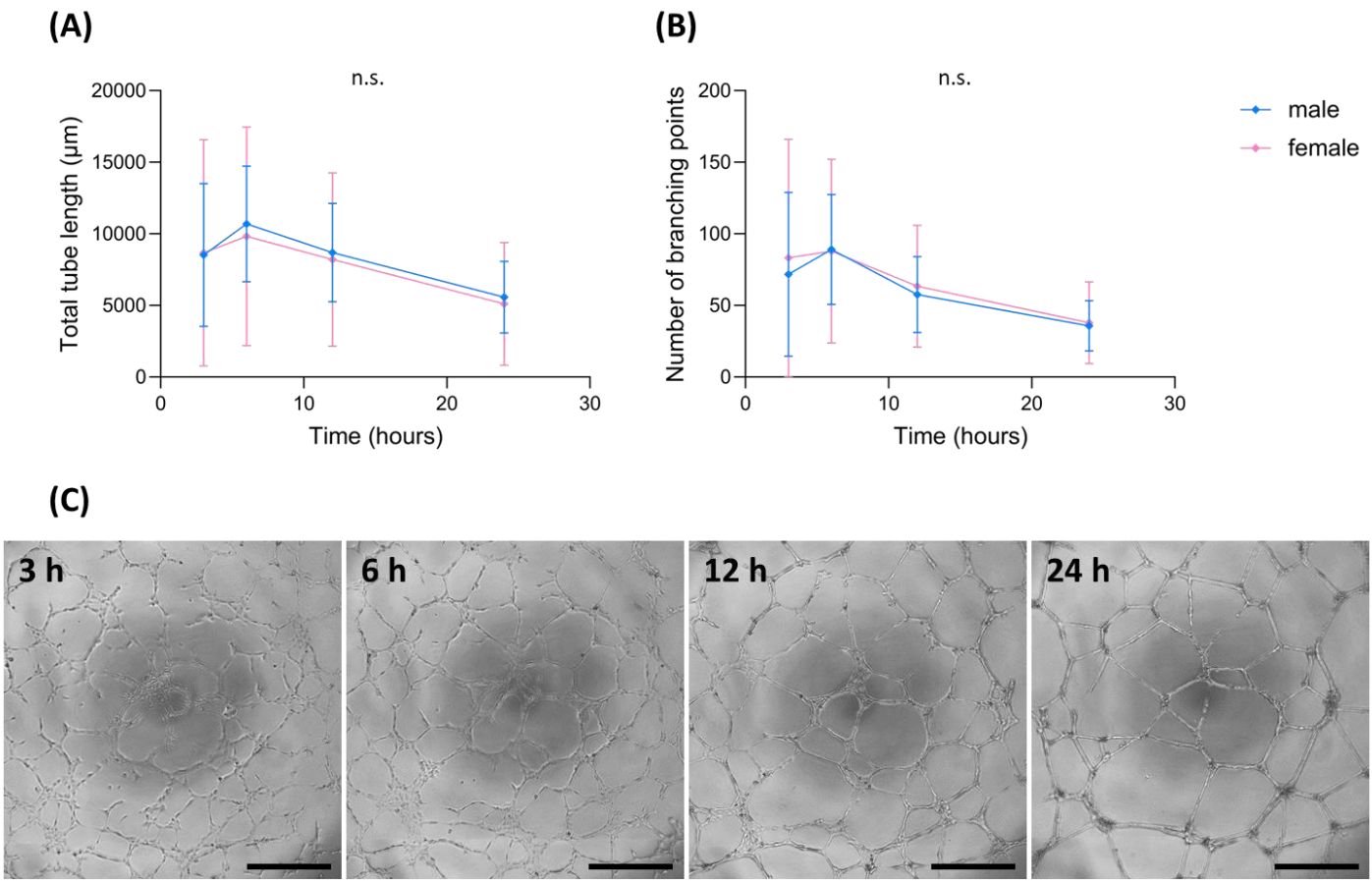
Supplementary Figure 1. Experimental study design.



Supplementary Figure 2. Phenotype of cord blood ECFC as determined by immunocytochemistry. Cells were analyzed for surface expression of endothelial cell markers (CD31 (**A**), VWF (**B**)), muscle cell markers (SMA (**C**), Desmin (**D**)) and fibroblast markers (CD90 (**E**), TE-7 (**F**)). Vimentin (**G**), a protein present in all cells, served as positive control, mouse IgG1 (**H**) as negative control. Depicted images represent a typical expression pattern and show specific antibody staining (red) vs staining of the nuclei (blue). Scale bar = 50 µm.



Supplementary Figure 3. Confounding factors for ECFC outgrowth. (**A**) Number of colonies in ECFC after vaginal delivery and C-section. (**B**) Days until colony outgrowth in ECFC after vaginal delivery and C-section. (**C**) Days until reaching confluency and passaging in ECFC after vaginal delivery and C-section. Data were analyzed via Mann-Whitney *U* test. n(vaginal delivery)=22, n(C-section)=35



Supplementary Figure 4. Network formation assay on Matrigel. (**A**) Total tube length in male vs female ECFC. (**B**) Number of branching points in male vs female ECFC. (**C**) Depicted images represent a typical network formation over time. Data were analyzed via Student's t-test. n(male)=12, n(female)=11; Scale bar = 500 µm.

Supplementary Table 1. Antibodies used for flow cytometry. All antibodies originate from mouse species.

Marker	Label	Manufacturer/Ord. No.
CD14	FITC	Miltenyi Biotec/130-080-701
CD31	FITC	BD Pharmingen/560984
CD34	PE-Cy7	Beckman Coulter/A21691
CD45	PE	BD Pharmingen/555483
CD90	APC	BD Pharmingen/561971
CD133	APC	Miltenyi Biotec/130-098-829
CD144	PerCP-Cy5.5	BD Pharmingen/561566
CD146	PE	BD Pharmingen/561013
CD309	PE	BD Pharmingen/560494
Tie-2	APC	R&D/FAB3131A
Isotype Controls		
	APC	BD Pharmingen/555751
	FITC	BD Pharmingen/555748
	PE	BD Pharmingen/556027
	PE-Cy7	Beckman Coulter/737662
	PerCP-Cy5.5	BD Pharmingen/550795

Supplementary Table 2. Antibodies used for immunocytochemistry.

Marker	Manufacturer/Ord.	Isotype
CD31	Monosan/MON6002-1	Mouse IgG1
VWF	Dako/A0082	Polyclonal
CD90	Dianova/DIA100	Mouse IgG1
TE-7	Millipore/CBL271	Mouse IgG1
SMA	Dako/M0851	Mouse IgG2a
Desmin	Dako/M0760	Mouse IgG1
Vimentin	Dako/M0725	Mouse IgG1
Negative	Dako/X0931	Mouse IgG1

Supplementary Table 3. Network formation assay on Matrigel.

		Branching points (number)				Total tube length (µm)			
		3h	6h	12h	24h	3h	6h	12h	24h
Male		71.8±57.2	89.0±38.5	57.6±26.6	35.8±17.6	8531±4989	10697±4033	8696±3446	5583±2511
Female		83.2±82.9	87.9±64.3	63.4±42.5	37.9±28.5	8668±7911	9826±7631	8215±6057	5115±4283
	p	0.701	0.959	0.699	0.830	0.960	0.740	0.820	0.756
FGP	r	0.059	0.112	0.066	0.087	0.056	0.038	-0.007	-0.018
	p	0.789	0.610	0.765	0.692	0.799	0.864	0.974	0.934

Data are presented as mean ± SD. Statistical differences are calculated by unpaired Student's t-test (male vs female) or by Pearson correlation (FPG). FPG: Fasting plasma glucose.

Supplementary Table 4. Correlation of outgrowth parameters with maternal metabolic parameters in the entire cohort and in the groups with male and female neonates separately.

	entire cohort		male		female	
	OR (CI)	p	OR (CI)	p	OR (CI)	p
Fasting plasma glucose (oGTT 0 h)						
Colonies/mL	0.97 (0.91; 1.03)	0.353	0.98 (0.90; 1.06)	0.604	0.97 (0.87; 1.07)	0.497
Days until outgrowth	**1.02 (1.00; 1.04)**	**0.030**	**1.03 (1.00; 1.05)**	**0.029**	1.01 (0.98; 1.04)	0.405
Days until passaging	1.00 (1.00;1.01)	0.189	1.01 (1.00; 1.02)	0.223	1.00 (0.99; 1.02)	0.599
Post-load glycemia (oGTT 1 h)						
Colonies/mL	0.99 (0.98; 1.00)	0.201	0.99 (0.97; 1.01)	0.518	1.00 (0.97; 1.02)	0.659
Days until outgrowth	1.00 (1.00; 1.01)	0.291	1.00 (1.00; 1.01)	0.727	1.00 (1.00; 1.01)	0.759
Days until passaging	1.00 (1.00; 1.00)	0.245	1.00 (1.00; 1.00)	0.849	1.00 (1.00; 1.00)	0.495
Post-load glycemia (oGTT 2 h)						
Colonies/mL	1.01 (0.99; 1.03)	0.200	1.02 (1.00; 1.04)	0.095	1.01 (0.98; 1.04)	0.502
Days until outgrowth	1.00 (0.99; 1.00)	0.241	1.00 (0.99; 1.00)	0.409	1.00 (0.99; 1.00)	0.164
Days until passaging	1.00 (1.00; 1.00)	0.114	1.00 (1.00; 1.00)	0.164	1.00 (0.99; 1.00)	0.131
Pre-pregnancy BMI						
Colonies/mL	1.01 (0.91; 1.11)	0.878	1.01 (0.90; 1.15)	0.806	1.02 (0.86; 1.20)	0.836
Days until outgrowth	1.00 (0.97; 1.02)	0.765	1.01 (0.97; 1.04)	0.701	0.97 (0.94; 1.01)	0.171
Days until passaging	1.00 (0.99; 1.01)	0.660	1.00 (0.98; 1.01)	0.572	1.00 (0.98; 1.02)	0.731
Gestational weight gain						
Colonies/mL	1.02 (0.95; 1.10)	0.494	1.03 (0.95; 1.12)	0.466	0.98 (0.84; 1.14)	0.755
Days until outgrowth	1.00 (0.98; 1.02)	0.734	0.99 (0.97; 1.02)	0.572	1.02 (0.98; 1.05)	0.396
Days until passaging	1.00 (0.99; 1.00)	0.324	1.00 (0.99; 1.01)	0.438	1.00 (0.98; 1.02)	0.707

Data were analyzed by linear regression analysis and are presented as unstandardized regression coefficient B, that requires interpretation as odds ratio (OR), and 95% confidence intervals (CI). Bolt letters indicate $p<0.05$. oGTT: oral glucose tolerance test.

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Article

Maternal Overweight Downregulates MME (Neprilysin) in Feto-Placental Endothelial Cells and in Cord Blood

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Abstract: Maternal overweight in pregnancy alters the metabolic environment and generates chronic low-grade inflammation. This affects fetal development and programs the offspring's health for developing cardiovascular and metabolic disease later in life. MME (membrane-metalloendopeptidase, neprilysin) cleaves various peptides regulating vascular tone. Endothelial cells express membrane-bound and soluble MME. In adults, the metabolic environment of overweight and obesity upregulates endothelial and circulating MME. We here hypothesized that maternal overweight increases MME in the feto-placental endothelium. We used primary feto-placental endothelial cells (fpEC) isolated from placentas after normal vs. overweight pregnancies and determined MME mRNA, protein, and release. Additionally, soluble cord blood MME was analyzed. The effect of oxygen and tumor necrosis factor α (TNFα) on MME protein in fpEC was investigated in vitro. Maternal overweight reduced MME mRNA (−39.9%, $p < 0.05$), protein (−42.5%, $p = 0.02$), and MME release from fpEC (−64.7%, $p = 0.02$). Both cellular and released MME protein negatively correlated with maternal pre-pregnancy BMI. Similarly, cord blood MME was negatively associated with pre-pregnancy BMI ($r = -0.42$, $p = 0.02$). However, hypoxia and TNFα, potential negative regulators of MME expression, did not affect MME protein. Reduction of MME protein in fpEC and in cord blood may alter the balance of vasoactive peptides. Our study highlights the fetal susceptibility to maternal metabolism and inflammatory state.

Keywords: MME; neprilysin; maternal overweight; feto-placental endothelial cells; umbilical cord blood

1. Introduction

Pregnancies complicated by maternal overweight are associated with an altered metabolic and endocrine environment and characterized by chronic low-grade inflammation [1,2]. These changes affect fetal development and, furthermore, program the offspring's health in the long term (reviewed by Ingvorsen et al. [3] and Zhou and Pan [4]). For instance, higher maternal pre-pregnancy BMI is associated with an increased childhood blood pressure [5,6] and an altered childhood cardiac structure [7]. Moreover, maternal overweight in pregnancy is positively associated with the development of cardiovascular disease in adult offspring [8]. Thus, although the mechanisms are not fully understood, there is a well-established association of maternal overweight and obesity with endothelial function and cardiovascular disease. Mechanisms may involve both direct mechanical effects of altered

fetal hemodynamics on fetal cardiac development, as well as programming effects of the changed intrauterine environment on endothelial cells, cardiomyocytes, and smooth muscle cells.

MME (membrane metalloendopeptidase, also termed neprilysin and CD10) is an integral membrane-bound zinc metallo-endopeptidase that cleaves various vasoactive peptides and thus participates in blood pressure regulation [9]. Specifically, MME catalyzes degradation of several vasodilator peptides, including ANP, BNP, and CNP (natriuretic peptides A, B and C), adrenomodulin and bradykinin, as well as vasoconstrictor peptides such as endothelin-1 and angiotensin II (reviewed by Corti et al. [10]). Thus, the overall effect of MME on vascular tone regulation depends on the presence and composition of substrates within the specific vascular system [10].

MME is expressed on various cell types including endothelial cells [9]. Besides the membrane-bound form, a soluble form of MME exists that retains catalytic activity [11]. In fact, endothelial cells are a source of soluble MME [12]. Under metabolic derangements, soluble MME increases in the systemic circulation in adults, and correlates positively with BMI [13,14]. Various factors of the altered metabolic and pro-inflammatory environment contribute to this upregulation—in vitro experiments revealed that hyperglycemia and hyperlipidemia increase MME production in endothelial cells [15]. Additionally, the correlation of circulating MME levels with CRP (C-reactive peptide) reveals the susceptibility of MME to the pro-inflammatory environment, and pro-inflammatory mediators such as IL-β1 (interleukin β1) and TGFβ (transforming growth factor β) increase MME in various cell types [16–18].

We here hypothesized that maternal overweight upregulates MME in the fetal endothelium. To this end, we isolated primary human feto-placental endothelial cells after pregnancies of normal weight and overweight women and measured MME mRNA and protein.

2. Results

2.1. Feto-Placental Endothelial Cells (fpEC) Expressed MME mRNA and Protein In Vivo and In Vitro

To confirm MME protein production in placental endothelium, we performed immunohistochemistry of placental tissue. In fact, there was a positive staining for MME protein in the endothelium of the feto-placental vessels (Figure 1A). Furthermore, there was an intense MME staining also in the syncytiotrophoblast, the classical placental barrier that contacts the maternal bloodstream. Immunocytochemistry of isolated primary fpEC also further revealed production of MME in cultured fpEC (Figure 1B). Quantitative RT-PCR was used to compare *MME* expression levels of primary fpEC to *MME* expression in various classical MME-producing human organs (Figure 1C) and revealed feto-placental *MME* levels comparable to brain and thyroid. Total placental tissue revealed the highest expression of all analyzed organs, due to the intense MME expression in the syncytiotrophoblast.

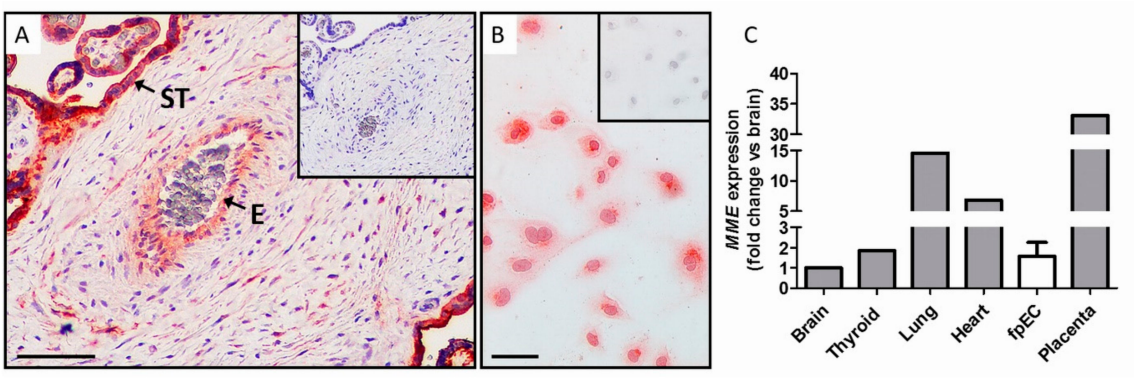

Figure 1. MME (membrane metalloendopeptidase) protein and mRNA expression in feto-placental endothelium. (**A**) In placental tissue, positive staining for MME (red) was detected in the syncytiotrophoblast (ST) facing the maternal circulation, as well as in the feto-placental endothelium (E) facing the fetal circulation. Nuclei were stained blue with DAPI (4′,6-diamidino-2-phenylindole). Scale bar: 100 µm. (**B**) Immunocytochemistry revealed that isolated primary feto-placental endothelial cells (fpEC) continued to express MME in culture. Scale bar: 200 µm. Negative controls using unspecific mouse IgG are shown in the inserts. (**C**) Comparison of *MME* mRNA expression in different classical MME-producing tissues and organs, and in fpEC and placenta. Data were normalized to the mean of the house-keeping genes hypoxanthine-guanine phosphoribosyltransferase (*HPRT1*) and peptidylprolyl isomerase A (*PPIA*) and represented in relation to the expression in brain.

2.2. Maternal Pre-Pregnancy Overweight Reduced MME mRNA and Protein in fpEC

Analysis of *MME* mRNA expression in primary fpEC isolated after pregnancies of women with normal vs. overweight BMI (Table 1) revealed a reduction of *MME* in fpEC exposed to overweight pregnancies (−39.9%, $p = 0.047$) (Figure 2A). This was paralleled by a reduction of cellular MME protein (−42.5%, $p = 0.02$) as well as secreted MME in the culture medium (−64.7%, $p = 0.02$) (Figure 2B,C). Whilst there was no significant correlation between fpEC *MME* mRNA expression and maternal pre-pregnancy BMI, cellular MME protein and MME secretion negatively correlated with BMI ($r = -0.42$, $p = 0.02$ and $r = -0.55$, $p = 0.02$, respectively) (Figure 2D–F).

Table 1. Characteristics of the fpEC donors.

Characteristics	Controls	Overweight Subjects
Number of cases	19	15
Pre-pregnancy BMI (kg/m^2)	21.1 ± 1.7	28.7 ± 3.4 ***
BMI at birth (kg/m^2)	26.9 ± 2.4	33.9 ± 2.6 ***
Maternal age (years)	34.0 ± 5.7	32.3 ± 4.1
oGTT (0 h)	80.6 ± 5.9	82.9 ± 4.7
oGTT (1 h)	105.9 ± 27.8	116.5 ± 27.8
oGTT (2 h)	95.6 ± 20.5	94.1 ± 16.3
Gestational age at delivery (weeks)	39.7 ± 1.0	39.2 ±1.6
Mode of delivery (vaginal/C-section)	8/11	6/9
Fetal weight (g)	3559 ± 351	3477 ± 402
Fetal height (cm)	51.5 ± 1.7	51.3 ± 2.5
Fetal sex (m/f)	12/7	8/7
Placental weight (g)	634 ± 117	602 ± 147

*** indicates $p < 0.001$. oGTT: oral glucose tolerance test.

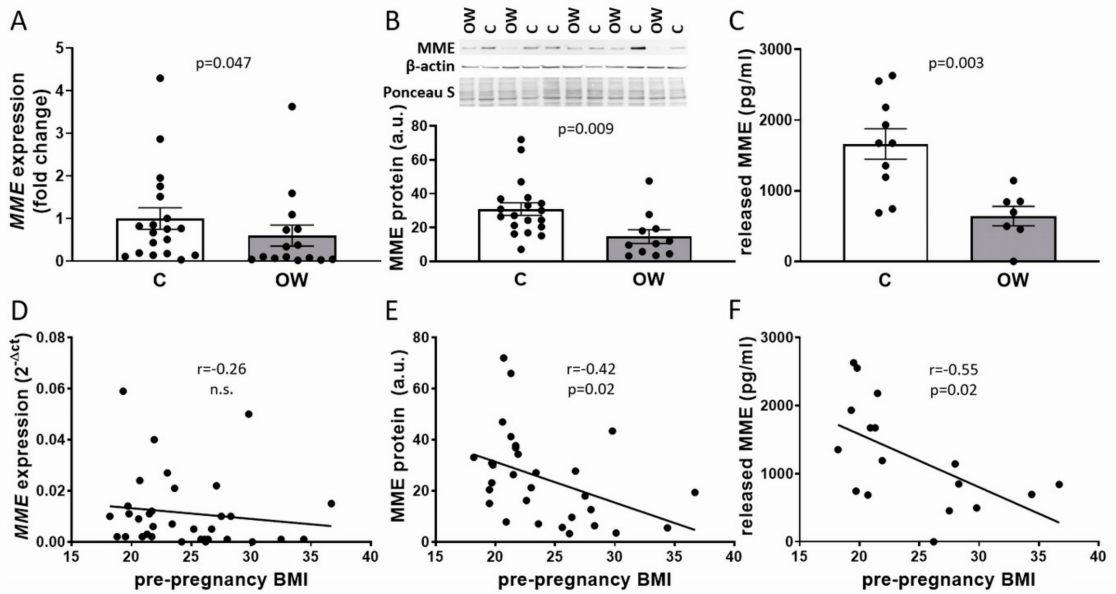

Figure 2. *MME* mRNA and protein in fpEC after normal and overweight pregnancy. *MME* mRNA (**A**), cellular protein (**B**), and released MME (**C**) was reduced in primary fpEC exposed to maternal overweight (*t*-test). When correlated to maternal pre-pregnancy BMI (Pearson correlation), this association was not significant for fpEC *MME* mRNA (**D**), but it was significant for fpEC protein production (**E**) and release (**F**). *MME* mRNA was normalized to the mean of the housekeeping genes *HPRT1* and ribosomal protein L30 (*RPL30*), respectively. A representative immunoblot for MME, β-actin, and the Ponceau S staining of the corresponding membrane are shown on top of the protein data in (B). C: controls; OW: overweight; a.u.: arbitrary units. *MME* mRNA: *n*(c) = 19; *n*(ow) = 15; cellular MME: *n*(c) = 19; *n*(ow) = 11; MME release: *n*(c) = 10; *n*(ow) = 7. The bars in (**A**–**C**) represent the mean ± SEM.

2.3. Maternal Pre-Pregnancy Overweight Reduced Umbilical Cord Blood MME Levels

Exposure to the intrauterine environment of overweight reduced MME release by fpEC in vitro. This raised the question as to whether maternal overweight also alters soluble MME in the fetal circulation. Thus, we collected a cohort of umbilical cord blood sera of pregnancies with normal vs. overweight pre-pregnancy BMI (Table 2). In parallel to the findings in isolated primary fpEC, MME in cord blood serum correlated negatively with maternal pre-pregnancy BMI (Figure 3).

Table 2. Characteristics of the cord blood donors.

Characteristics	Controls	Overweight Subjects
Number of cases	20	12
Pre-pregnancy BMI (kg/m^2)	21.4 ± 1.2	28.6 ± 2.4 ***
BMI at birth (kg/m^2)	26.7 ± 2.1	33.1 ± 2.2 ***
Maternal age (years)	31.1 ± 5.3	27.8 ± 3.1
oGTT (0 h)	81.1 ± 5.7	83.6 ± 4.8
oGTT (1 h)	123.5 ± 34.7	112.2 ± 20.4
oGTT (2 h)	98.8 ± 24.4	102.4 ± 20.5
Maternal CRP at delivery	2.8 ± 1.9	4.9 ± 2.6 *
Gestational age at delivery (weeks)	39.1 ± 0.9	38.9 ± 0.9
Mode of delivery (vaginal/C-section)	3/17	1/11
Fetal weight (g)	3358 ± 370	3493 ± 385
fetal height (cm)	50.5 ± 2.1	51.8 ± 2.0
Fetal sex (m/f)	10/10	8/4
Placental weight (g)	667 ± 89	661 ± 100

* indicates $p < 0.5$, *** indicates $p < 0.001$.

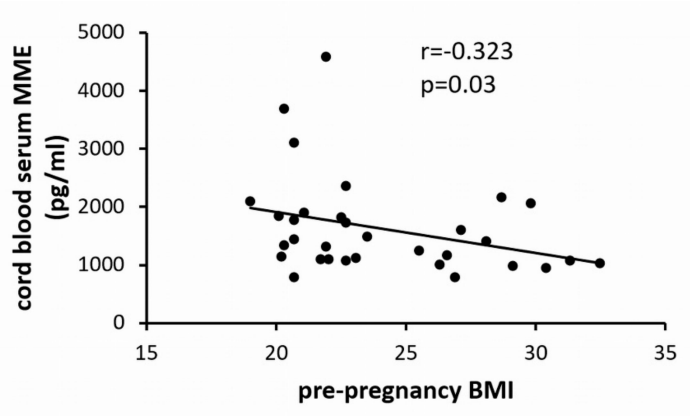

Figure 3. Correlation of umbilical cord blood serum MME levels with maternal pre-pregnancy BMI (n = 32).

This opposes findings in adults demonstrating upregulation of circulating MME with increasing BMI [13,14]. We therefore investigated whether the reduction of MME in cord blood serum is determined not by maternal BMI, but by fetal weight. However, similar to maternal BMI, neonatal weight also correlated negatively with cord blood MME (Figure 4).

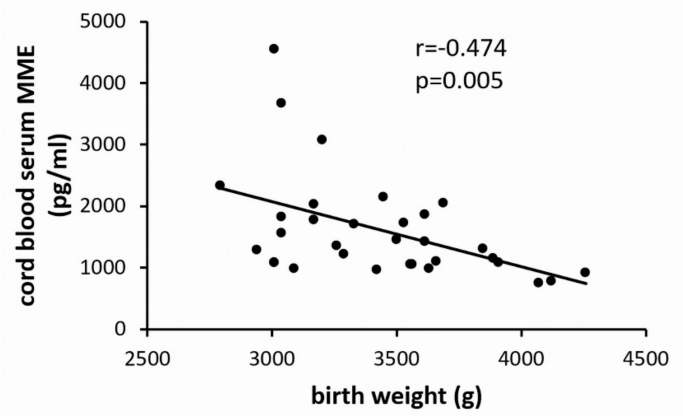

Figure 4. Correlation of umbilical cord blood serum MME levels with birth weight (n = 32).

2.4. MME Protein in fpEC was not Regulated by Oxygen and Tumor Necrosis Factor α (TNFα)

Hypoxia and NF-κB (nuclear factor kappa B) signalling downregulate MME in other cell types [19–21], and TNFα (tumor necrosis factor α) is an activator of NF-κB signaling [22]. Thus, we tested whether oxygen or TNFα altered MME in fpEC from control pregnancies. However, after 48 h, MME protein did not differ between cells grown at 5%, 12%, and 21% oxygen (Figure 5A). Additionally, TNFα treatment (5 and 50 ng/mL) did not affect MME after 24 h (Figure 5B).

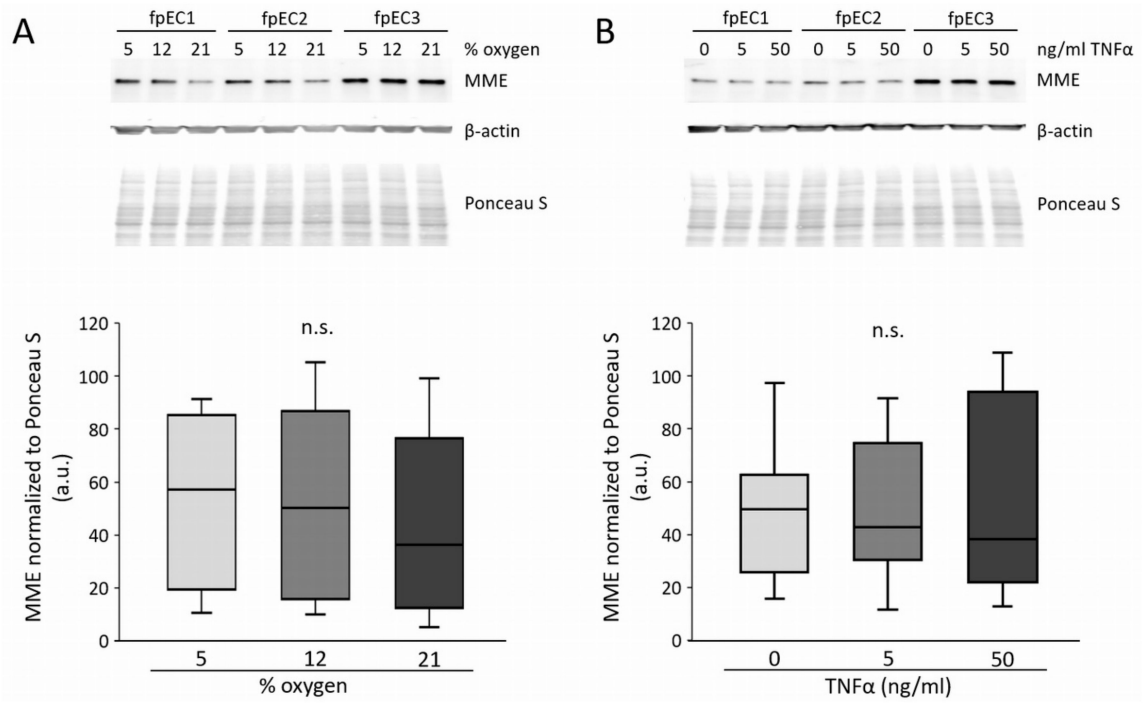

Figure 5. Effect of oxygen and tumor necrosis factor α (TNFα) on MME protein in fpEC. (**A**) MME protein after culture at 5%, 12%, and 21% oxygen for 48 h. (**B**) MME protein after TNFα treatment (0, 5, and 50 ng/mL) for 24 h. Protein levels of β-actin were used as loading controls and protein was normalized to total protein staining with Ponceau S. Experiments were performed in $n = 7$ different fpEC isolations. Representative immunoblots of three fpEC isolations are shown on top. a.u.: arbitrary units; *n*.s.: not significant.

3. Discussion

Obesity and metabolic syndrome in adults are associated with increased levels of MME [13,14], and this may modulate vascular tone regulation. Upregulation of MME by fatty acids, glucose, and the pro-inflammatory environment represent a possible underlying reason [15–17]. In utero exposure to maternal overweight primes the offspring to a disturbed vascular tone regulation in childhood with elevated blood pressure and an adverse cardio-metabolic risk profile [3–8]. This study tested the hypothesis that the moderate metabolic derangement of maternal overweight in pregnancy increases the expression and release of MME by primary fpEC isolated from the feto-placental vasculature.

Surprisingly, maternal overweight reduced MME levels in endothelial cells and in the fetal circulation. In addition, fetal weight was negatively correlated with circulating cord blood serum MME. Despite normal maternal oGTT (oral glucose tolerance test) levels, maternal overweight and obesity in pregnancy are characterized by insulin resistance and increased levels of inflammatory markers, i.e., IL-6 [23] and CRP [24]. Additionally, in our overweight samples revealing only moderate metabolic derangement with normal oGTT, maternal CRP was increased (Table 2) and positively correlated with maternal pre-pregnancy BMI (Supplementary Figure S1). This altered maternal metabolic and pro-inflammatory environment translates also to the fetal circulation, as fetal insulin resistance, increased cord blood TNFα, and CRP arise [25–28]. However, whilst insulin resistance and inflammatory status are associated with elevated MME in adults [16–18], other mechanisms seem to account for MME regulation in the fetal compartment.

Possible insults downregulating MME include hypoxia and NF-κB (nuclear factor kappa B) signaling. In rodents, hypoxia downregulates MME expression in the lungs, kidneys [20], and in the brain [21]. In fact, several studies have revealed a subtle hypoxic fetal environment in pregnancy complicated by maternal overweight [29,30]. Additionally, MME is downregulated by NF-κB pathway

via microRNA miR155 in B-lymphoma cells [31]. The pro-inflammatory cytokine TNFα activates NF-κB pathway [22], which subsequently upregulates ICAM-1 (intercellular adhesion molecule 1) expression [32]. TNFα is increased in cord blood when mothers are overweight [27], and fpEC also respond to TNFα with an induction of ICAM-1 expression [33]. Thus, we further tested whether hypoxia or TNFα reduce MME protein in fpEC in vitro. However, both conditions did not affect MME protein, suggesting that neither hypoxia nor NF-κB participate in the overweight-associated reduction of MME. Fitzpatrick et al. [34] discovered that shear stress downregulates MME in aortic endothelial cells by a mechanism involving NADPH oxidase-dependent production of reactive oxygen species (ROS). Nonetheless, the unaffected expression of MME in fpEC exposed to different oxygen concentrations does not point towards a ROS-dependent signaling event accounting for MME reduction in overweight pregnancies. Besides signaling events, various studies have revealed epigenetic mechanisms underlying changes in fetal gene expression in response to an altered intrauterine environment. Such epigenetic mechanisms include DNA methylation and translational repression by miRNAs (reviewed by [35]). In fact, both mechanisms occur in MME regulation: Increased DNA methylation of the *MME* promoter region was observed in Alzheimer's disease brain [36] and in several types of cancer, such as leukemia [37] and breast cancer [38]. Additionally, MME is a target of miRNA-mediated regulation, as shown for the abovementioned miR155 [31]. Moreover, the miRNA target prediction database miRDB (www.mirdb.org) revealed that 156 different miRNAs potentially target *MME* mRNA, and thus may participate in its translational repression.

The physiological outcome of reduced endothelial and circulating MME can only be hypothesized and may be—in parallel with MME's multifunctional action—versatile. Reduced MME levels may affect the balance of vasoactive peptides, therefore affecting vascular tone regulation [10]. In fact, in human umbilical vein endothelium, MME effectively inactivates bradykinin, and hence impairs bradykinin-mediated vasodilatation [39]. However, depending on the presence and composition of MME vasoactive substrates within a certain vascular bed, and depending on the phenotype and responsiveness of the specific endothelial cells to these substrates [10], MME effects may differ in the fetus or neonate.

Moreover, recent literature suggests that elevated MME participates in the development of insulin resistance. On one hand, MME cleaves peptides stimulating insulin secretion, i.e., glucagon [40] and GLP1 (glucagon-like peptide 1) [41], and thus MME may reduce systemic insulin secretion [42]. On the other hand, membrane-associated MME modulates internalization and cellular localization of the insulin receptor, as shown by *MME* knockdown and overexpression in insulin target cells [43]. Hence, decreased levels of MME, as we observed in the overweight cohort, may play a role in the deregulation of insulin response and insulin resistance. In mice *MME*, knockout results in increased insulin sensitivity [43]. However, neonates from pregnancies complicated by maternal obesity have increased insulin resistance [24]. Thus, the role and specific effect of reduced MME in feto-placental endothelium and cord blood on insulin secretion and resistance is difficult to predict.

Besides the regulation of vascular tone and insulin sensitivity, a role of MME in the regulation of angiogenesis was suggested. On one hand, MME cleaves and inactivates pro-angiogenic [44,45] as well as anti-angiogenic [46,47] acting growth factors and peptides. On the other hand, MME located at the cell membrane seems to participate in signaling events and has been shown to prevent FAK (focal adhesion kinase) activation and attenuates PKB (protein kinase B) signaling, causing reduced migration and angiogenesis (reviewed by Maguer-Satta et al. [48]). Several reports describe distinct placental vascular structure as a result of obesity in human and animals, with either increased [49–51] or decreased [52–54] vascularity, suggesting that placental angiogenesis and vascular development is susceptible towards maternal metabolic and pro-inflammatory changes, but the effective result may depend on the specific situation, i.e., moderate vs. severe metabolic changes. In any event, altered angiogenesis in the placenta will generate distinct placental vascular structure and architecture, ultimately affecting hemodynamics. This may contribute to functional programming of the fetal cardiovascular system.

We see it as a limitation of our study that we do not have further metabolic and inflammatory information on the subjects, both from the maternal and from the fetal side. Data on maternal insulin resistance, fetal C-peptide, erythropoietin, and inflammatory markers would add significantly to the knowledge about the specific intrauterine stimulus downregulating MME and should be included in further studies.

Our study demonstrates for the first time that the moderate metabolic derangements of maternal overweight decreases feto-placental endothelial and fetal circulating MME, thus highlighting the susceptibility of the fetus to maternal metabolism and low-grade inflammation.

4. Materials and Methods

4.1. Sample Collection

Ethical approval was obtained from the Medical University of Graz (approval reference number 29–319 ex 16/17, 29.06.2017) and all women provided written informed consent. Placentas for feto-placental endothelial cell (fpEC) isolation were collected from pregnancies of non-smoking (self-reported) women with a negative 75 g oral glucose tolerance test (oGTT) performed at 25–28 weeks of gestation, free from any medical disorders or pregnancy complications. Control placentas were obtained from women with a pre-pregnancy BMI < 25, overweight placentas were collected from women with a pre-pregnancy BMI > 25. Table 1 shows the characteristics of the fpEC donors.

For the collection of umbilical cord blood serum, the same inclusion and exclusion criteria were used. Umbilical cord blood was collected directly after delivery, centrifuged at 3000 rpm for 10 min at 4 °C, and the serum was then stored at −80 °C. Table 2 shows the characteristics of the cord blood donors.

4.2. Cell Culture

Primary arterial feto-placental endothelial cells (fpEC) were isolated from the collected placentas following a standard protocol [55]. Briefly, chorionic arteries were dissected and endothelial cells isolated by perfusion with a collagenase/dispase (Roche, Mannheim, Germany) solution. Cells were resuspended in endothelial basal medium (EBM, Lonza, Walkersville, MD, USA) supplemented with the EGM-MV BulletKit (Lonza) on 1% (*v*/*v*) gelatin-coated flasks. Isolated fpECs were grown at 37 °C and 12% oxygen, and used up to passage 10. Cells were characterized by immunocytochemical analysis (c.f. below) with positive staining for the endothelial cell markers VWF (von Willebrand factor) and CD31, and negative for the SMA (smooth muscle actin) and the fibroblast marker CD90. For isolation of RNA, protein, and collection of supernatant, cells were grown in 75 cm^2 flasks to approximately 90% confluency, washed with ice-cold Hank's balanced salt solution (HBSS; Gibco, Thermo Fisher Scientific, Runcorn, United Kingdom), and harvested as described below.

4.3. Immunohistochemistry

Immunohistochemical staining of MME was performed on standard formalin-fixed paraffin embedded term placenta sections (5 µm). Standard deparaffinization procedure was followed by boiling slides in Epitope Retrieval Solution pH 9.0 (Novocostra, Leica, Vienna, Austria) for 7 min at 120 °C in a decloaking chamber (Biocare Medical, Pacheco, CA, USA). Sections were immunostained using the UltraVision horseradish peroxidase (HRP) Polymer Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Briefly, endogenous peroxidase was blocked using the hydrogen peroxidase block for 10 min. Three washing steps with Tris-buffered saline (TBS) were followed by background blocking using Ultra Vision Protein Block for 5 min. Monoclonal mouse anti-CD10 antibody (Thermo Fisher Scientific) was diluted at 1:2000 in Antibody Diluent (Dako, Glostrup, Denmark) and incubated on slides for 45 min at RT (room temperature). Slides were washed and detection achieved by incubation with the anti-mouse/rabbit UltraVision HRP-labelled polymer system (15 min) and 3-amino-9-ethylcarbacole (AEC, Thermo Fisher Scientific), according to the manufacturer's instructions.

Nuclei were stained with Haemalaun solution (Sigma, St. Lois, MO, USA) and slides were mounted with aqueous mounting agent Aquatex (Merk Millipore, Darmstadt, Germany). For negative controls, slides were incubated with the same concentration of unspecific mouse IgG1 (Dako) as the primary antibody. Images were acquired using a Zeiss Axiophot microscope equipped with an AxioCamHRc digital camera.

4.4. Immunocytochemistry

Immunocytochemistry for quality control of fpEC and for MME was performed according to the same protocol. Cells (100,000 cells per 1.7 cm^2 chamber) were grown on chamber slides for 48 h, washed with HBSS, and fixed with ice-cold acetone (Merck, Darmstadt, Germany) for 3 min. Slides were rehydrated in Tris-borate EDTA (TBE) pH 7.5 with 0.1% Tween (Sigma) for 3 min, which was also used as a washing puffer. Non-specific binding sites were blocked with UltraVision Protein Block for 10 min. Subsequently, the primary antibody for endothelial cell markers VWF (anti-VWF A0082, Dako; 1:3000) and CD31 (anti-CD31 MON6002-1, clone EN4, Monosan, Uden, the Netherlands; 1:300), smooth muscle cell marker SMA (anti-SMA M0851, clone 1A4, Dako, 1:200), fibroblast marker CD90 (anti-CD90, DIA100, clone AS02, Dianova, Hamburg, Germany; 1:200), and MME (anti-CD10; Thermo Fisher Scientific; 1:100) diluted in Dako antibody diluent was applied for 30 min. Negative controls were of the same isotype and in the same dilution (Dako). After three washings, slides were incubated with HRP polymer for 15 min in the dark and washed again. Then, chromogenic reaction was started by addition of peroxidase-compatible chromogen (Thermo Fisher Scientific) for 5 min. After washing in distilled water, nuclei were stained with hematoxylin and mounted with Aquatex.

4.5. Quantitative Reverse Transcription PCR (RT-qPCR)

Total RNA was isolated using the miRNeasy mini kit (Qiagen, Hilden, Germany). The quality and integrity of the RNA was determined by the ratio of spectrophotometric absorbance 260 nm/280 nm measured with the Scandrop 250 (Analytik Jena AG, Jena, Germany). Complementary DNA was transcribed from 1 µg of total RNA using miScript II RT kit and 5× miScript HiFlex Buffer (Qiagen) according to the manufacturer's instructions.

For the quantification of *MME* mRNA in primary fpEC, 3 ng/µL of cDNA was used per reaction in a total reaction volume of 20 µL in a CFX96 cycler (BioRad, Hercules, CA, USA). RT-qPCR for *MME* was performed using the TaqMan assay Hs00155310_m1 (Applied Biosystems, CA, USA) for *MME*, and Hs02800695_m1 and Hs00265497_m1 (hypoxanthine-guanine phosphoribosyltransferase (*HPRT1*) and ribosomal protein L30 (*RPL30*), respectively) were used as housekeeping genes. For the quantification of *MME* mRNA in different human organs, 40 ng of cDNA was used, which was transcribed from fpEC and purchased RNA from human tissues (Clonetech; Thermo Fisher Scientific). For the comparison of gene expression between different organs, the housekeeping genes *HPRT1* (Hs02800695_m1) and peptidylprolyl isomerase A (*PPIA*, Hs04194521_s1) were used [56]. Mean expression of the housekeeping genes was used to normalize gene expression with $2^{-\Delta\Delta Ct}$ method.

4.6. Immunoblot

Total cellular protein was extracted with RIPA buffer containing proteinase inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche). Cell lysates (8 µg per lane) were applied to a gradient 4–20% SDS-PAGE, and transferred to 0.2 µm nitrocellulose membranes (Trans-Blot Turbo Mini Nitrocellulose Transfer Membrane, BioRad) using the Trans-Blot Turbo Transfer System (BioRad). After transfer, membranes were incubated with Ponceau S solution (Sigma), which stained all transferred proteins. The membranes were photographed for protein normalization later. For detection of MME, mouse monoclonal anti-CD10 antibody (clone SN5c, Abcam, CA, USA) was used at a 1:1000 dilution. The secondary antibody was HRP-conjugated goat anti-mouse antibody (1:1000; R&D Systems). Signals were detected using the SuperSignal West Pico (Pierce, Thermo Fisher Scientific). For control of antibody detection, membranes were incubated with mouse monoclonal anti-β-actin (1:20,000; clone

AC-15, Abcam) followed by incubation with the HRP-conjugated secondary antibody (1:20,000). MME signals were detected at 85 kDa and normalized to the Ponceau S-stained proteins in the molecular range between 40 and 100 kDa. Normalization of signals was performed with the signal intensity and was calculated by DigiDoc 1000 software.

4.7. Enzyme-Linked Immunosorbent Assay (ELISA)

For the collection of supernatants, fpEC were seeded (15,000 cells/cm^2) in T25 flasks and cultured for 48 h. Then, supernatants were centrifuged at 3000 rpm for 10 min and frozen at −80 °C until the experiment. Absorbance of medium without cells incubated the same way was substracted from MME levels measured in the conditioned medium. Cord blood serum was obtained as described above. The human Neprilysin (MME) ELISA kit (Abnova, Taoyvan City, Taiwan) was performed according to the manufacturer's instructions. A total of 100 µL of cell culture supernatants diluted at 1:4 and 100 µL of cord blood serum were applied. Optical density was determined at 450 nm using a spectrophotometer (SPECTRO Analytical Instruments, Kleve, Germany).

4.8. Hypoxia and TNFα Treatments

To test the effect of hypoxia or TNFα on MME protein expression, fpEC (n = 7 different cell isolations, in duplicates) were seeded in gelatin coated 6-well plates (200,000 cells/well). Then, culture plates were placed at different oxygen concentrations (21%, 12%, and 5%) for 48 h. For TNFα treatment, after overnight culture, cells were treated with TNFα (5 and 50 ng/mL, Reliatech, Wolfenbüttel, Germany) for 24 h. Cells without TNFα treatment served as the control. Protein was extracted with RIPA buffer containing proteinase inhibitors (complete), and immunoblotting for MME was performed as described above.

4.9. Statistical Analysis

Data were analyzed using GraphPad Prism software Version 5.01 (GraphPad Software, Inc). After testing for normal distribution (Kolmogorov–Smirnov test), Student's t-test and Pearson's test were applied to detect differences between the control and overweight samples and correlations, respectively. p-values below 0.05 were considered statistically significant.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/21/3/834/s1.

Author Contributions: Conceptualization, U.H.; methodology, E.W., H.M.B., W.T.B., J.S., B.H., V.S., C.T.-A.; formal analysis, E.W., S.C.; investigation, E.W., S.C.; writing—original draft preparation, U.H., E.W.; writing—review and editing, E.W., S.C., U.H. All authors have read and agreed to the published version of the manuscript.

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

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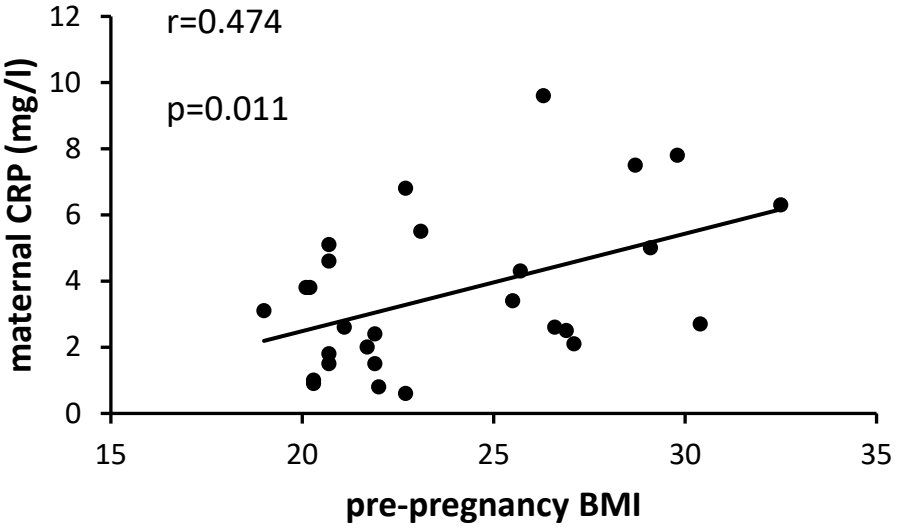

Figure S1. Maternal CRP at delivery correlates with maternal pre-pregnancy BMI (N=28).

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Transcriptomic Remodelling of Fetal Endothelial Cells During Establishment of Inflammatory Memory

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Inflammatory memory involves the molecular and cellular 'reprogramming' of innate immune cells following exogenous stimuli, leading to non-specific protection against subsequent pathogen exposure. This phenomenon has now also been described in non-hematopoietic cells, such as human fetal and adult endothelial cells. In this study we mapped the cell-specific DNA methylation profile and the transcriptomic remodelling during the establishment of inflammatory memory in two distinct fetal endothelial cell types – a progenitor cell (ECFC) and a differentiated cell (HUVEC) population. We show that both cell types have a core transcriptional response to an initial exposure to a viral-like ligand, Poly(I:C), characterised by interferon responsive genes. There was also an ECFC specific response, marked by the transcription factor ELF1, suggesting a non-canonical viral response pathway in progenitor endothelial cells. Next, we show that both ECFCs and HUVECs establish memory in response to an initial viral exposure, resulting in an altered subsequent response to lipopolysaccharide. While the capacity to train or tolerize the induction of specific sets of genes was similar between the two cell types, the progenitor ECFCs show a higher capacity to establish memory. Among tolerized cellular pathways are those involved in endothelial barrier establishment and leukocyte migration, both important for regulating systemic immune-endothelial cell interactions. These findings suggest that the capacity for inflammatory memory may be a common trait across different endothelial cell types but also indicate that the specific downstream targets may vary by developmental stage.

Keywords: endothelial progenitor cell, endothelial cells, inflammation, trained immunity, inflammatory memory, innate immune memory, HUVEC (human umbilical vein endothelial cells), transcriptome (RNA-seq)

INTRODUCTION

Immune memory is traditionally associated with adaptive immunity, mediated by specific antibody producing lymphocytes (1). This enables enhanced immune responses upon re-infection with a pathogen (2). In contrast to the adaptive system, innate immunity is traditionally described as the rapid, non-specific response to pathogens, involving the engagement of neutrophils, natural killer cells, monocytes, macrophages, the complement system and cytokines (1), in the absence of any capacity for memory. This dogma, ascribing memory solely to the adaptive immune compartment, has now been challenged, as an unequivocal body of literature has confirmed the memory capacity of specific cells within the innate immune system (3). A heightened response, relative to an initial stimulation, is often termed 'trained immunity' while a dampened or attenuated response is referred to as 'tolerance', phenomena that are on opposing ends of 'inflammatory memory' (4). Endotoxin tolerance is observed in sepsis patients (5), where an initial hyper-inflammatory response leads to a transient unresponsive state, usually characterised as inability to release proinflammatory cytokines (6). Trained immunity, on the other hand, is associated with stronger release of proinflammatory cytokines and has been associated with the off-target effects of the BCG vaccine (7) and is observed in monocytes in chronic conditions, such as obesity (8). Both phenomena are specified by epigenetic and metabolic remodelling upon initial microbial exposure, resulting in altered capacity for transcription of genes on subsequent challenge (9).

It has become clear that endothelial cells (ECs) play a key function in innate immune responses (10), becoming activated by inflammatory signals in association with increased capacity for cytokine production (11), cell permeability, leukocyte adhesion and transendothelial migration, as well as pro-coagulant features (12, 13). A growing body of evidence suggests that ECs are also capable of presenting antigens *via* MHC class II molecules (14). Thus, the immune system depends on the vascular response triggered by ECs (12). As with innate immune cells, recent data have revealed that ECs possess the capacity to establish inflammatory memory (15). Hence, modulation of endothelial immune response by immune memory effects may enable ECs to sense and respond more effectively, potentially to improve transendothelial guidance of immune cells to a site of inflammation (11). However, disruption of this process may also increase the susceptibility to develop endothelial dysfunction and chronic inflammation.

Initial exposure of murine adult ECs to pathogens increases leukocyte adherence following a subsequent challenge (16). Similarly, human adult aortic ECs exposed to oxidized low density lipoprotein (oxLDL) show a trained immune response following a second stimulation (15). In fetal ECs, such as HUVECs, an initial lipopolysaccharide (LPS) exposure leads to tolerance in response to a second LPS hit, while an exposure to the viral mimic Poly(I:C) induces a trained phenotype, and stronger cytokine (IL6 and CXCL10) response to a second LPS hit (17, 18). As ECs are highly heterogeneous in terms of the vascular bed from which they are derived (arterial *vs* venous, macro- *vs* microvascular), their developmental stage, and response to inflammatory stimuli, it remains unclear how generalisable the phenomenon of inflammatory memory is in ECs. We, and others, have also shown that endothelial cell heterogeneity is associated with epigenetic variation, including differences in DNA methylation profile (19, 20).

Although not widely studied in ECs it is clear that the capacity for training of innate immune cells also involves distinct epigenetic processes, such as active histone marks, open chromatin, and DNA methylation (21–24). Here, we hypothesised that the capacity for inflammatory memory is widespread in ECs of different tissue origin and developmental stage and is underpinned, at least in part, by common downstream molecular changes. In order to test this, we measured the capacity of fetal progenitor ECs (endothelial colony forming cells; ECFC) (25, 26) and more differentiated human umbilical vein ECs (HUVEC) (17, 18, 27) to establish inflammatory memory in response to a viral TLR3 ligand and characterised the transcriptional and DNA methylation profiles associated with this process in both cell types.

MATERIALS AND METHODS

Isolation of HUVECs and ECFCs

HUVECs were extracted from umbilical cords, which were collected at delivery as part of the Peri/Postnatal Epigenetic Twins Study (PETS) (28). Briefly, type 2 collagenase (1 mg/mL, Worthington Biochemical Corporation, Lakewood, NJ, USA) was used to detach endothelial cells which were then further purified using CD31 MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Explant cultures were established and viably frozen in Fetal Calf Serum (FCS) with 10% DMSO at passage 2-4. Fetal ECFC isolation was approved by the ethics committee of the Medical University of Graz, Austria, (29-319 ex 16/17) and written informed consent was obtained. ECFCs were isolated from umbilical cord blood *via* density gradient centrifugation and characterized as described (29). Samples were frozen in culture media with 20% FCS and 10% DMSO at passage 3. All primary lines used in this study are from healthy term pregnancies of women in normal weight range without any pregnancy complications. Exclusion criteria were overweight/obesity, diabetes, hypercholesterolemia, and acute/chronic diseases. Placental and fetal weight and height are in normal range, revealing no sign of any inflammatory disease.

Inflammatory Memory Model

Endothelial cells were passaged at 70-90% confluency and seeded in 6-well plates coated with 1% gelatine at a density of 1.5-2.5 x10^5 (Nunclon Delta, Thermofisher). Cells were then left to attach for 24h at 37°C, 21% O_2, 5% CO_2 in EGM-2 media (Lonza, Basel, Switzerland). Following attachment, cells were stimulated with Poly(I:C) (10 μg/mL, Sigma, Burlington, MA, USA) or media only for 24h. Poly(I:C) was removed after 24h, replaced

with EGM-2 only, and cells were left to rest for a further 24h. Cells were then re-stimulated with 100 ng/mL LPS (Sigma) for 4h (**Figure 2A**). Endothelial cells in 6-well culture plates were collected at T0, 4h, 24h, 48h, and 52h in 500 µL RLT+β-mercaptoethanol (Qiagen, Venlo, Netherlands). All experiments were performed in duplicate, using cells between passages 4-6.

RNA Collection and Sequencing

Total RNA was extracted from cells using the RNeasy RNA extraction kit (Qiagen) with on-column DNaseI treatment. RNA quality (RIN) scores were determined using the RNA Tapestation system (Agilent). Libraries were prepared by the Victorian Clinical Genetic Services (VCGS) Sequencing Service (Melbourne, Australia) using the TruSeq stranded mRNA kit (Illumina). Libraries were sequenced on the NovaSeq 6000 (Illumina) at ~20 M reads per sample, using 2x150 bp reads.

cDNA Synthesis and Quantitative Reverse Transcription PCR

cDNA was synthesised using the Tetro cDNA Synthesis Kit (Meridian Bioiscience) as per the manufacturer's protocol. Gene expression levels were analysed by quantitative real-time PCR using LightCycler 480 (Lifesciences, Roche). cDNA was amplified using primers ordered from Integrated DNA Technologies (IDT), reconstituted to 100 µM as per the manufacturer's instructions, then diluted to a 10 µM working solution (20µL Forward primer, 20 µL Reverse primer, 160 µL Nuclease free H2O) for IL-6 ([F-AAAGAGGCACTGGCAGAAAA] [R-AGCTCTGGCTTGTTCCTCAC]), MX1 [F-GTGCATTGCAGAAGGTCAGA] [R-GGATGATCAAAGGGATGTGG], CCL2 [CCCCAGTCACCTGCTGTTAT] (F) [TGGAATCCTGAACCCACTTC] (R), and GAPDH ([F-TTCGACAGTCAGCCGCATCTT] [R-CCCAATACGACCAAATCCGTT]). qPCR mixtures were prepared in 10 µL reactions comprised of 5 µL of SensiFast SYBR Green I mix (BIO-98050) (Meridian Bioscience/BIOLINE), 1 µL of combined forward and reverse primers (1 µM final concentration), 2 µL of nuclease-free H_2O, and 2 µL of diluted cDNA template (diluted 1:10 with nuclease-free H_2O). Samples and no template controls (NTCs) were amplified in triplicate, and HUVEC and ECFC expression levels were determined using the ΔΔCt method, with *GAPDH* as the control gene.

RNA Sequencing Analysis

To infer gene expression levels, RNA-seq reads were aligned to hg19 human transcriptome using Bowtie (30). Quantification of gene expression was performed using MMSEQ (31). Counts per gene were normalised using DESeq2, while RPKM values were used for plotting gene expression in heatmaps and line graphs. DESeq2 was used to identify differentially expressed genes (DEGs) using logistical regression (32), with p value <0.05, fold change >2 and RPKM >1 considered significant. Pairwise comparisons were performed over time (time 0, 4 hours, 24 hours, 48 hours and 52 hours) and between stimulations [media *vs* Poly(I:C)]. The gene lists were then merged, and duplicates removed, to understand the time-course dynamics using PCA plots using prcomp in R. For 4h LPS exposure experiments, DEGs were identified as p value <0.05, fold change >2 and RPKM >1. Trained, unaffected and attenuated genes were identified by comparing media+LPS to Poly(I:C)+LPS, with fold change >1.5 higher in Poly(I:C)+LPS designated as trained, fold change >1.5 higher in media+LPS as attenuated, and all other genes assigned as unaffected. Gene ontology and promoter motif enrichment was performed using HOMER (33).

DNA Methylation Analysis

Raw Infinium HumanMethylation.idat files (Illumina, San Diego, CA) were downloaded from the Gene Expression Omnibus (GEO) for healthy HUVECs (GSE103253), ECFCs (GSE180355), placental arterial endothelial cells (AEC) and venous endothelial cells (VEC) (GSE106099) (34, 35). Raw.idat files were processed and analyzed using the MissMethyl and minfi packages for R (36, 37). Samples were checked for quality and those with a mean detection p-value of >0.01 were removed. Data were normalized for both within and between array technical variation using SWAN (Subset-quantile Within Array Normalization) (38). Probes with poor average quality scores (detection p-value > 0.01), those associated with SNPs (MAF > 0%) and cross-reactive probes (39) were removed from further analysis. Differential methylation analysis by linear regression modelling was performed using limma (40). DMPs were assigned to the nearest gene within 1Mb using the GREAT tool (41).

Data Availability Statement

The data sets generated and analyzed for the current study are deposited in the Gene Expression Omnibus repository with the accession number GSE180881.

RESULTS

Fetal Endothelial Cells of Different Origins Display Distinct Immune-Related Genome-Wide DNA Methylation Patterns

To test our hypothesis, we first mapped the epigenetic and transcriptional landscape of immune-related genes in a range of ECs at rest (unstimulated; **Figure 1**). Using published genome-wide methylation data from HUVECs (n=80), cord blood-derived ECFCs (n=60), placental arterial ECs (AECs) (n=15) and placental venous ECs (VECs) (n=15), we extracted methylation data for 15,072 probes within 100kb of genes related to the KEGG section '5.1 - Immune system' (**Figure 1A**). These probes were chosen because we wanted to specifically focus on DNA methylation differences that may influence inflammatory gene expression responses in cis. Principal component analysis (PCA) clustering according to methylation data of these probes clearly separated the four cell types, HUVECs from the rest (PC1) and ECFCs from the rest (PC2) (**Figure 1B**). Clustering of ECFCs and HUVECs alone shows a strong signature (**Figure 1C**), which we postulated will result in differing responses to microbial ligands and capacity for

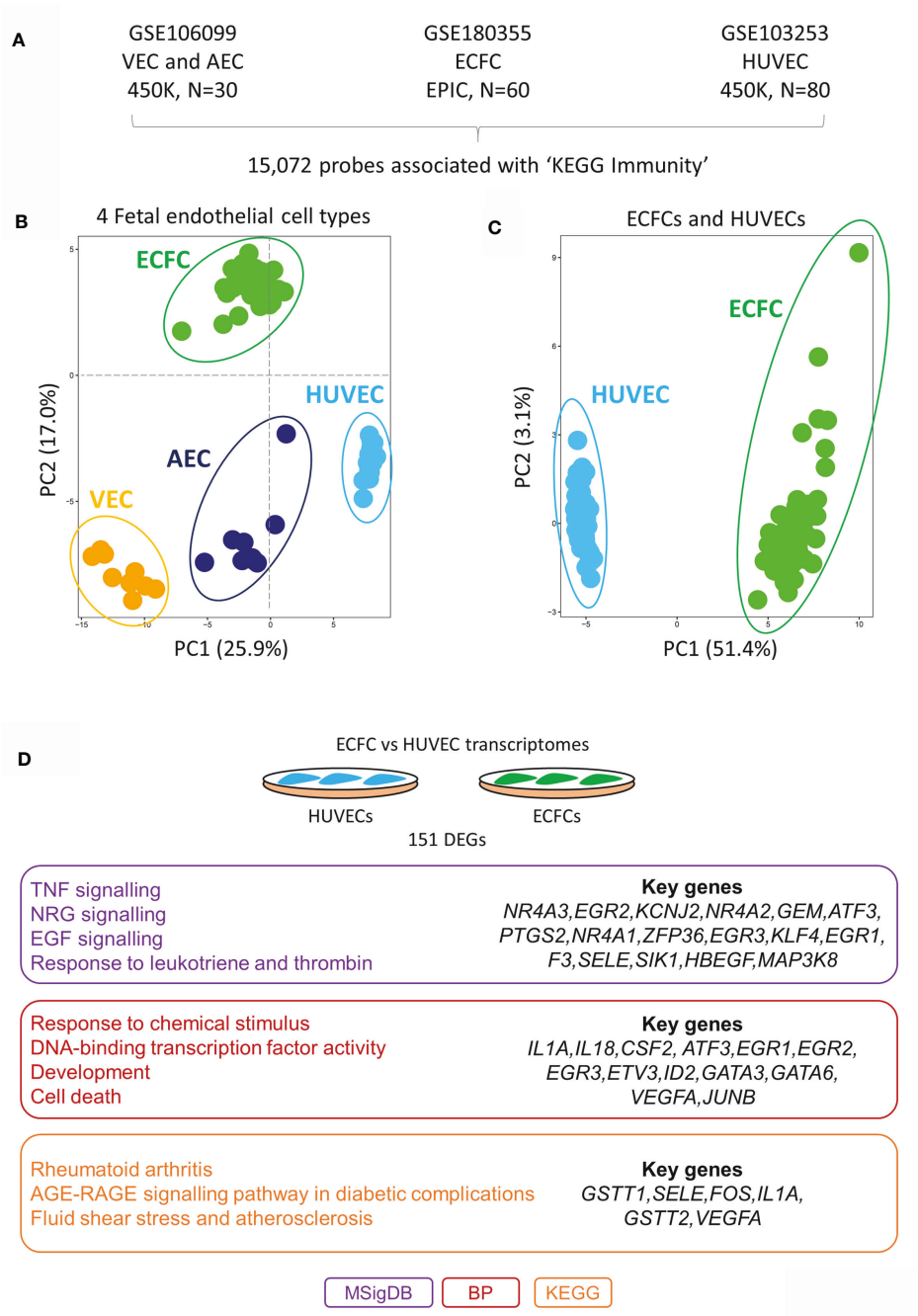

FIGURE 1 | DNA methylation and transcriptomic profiles of immunity-related genes in different placental endothelial sub-types. **(A)** Immunity-related probes were extracted from publicly available Infinium HumaMethylation datasets for VEC (450K platform), AEC (450K platform), ECFC (EPIC platform) and HUVECs (450K platform). **(B)** PCA plot of immune-related probes at rest. HUVECs are separated *via* PC1 and ECFCs *via* PC2 from the other fetal endothelial cell types. **(C)** PCA plot of immune-related genes at rest in ECFCs and HUVECs. **(D)** Differentially expressed genes between ECFCs and HUVECs at baseline. Gene ontology terms associated with the differentially expressed genes. MSigDB, GSEA Molecular Signatures Database; BP, Gene Ontology Biological Process; KEGG, Kyoto Encyclopedia of Genes and Genomes.

inflammatory memory. Next, we compared the baseline transcriptomes of HUVECs and ECFCs, revealing a total of 151 genes with differential expression (DEGs) (**Figure 1D** and **Table S1**). Amongst the differentially expressed genes were those involved in inflammatory response, EGF signalling, transcriptional regulation, and response to environment (**Figure 1D**). These epigenetic and transcriptional signatures suggest that the two cell types may mount a distinct transcriptional inflammatory response.

Poly(I:C) Induces Large-Scale Transcriptional Remodeling in Fetal Endothelial Cells

The viral dsRNA mimic, Poly(I:C), was chosen as the acute stimulus due to its ability to induce inflammatory memory in HUVECs (17, 18). Each of ECFCs (n=2) and HUVECs (n=2) were exposed to Poly(I:C) in culture for 24 hours with RNA isolation and sequencing (RNA-seq) at baseline (T0), 4 hours (4h), and 24 hours (24h) (**Figure 2A**). We performed pairwise comparisons across time for each exposure [e.g. ECFC 4h Poly(I:C) *vs* ECFC 24h Poly(I:C)], and between exposures at matched time-points (e.g. HUVEC 4h media *vs* HUVEC 4h Poly(I:C)). A total of 4,390 protein-coding genes were differentially expressed in our model across all comparisons (FC >2, p <0.05), which we visualised using a PCA plot, with the Poly(I:C) response being highest at 24h post exposure (PC1; **Figure 2B**). This Poly(I:C) response trajectory was similar between the two cell types, even if the cell-type specific transcriptional profiles persisted (PC2; **Figure 2B**).

In order to determine the trajectory of Poly(I:C) induced genes following the removal of the stimulus, we profiled the 4,390 dynamic genes at the 48h time-point, 24h after the removal of Poly(I:C) (**Figure S1**). We separated Poly(I:C) induced genes into 4 quartiles (Q1 to Q4) based on their expression at the 48h time-point. Q1 genes showed the most persistent elevated expression at 48 hours, while Q4 genes returned to basal levels (**Figure S1A**). We confirmed that this was a direct result of Poly(I:C) exposure, and not simply a persistence of low levels of Poly(I:C) remaining after media replacement (**Figure S2**). Promoters of genes showing persistent expression were enriched for the interferon stimulated response element (ISRE) and viral response pathways. The viral response gene, *MX1*, was the top gene in the persistent expression (Q1) quartile in both ECFCs and HUVECs, while *TNF* expression returned to baseline by 48h (Q4) (**Figure S1B**). There was no clear promoter-associated TF signature in genes that returned to baseline, with no motif reaching a p-value or fold change threshold relative to background (**Figure S1C**).

ECFCs and HUVECs Show Distinct Transcriptional Responses to Poly(I:C)

In total, 1,327 genes were specifically upregulated following 24h of Poly(I:C) exposure in either ECFCs or HUVECs (**Figure 2C** and **Table S2**). Of these, 1,039 were induced in both cell types ('equally responsive'), indicating that the two endothelial cell types share common inflammatory pathways (**Figure 2C**). These genes are involved in cytokine receptor signalling and TNF signalling pathways (**Figure 2D**) and their promoters were enriched for viral transcription factor motifs, such as the ISRE, IRF1-3, and NFKB (**Figure 2E**). This motif signature was particularly strong at genes that were slightly more responsive in HUVECs (bottom half of heatmap, **Figure 2C**). Expression of genes coding for transcription factors associated with the motif signature was higher in HUVECs than ECFCs (**Figure S3**). There was also an ECFC-specific gene signature, containing 288 genes that were non-responsive in HUVECs at 24h Poly(I:C) exposure (**Figure 2C**). This set of genes was enriched for 'oxidative phosphorylation', 'immunoproteasome' and 'antigen presentation' (**Figure 2D**), and their promoters were marked by motifs recognised by ELF1 (**Figure 2E**). Due to the fundamental role of metabolic remodelling in endotoxin tolerance and trained immunity in macrophages (42, 43), we specifically looked at genes in the 'glycolysis', 'oxidative phosphorylation' and 'metabolism' GO categories (**Table S3**). In total, 4,390 (21.9%) protein coding genes were dynamically expressed in ECFCs or HUVECs during the 48-hour experimental set-up in response to media or Poly(I:C). Metabolic genes overall were not more dynamic (22.1%, 380/1,714 genes), however 29.9% (32/107) of genes involved in oxidative phosphorylation were dynamic (**Table S3**). The same increase was observed for genes responsive to LPS re-stimulation, with 3.4% of all protein coding genes dynamic, while 10.3% of oxidative phosphorylation genes were altered.

Specific Poly(I:C) Responses Are Associated With Minimal DNA Methylation Variation

Next, we tested our hypothesis that the expression difference to the primary Poly(I:C) response between ECFCs and HUVECs (**Figure 2C**) is associated with the underlying DNA methylation differences between the two EC types (**Figure 3**). We restricted this analysis to promoter differential methylated probes (DMPs) to ensure gene-specificity. To do this, we extracted all CpG probes from the gene promoter regions that mapped to within 500bp of the transcriptional start site (TSS) that showed 'ECFC-specific' or 'equally responsive' patterns (**Figure 3A**). Only 1.1% (26/2,335) of probes associated with 'ECFC-specific' genes, and 0.8% (68/8,753) of probes mapping to 'equally responsive' genes showed differential methylation between the two cell types at a stringent 20% absolute cut-off (**Figure 3B**). In each of these rare instances, only a single DMP was present at the promoter. This indicates that the underlying DNA methylation profile within the promoter regions is a poor predictor of response to Poly(I:C). Nevertheless, the top ranked ECFC-specific gene, *KLRD1* (**Figure 2C**), had a single DMP in its promoter region (**Figure 3C**). This gene is inducible by Poly(I:C) in ECFCs only and is not expressed in HUVECs at any time, or in response to stimulation (**Figure 3D**). The DMP is in the promoter region, showing complete hypomethylation in ECFCs and overlapping an ENCODE TF binding site (**Figure 3E**).

Evidence of Inflammatory Memory in HUVECs and ECFCs

Next, we explored the influence of Poly(I:C) primary exposure on a secondary unrelated bacterial, lipopolysaccharide (LPS), transcriptional response (**Figure 4A**). After an initial 24h Poly(I:C) exposure, cells were allowed to rest for another 24h in media alone, after which they were stimulated for 4h with LPS (17, 18). A total of 352 genes showed expression changes induced by LPS in media-ECFCs or Poly(I:C)-ECFCs (**Figures 4B, C** and **Table S4**), and 242 genes were induced by LPS in media-HUVECs or Poly(I:C)-HUVECs (**Figures 4B, D** and **Table S5**). Each EC type displayed examples of both trained (heightened induction) and tolerized

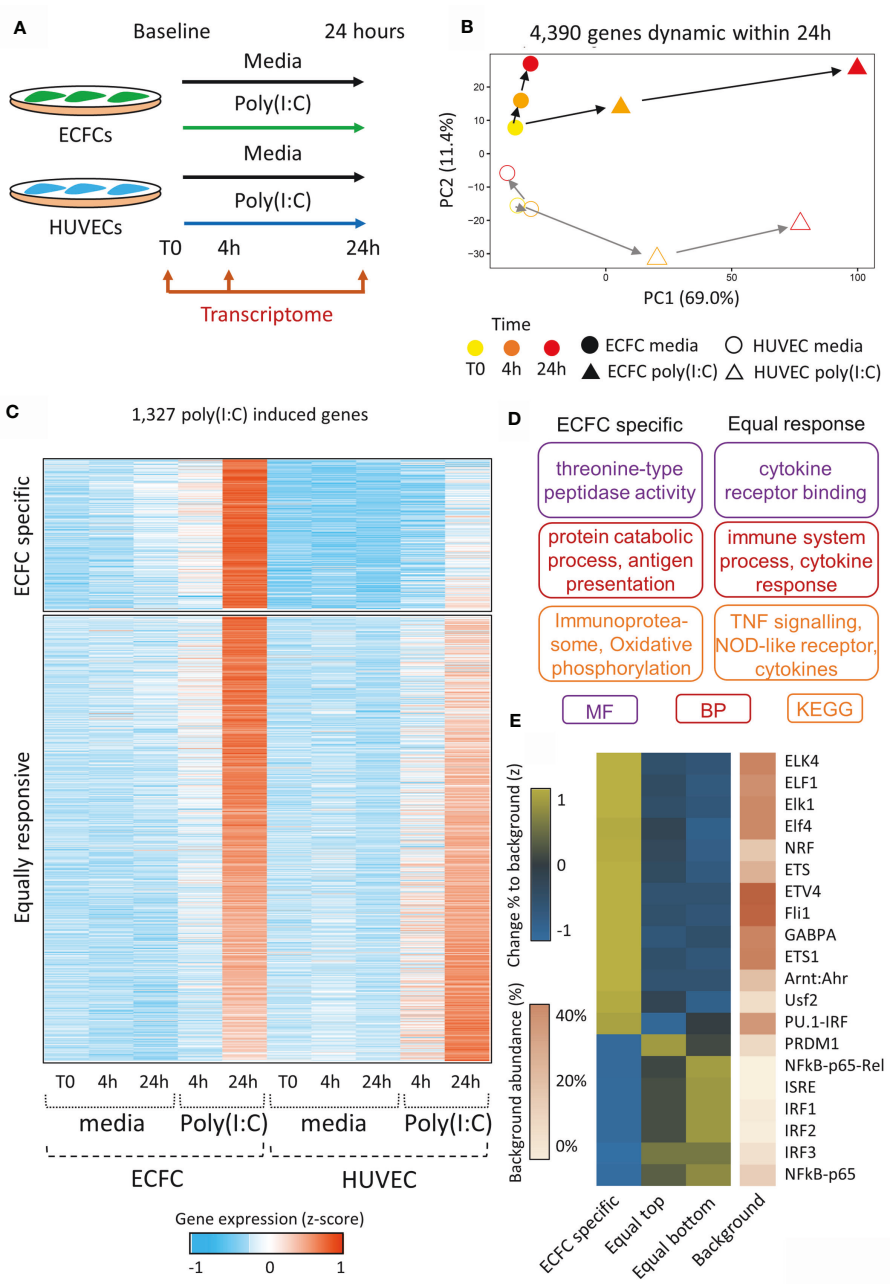

FIGURE 2 | Transcriptional reprogramming of ECFCs and HUVECs in response to viral mimic Poly(I:C). **(A)** Experimental model. **(B)** PCA plot of transcriptomic changes that occur 4 hours and 24 hours following Poly(I:C) exposure. PC1 shows the response to Poly(I:C), which peaks at 24h, while PC2 represents the difference between ECFCs and HUVECs. **(C)** Heatmap showing genes upregulated by Poly(I:C) at 24h. A subset of genes is only induced in ECFCs, while the majority are equally induced in both cell types. **(D)** Gene ontology terms associated with ECFC-specific and equally induced genes. **(E)** Heatmap showing enrichment of transcription factor motifs at promoters of ECFC-specific and equally induced genes.

(reduced induction) gene expression in response to LPS, with HUVECs having more tolerized genes (**Figure 4D** and **Figure S4A**), and ECFCs more trained genes (**Figure 4C**). A total of 163 common genes were induced in both cell types (**Figure 4B**), with a general concordance in the direction of effect on gene expression (**Figures 4C, D**).

In general, the level of induction by the initial Poly(I:C) response determined the LPS response. For example, genes that were trained for LPS response by Poly(I:C) were more strongly induced by Poly(I:C) than those that were tolerized (**Figure 5A** and **Figure S4B**). To understand the underlying pathways involved in inflammatory memory in endothelial cells,

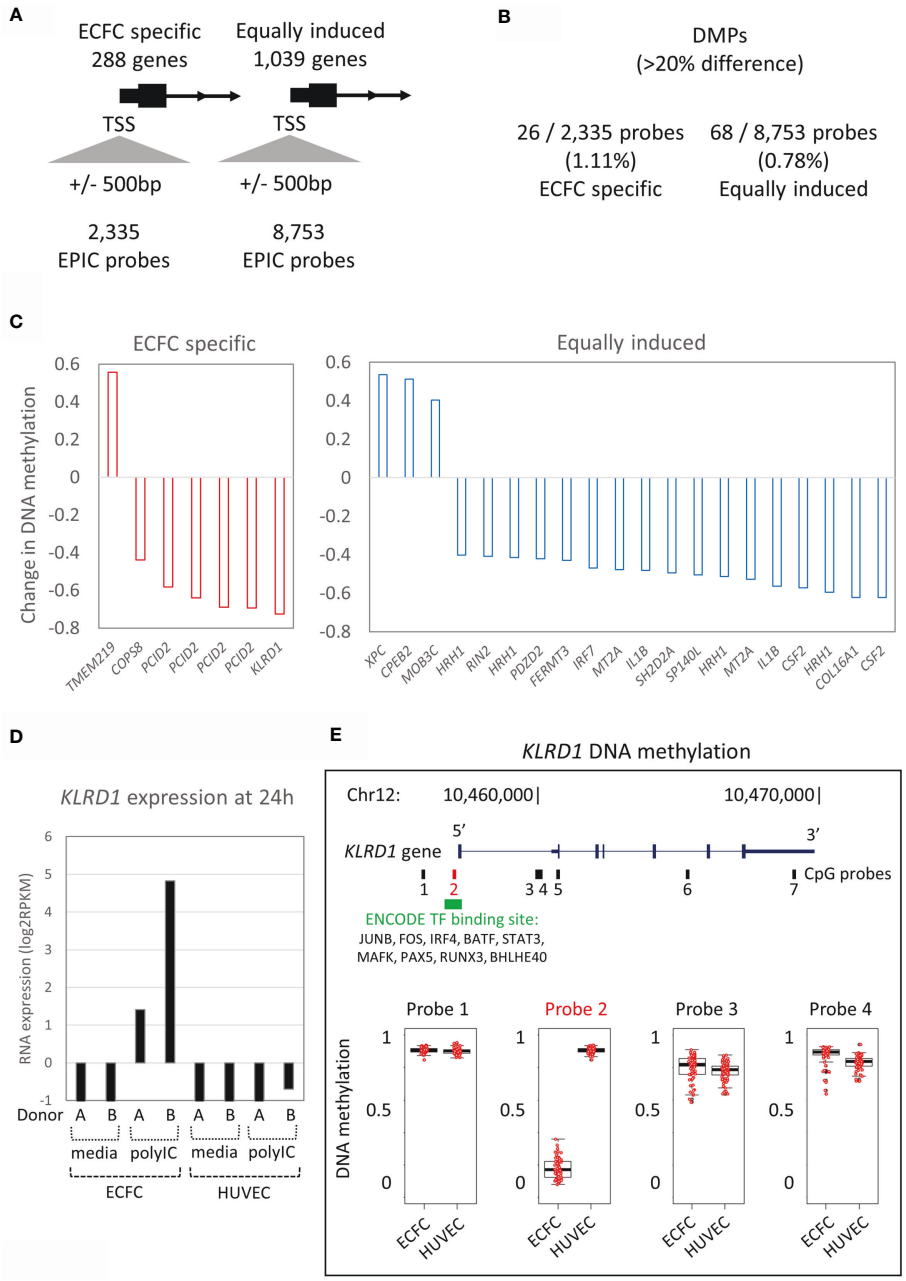

FIGURE 3 | DNA methylation patterns at promoters of Poly(I:C) induced genes. **(A)** The 288 ECFC-specific Poly(I:C) induced genes have 2,335 EPIC probes at their promoters, while the 1,039 equally induced genes have 8,753 EPIC probes. **(B)** Only 1.1% and 0.8% of probes are differentially methylated (DMPs) between ECFCs and HUVECs. **(C)** Bar plot showing mean difference in DNA methylation between ECFCs and HUVECs at top promoter DMPs. **(D)** Bar plot showing expression of *KLRD1* in ECFCs and HUVECs after 24h exposure to media or Poly(I:C). **(E)** Map of the KLRD1 locus, showing EPIC probes and TF binding tracks. Of the 7 EPIC probes, only one is differentially methylated between ECFCs and HUVECs. Probe 2 is present at the transcriptional start site (TSS), overlaps TF binding sites and shows complete hypomethylation in ECFCs, predicting responsiveness.

we performed gene ontology analysis on genes that showed trained, unaffected or tolerized responses to re-stimulation with LPS (**Figure 5B**). Genes involved in inflammation were enriched in all three groups. Interestingly, the attenuated gene set was enriched for leukocyte transendothelial migration, with *SELE*, *ICAM1*, and *VCAM1* genes in the list (**Figure 5C**).

DISCUSSION

Innate immune memory is a relatively novel phenomenon with implications for a range of human inflammatory disorders, infectious disease, and vaccinology (4, 9). The establishment of innate immune memory involves metabolic, epigenetic and

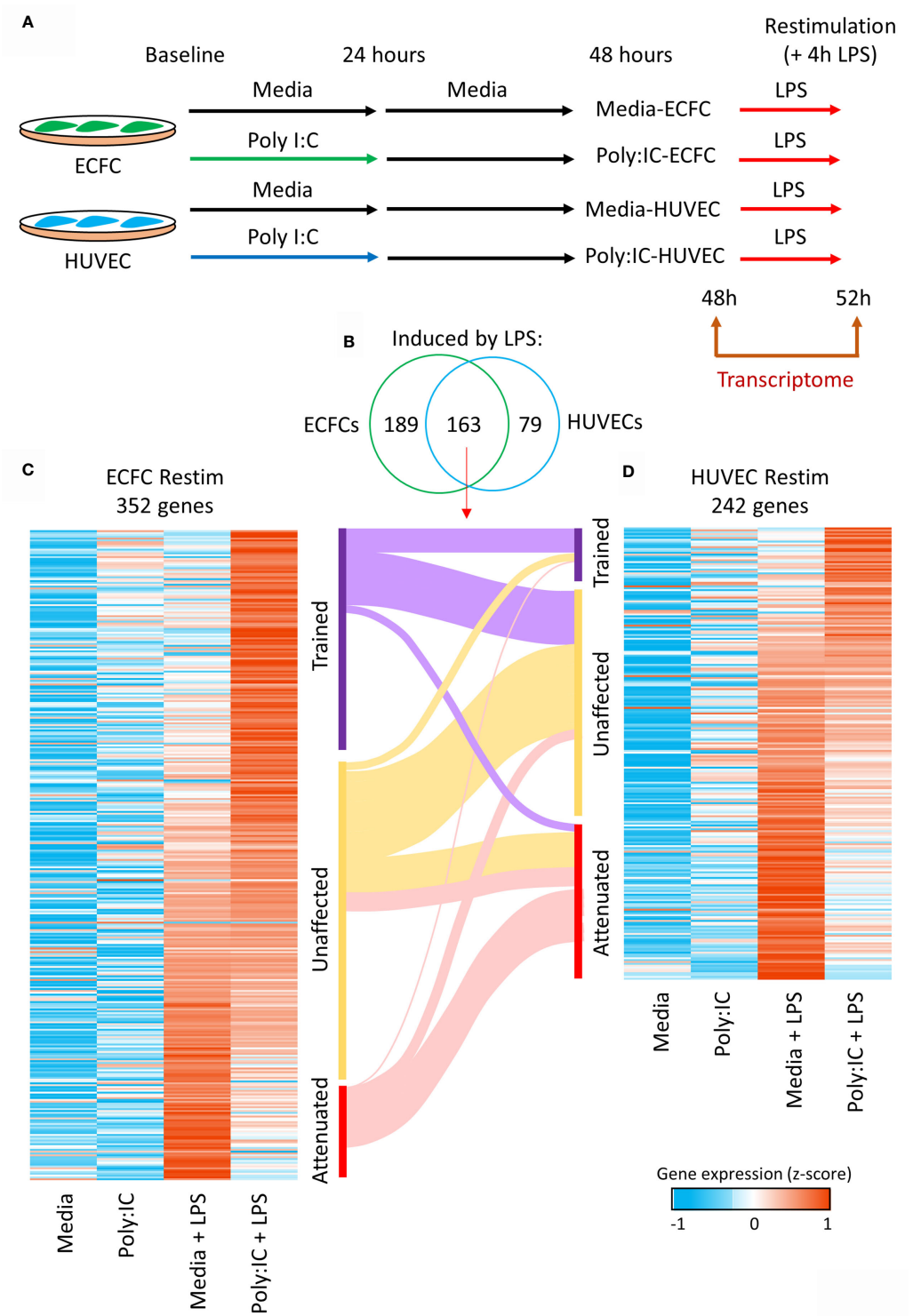

FIGURE 4 | Trained immunity in ECFCs and HUVECs. **(A)** *Ex vivo* model to test Poly(I:C) induced trained immunity in fetal endothelial cells. Cells were exposed to Poly(I:C) for 24h, followed by 24h rest, and re-stimulation with LPS, an unrelated microbial compound. RNA-seq data was generated at 48h and 52h (4h after LPS exposure). **(B)** A total of 352 and 242 genes are induced by LPS in ECFCs and HUVECs, respectively, of which 163 are induced in both. **(C, D)** Heatmap of the LPS inducible genes in ECFCs and HUVECs, ranked from trained to tolerized by Poly(I:C). A Sankey plot shows the overlap between trained, equal and tolerized genes in ECFCs and HUVECs.

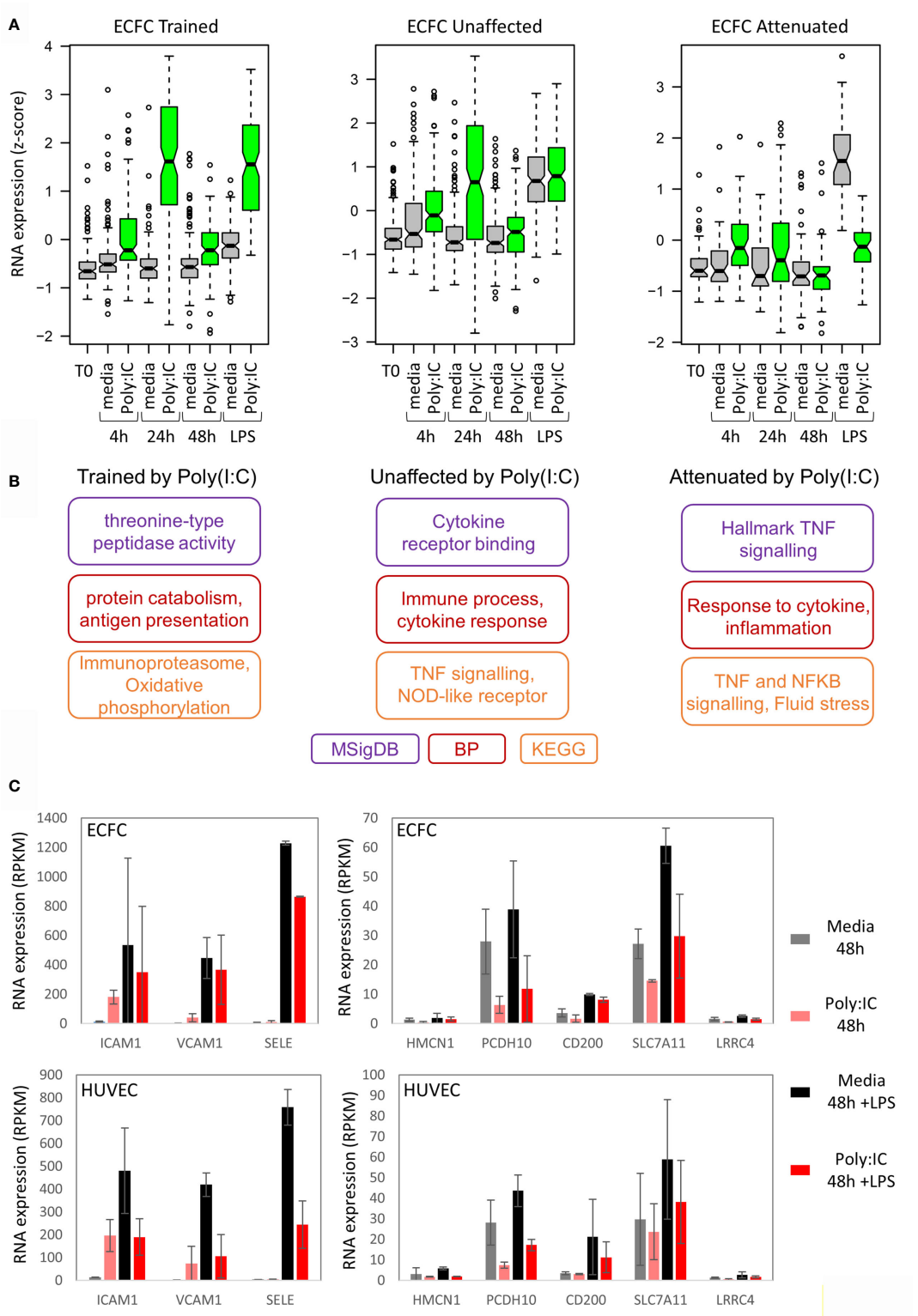

FIGURE 5 | Poly(I:C) tolerizes genes involved in endothelial cell adhesion. **(A)** Expression of trained, unaffected and tolerized genes over time in ECFCs. This indicates that genes trained for LPS response are more strongly induced by Poly(I:C) initially compared to genes that are tolerized for LPS response. **(B)** Gene ontology analysis for each group of genes. **(C)** Expression of cell-adhesion genes that are tolerized for LPS response by Poly(I:C).

transcriptional remodelling, which subsequently influences responses to secondary stimuli (23, 42, 44). This phenomenon is not restricted to cells of the hematopoietic lineage, indicating that memory to microbial compounds, danger signals or injury can be established in a range of cell types (45).

In this study we selected two human fetal endothelial cell types, HUVECs and umbilical cord blood ECFCs, which derive from circulating endothelial progenitor cells, to investigate transcriptome remodelling during development of inflammatory memory. Two biological replicates was based on our previous work on monocyte inflammatory memory (23) and allows us to only detect robust changes in gene expression. However, we did not have enough statistical power to contrast the memory signature in ECFCs to that in HUVECs (**Figure 5**). ECFC are recruited for repair, vascular growth and angiogenesis (46) and their high abundance perinatally (26) suggests a function in postnatal vasculogenesis and angiogenesis. Our data shows that both cell types can mount a transcriptional response to Poly(I:C) exposure (**Figure 2**), which influences the transcriptional output to a second stimulation with LPS (**Figure 4**). Similarly to trained macrophages, genes involved in oxidative phosphorylation were more likely to be transcriptionally dynamic in response to Poly(I:C) or LPS in endothelial cells (**Table S3**). This indicates that metabolic remodelling is a common event in the establishment of inflammatory memory in different cell types.

Due to inexperienced adaptive immunity, innate immunity is critical for neonatal survival. The fact that stimulation of TLR3 with agonists induces inflammatory gene expression and interferon production in human adult and fetal endothelial cells highlights a common role of fetal and adult endothelial cells in innate immunity (17). Our study specifically identified upregulation of genes encoding interferon-inducible proteins (e.g. *MX1*, *MX2*) and interferon inducible transmembrane protein (e.g. *IFITM1*, *IFITM2*) which are known anti-viral genes. Particularly, interferon-inducible transmembrane (IFITM) proteins inhibit viral entry, transcriptional processes and protein synthesis (47). This was shown in both HUVECs (fetal ECs) and adult endothelial cells (48). The TLR3-mediated expression of antiviral genes in HUVEC and ECFC suggests that neonatal endothelial cells contribute to the vital/essential innate immunity of the newborn.

The participation of ECs in immune response is linked to the development of endothelial dysfunction and cardiovascular disease (CVD). For instance, anti-inflammatory cytokines can inhibit endothelial dysfunction (49) whilst endothelial dysfunction represents a shift towards a pro-inflammatory state, inducing CVD (50). Early development is particularly sensitive towards programming events. In fact, environmental influences and maternal pathologies in pregnancy affect long-term health and disease of the offspring in later life (51). Moreover, inflammatory situations such as infections in early life could increase the risk of CVD in adulthood (52). The fact that the immune response of fetal ECs can be programmed by exposure to pyrogens suggests that endothelial inflammatory memory *in utero* may participate in this programming event.

Environmental influences of various kinds alter the epigenome of cells, leading to memory effects and programming. In fact, primary ECs are modified by, for instance, inflammatory environment and hyperglycemia *in vitro* (53) and *in vivo* (34). Moreover, programming effects have not only been observed in mature ECs, but also in cord blood ECFCs (54). Thus, when using primary cells in the field of cell engineering, epigenetic and transcriptional adaptations to the donors' environment are to be expected. These adaptations cannot be avoided but can probably be minimized by using cells from donors that do not suffer from active infections as well as chronic diseases. The advantage of using cord blood ECFCs for cell therapy is also highlighted by a study that showed a reduced pro-inflammatory signature in cord blood ECFCs compared to adult ECFCs (55). In addition to transcriptomics, future studies should explore the differences between cord blood and adult ECFCs at the level of epigenetics and metabolomics, as all of these are known to influence inflammation and trained immunity in general (42).

In support of the theory that the heterogeneous functions of ECs are specified by underlying molecular differences, we confirmed that ECFCs and HUVECs represent two epigenetically distinct fetal endothelial cell populations (**Figure 1**). Further, the differential response of these cells to stimuli [Poly(I:C)] was also characterised by differential gene expression patterns (**Figure 2**). Interestingly, Poly(I:C) induced the expression of 288 genes exclusively in ECFCs (**Figure 2**), which were related 'oxidative phosphorylation', 'immunoproteosome' and 'antigen presentation'. Threonine-type peptidases are central components of the immunoproteasome. It is still controversial whether ECs are capable of antigen presentation. However, the fact that in microvasculature and small vessels, in addition to MHC class I antigens, ECs express MHC class II antigens *in vivo* points to this ability (56, 57). *In vitro*, endothelial MHC expression diminishes and requires cytokine stimulation to recover, and cultured ECs pretreated with interferon gamma (IFN-γ) to express MHC II, activate CD4+ central memory (T_{CM}) and effector memory (T_{EM}) T-cells (58). This ECFC-specific signature was marked by enrichment for ELF promoter motifs, as opposed to the IRF signature in the equally induced genes (**Figure 2E**). ELF1 is an ETS transcription factor that regulates a viral response that is distinct from interferon (59), suggesting that this pathway is active in ECFCs, but not HUVECs.

Only a small proportion of observed transcriptional responses in ECFC *vs* HUVEC could be explained by differences in baseline DNA methylation profile, suggesting that other molecular mechanisms, such as histone modifications, may underlie their distinct immunological phenotype and function. Histone modification have a shorter half-life than DNA methylation and are more responsive to acute stimuli (44). In particular histone 3 lysine 4 tri-methylation (H3K4me3) is enriched at promoters of pro-inflammatory cytokines in trained monocytes (43). Therefore, future studies should explore genome-wide histone modifications to identify regulatory elements that control inflammatory transcriptional responses in fetal ECs.

Nor can the differences be explained by differences in TLR3 and TLR4 expression levels, which are expressed at similar levels in the two cell types. Interestingly, one CpG site in the promoter for *KLRD1* (killer cell lectin like receptor D1) was completely hypomethylated in ECFC, enabling the induction of *KLRD1* gene expression, whilst *KLRD1* transcripts were virtually absent in HUVECs before or after Poly(I:C) exposure. Interestingly, *KLRD1* (CD94) is reported to encode an NK cell-specific receptor, though some evidence of non-NK expression also exists, including ECs (Human protein atlas). Binding of HLA-E to KLRD1 prevents the cytotoxic activity of NK cells (60). The function of KLRD1 in ECFC however, remains topic of further studies.

Inflammatory memory in HUVECs has recently been reported at the level of cytokine release (17, 18). Our data demonstrates that both ECFCs and HUVECs can establish transcriptional inflammatory memory, with some differences in terms of degree of memory and the number of genes that are trained or tolerized (**Figure 4**). In general, there was concordance in direction with genes trained in one cell type likely to be trained in the other (**Figures 4C, D**). ECFCs induced a higher number of genes after LPS re-exposure, which is in line with these cells being microvascular ECs (61). For instance, lung microvascular endothelial cell produce larger amounts of cytokines upon stimulation (62) and L-selectin dependent adhesion of leukocytes occurring on microvascular but not macrovascular ECs of the human coronary system (63).

An interesting pathway that was tolerized in both ECFCs and HUVECs was leukocyte transendothelial migration (**Figure 5C**). Genes that were tolerized in both cell types in this pathway include *SELE*, *ICAM1* and *VCAM1*, which code for adhesion molecules and are induced by LPS in pericytes, a cell type that interacts with ECs (64). This finding suggests that the initial Poly(I:C) exposure induced genes necessary for the recruitment of phagocytes in the circulation, and that the subsequent tolerization of these genes is required to prevent excessive recruitment and inflammation. This is of course speculative and requires functional validation in co-culture experiments or *in vivo*.

It is important to note that culturing induces epigenetic and transcriptional changes in endothelial and other cell types, and that the media used for both cell types contains growth factors that partially activate the cells. As a result, ECFCs and HUVECs in culture may not represent the *in vivo* condition. Nevertheless, the different initial response to Poly(I:C) indicates that cell-intrinsic responses remain. Further, our model has a 24 hour rest period, which is similar to that used to study innate immune tolerance (5, 65), but not as long as that used to study trained immunity (66). As described in Koch et al. (17), there will still be some circulating cytokines in the supernatant after the 24 hour rest period, which would have a polarising effect on the cells. To address this, in our analysis we removed genes that show a persistent expression after Poly(I:C) and only designated genes as trained or attenuated if their basal expression at 48 hours was similar to that of media-exposed cells, in keeping with the definition of trained immunity and tolerance (4).

In summary, our data show that two distinct fetal endothelial cell types can both remodel their transcriptome in response to an inflammatory stimulus, with ECFCs inducing a distinct subset of genes from HUVECs in response to the viral ligand Poly(I:C), linked to the transcription factor ELF1. This remodelling in response to Poly(I:C) results in training or attenuation of specific gene sets in response to subsequent LPS stimulation, with a strong overlap between the two cell types. Genes that were tolerized in both cell types are involved in leukocyte recruitment, suggesting a mechanism that prevents excessive recruitment. Baseline genome-wide DNA methylation differences were a poor predictor of inflammatory transcriptional response, which is in line with previous work showing that acute inflammatory responses are regulated by histone modifications and nucleosome occupancy.

DATA AVAILABILITY STATEMENT

The data sets generated and analyzed for the current study are deposited in the Gene Expression Omnibus repository with the accession number GSE180881.

AUTHOR CONTRIBUTIONS

EW, AV, BK, DS, SW, and TA performed the cell culture experiments. J-HJ, RSh, JC, EW and UH provided primary endothelial cells. UH, MG, RS and BN supervised the students performing the experiments. RS and BN conceptualised the study. BN SW and SI performed the bioinformatic analysis. EW, UH and BN wrote the original draft manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.757393/full#supplementary-material

Supplementary Figure 1 | Persistent Poly(I:C) induced gene expression. **(A)** Bar plot showing median expression of genes following Poly(I:C) exposure. Genes were separated into 4 quartiles based on expression difference at 48h between Poly(I:C) and media exposed cells (green line – ECFCs, black line – media). This time-point is 24h after removal of the stimulus. Q1 shows the most persistent expression, while genes in Q4 return to basal levels. **(B)** Expression of example genes in ECFCs and HUVECs exposed to Poly(I:C) or media. MX1 is the top gene showing persistent

expression after Poly(I:C), CXCL10 is in Q2, and is showing reduced expression at 48h, while TNF is in Q4 and shows complete return to basal levels by 48h. **(C)** Bar plot showing abundance of TF motifs at the 4 quartiles. Q1 is enriched for IRF, NFKB and STAT motifs, with no clear enrichment of motifs at the other quartiles.

Supplementary Figure 2 | Poly(I:C) associated transcriptional programs are dependent on direct exposure. **(A)** Experimental model. **(B)** Gene expression after indirect Poly(I:C) exposure, i.e. cells were seeded after incubating wells with stimulation media following wash out, is comparable to media control.

Supplementary Figure 3 | Motif enrichment scores and expression of associated transcription factors involved in the initial Poly(I:C) response in ECFCs and HUVECs. **(A)** Abundance of transcription factor motifs in promotors of Poly(I:C) induced genes. **(B)** Transcription factor genes related to the motif signature are higher in HUVECs than ECFCs.

Supplementary Figure 4 | Summary of genes trained or tolerized for LPS response by Poly(I:C) in HUVECs and ECFCs. **(A)** PCA plot of transcriptomic changes that occur 4 hours following LPS exposure after previous stimulation with Poly(I:C) or media only. **(B)** Expression of trained, unaffected and tolerized genes over time in HUVECs. Genes trained for LPS response are more strongly induced by Poly(I:C) compared to genes that are tolerized for LPS response. **(C)** Line plot showing expression of example trained, unaffected and tolerized genes (green line – ECFCs, blue line – HUVECs, black line – media control).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

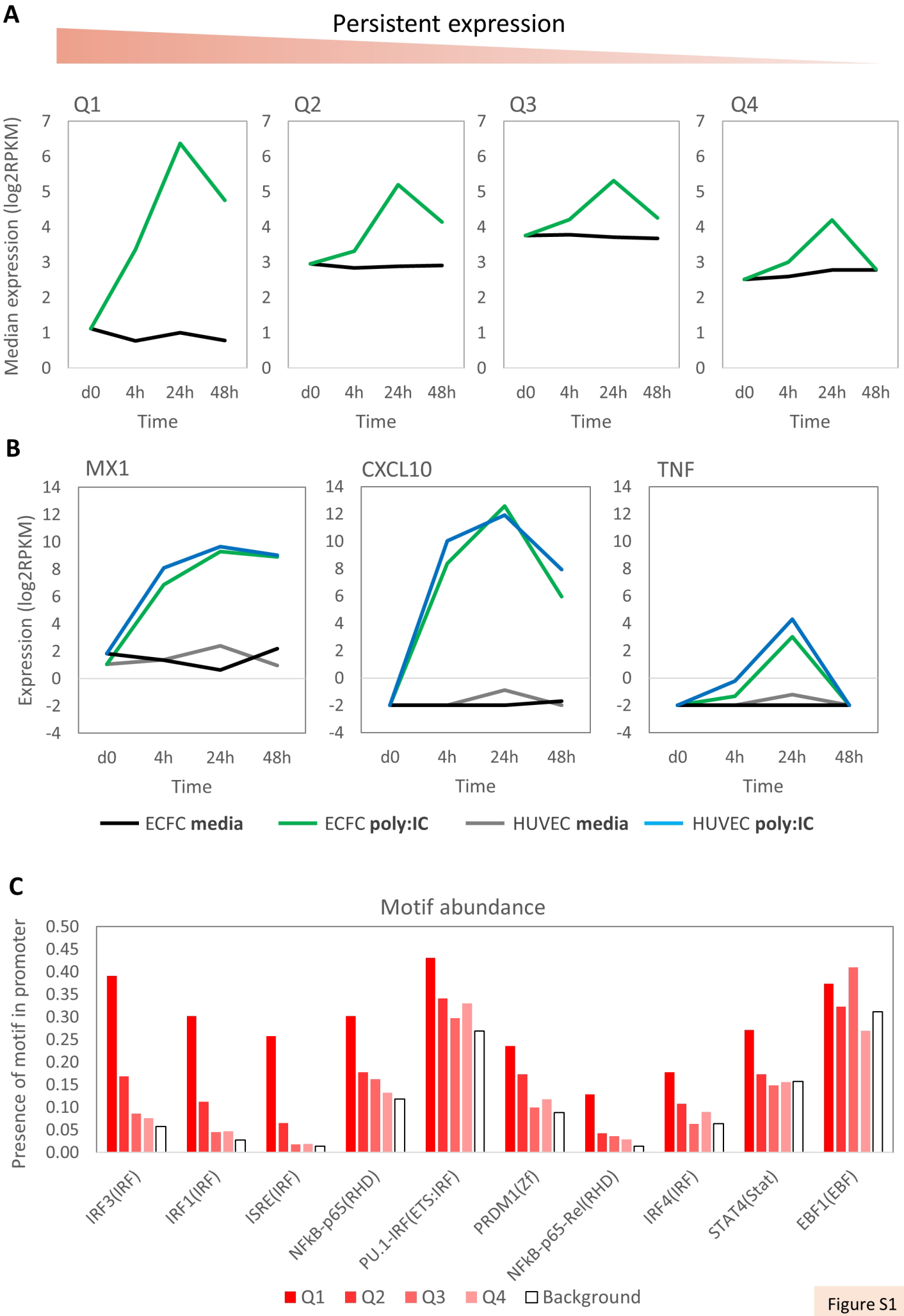
A
Persistent expression
Q1
Q2
Q3
Q4
Median expression (log2RPKM)
0
1
2
3
4
5
6
7
d0
4h
24h
48h
Time
B
MX1
CXCL10
TNF
Expression (log2RPKM)
-4
-2
0
2
4
6
8
10
12
14
ECFC media
ECFC poly:IC
HUVEC media
HUVEC poly:IC
C
Motif abundance
Presence of motif in promoter
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
0.50
IRF3(IRF)
IRF1(IRF)
ISRE(IRF)
NFkB-p65(RHD)
PU.1-IRF(ETS:IRF)
PRDM1(Zf)
NFkB-p65-Rel(RHD)
IRF4(IRF)
STAT4(Stat)
EBF1(EBF)
Q1
Q2
Q3
Q4
Background

Figure S1

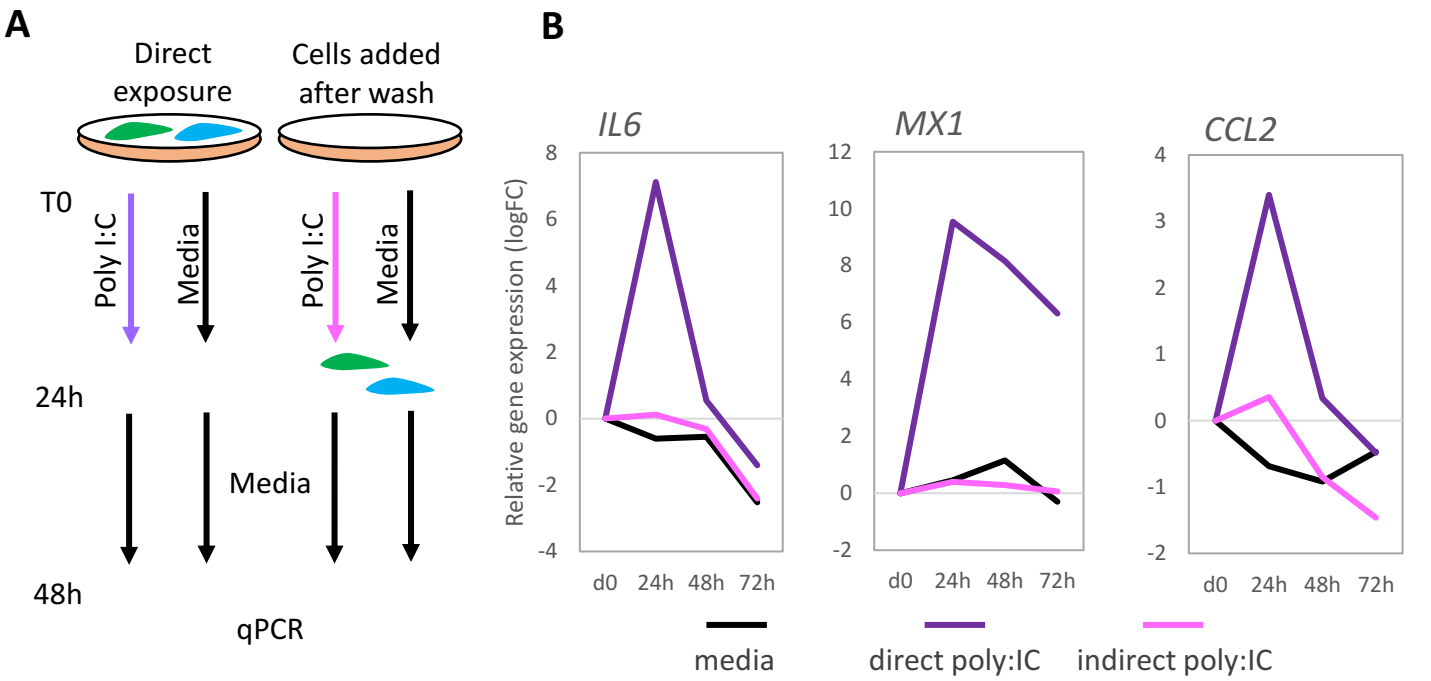
A
Direct exposure
Cells added after wash
T0
Poly I:C
Media
Poly I:C
Media
24h
Media
48h
qPCR
B
IL6
MX1
CCL2
Relative gene expression (logFC)
d0 24h 48h 72h
media
direct poly:IC
indirect poly:IC

Figure S2

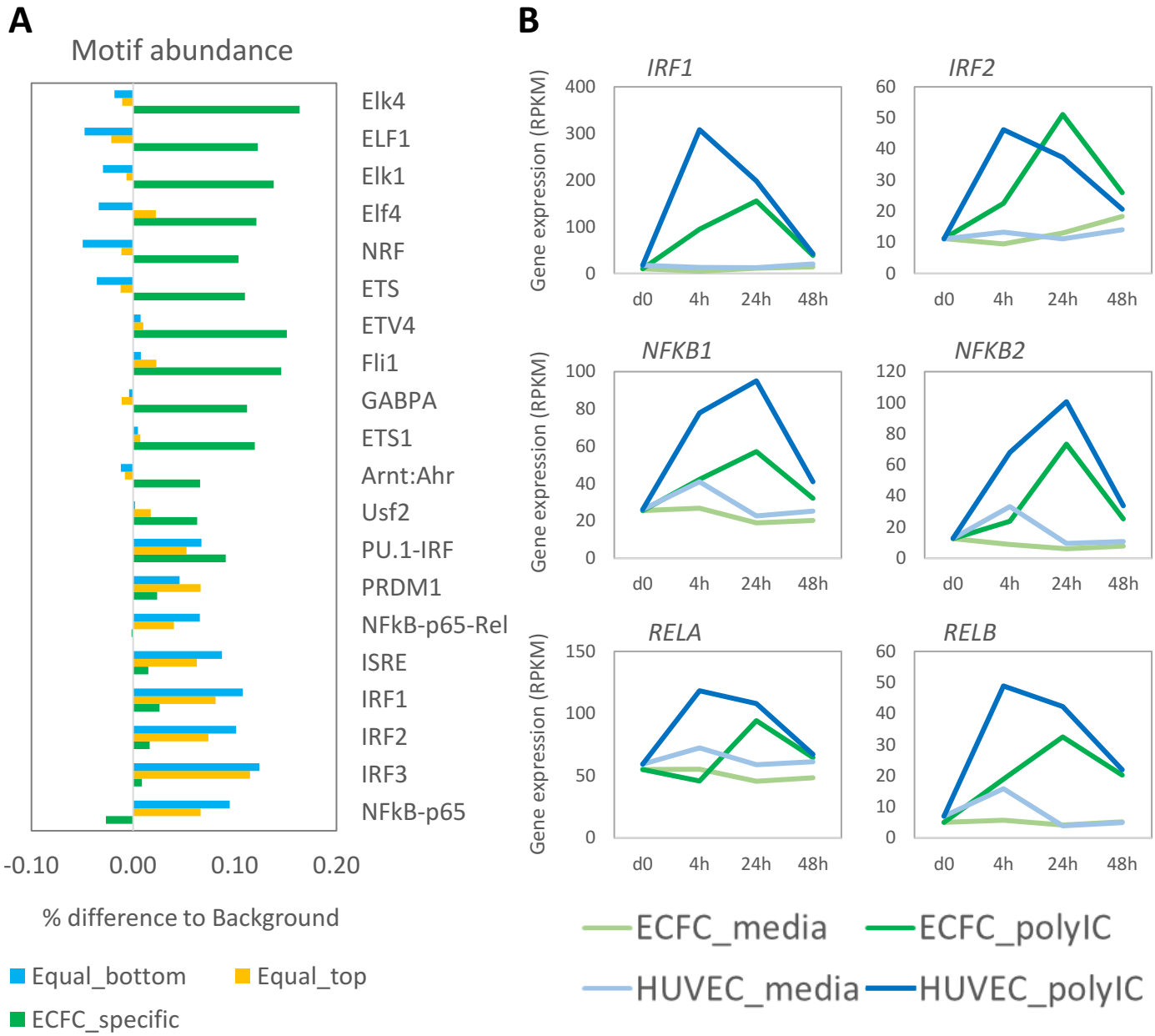
A
Motif abundance
Elk4
ELF1
Elk1
Elf4
NRF
ETS
ETV4
Fli1
GABPA
ETS1
Arnt:Ahr
Usf2
PU.1-IRF
PRDM1
NFkB-p65-Rel
ISRE
IRF1
IRF2
IRF3
NFkB-p65
-0.10
0.00
0.10
0.20
% difference to Background
Equal_bottom
Equal_top
ECFC_specific
B
IRF1
IRF2
NFKB1
NFKB2
RELA
RELB
Gene expression (RPKM)
d0
4h
24h
48h
ECFC_media
ECFC_polyIC
HUVEC_media
HUVEC_polyIC

Figure S3

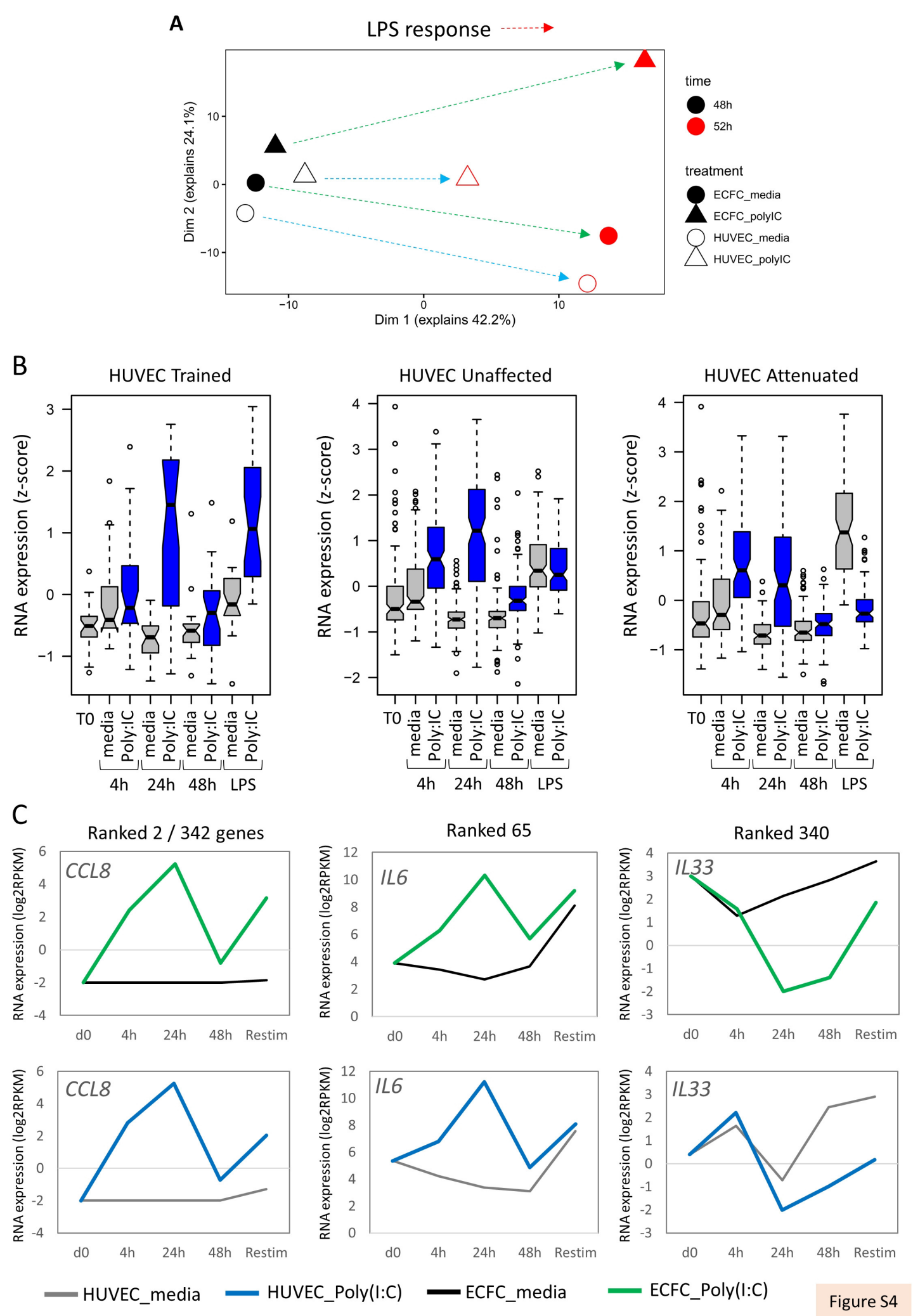
A
LPS response
Dim 2 (explains 24.1%)
Dim 1 (explains 42.2%)
time
48h
52h
treatment
ECFC_media
ECFC_polyIC
HUVEC_media
HUVEC_polyIC
B
HUVEC Trained
HUVEC Unaffected
HUVEC Attenuated
RNA expression (z-score)
T0
media
Poly:IC
4h
24h
48h
LPS
C
Ranked 2 / 342 genes
Ranked 65
Ranked 340
CCL8
IL6
IL33
RNA expression (log2RPKM)
d0
4h
24h
48h
Restim
HUVEC_media
HUVEC_Poly(I:C)
ECFC_media
ECFC_Poly(I:C)

Figure S4