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Ex vivo perfusion of the human placenta to investigate pregnancy pathologies

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ABSTRACT

Pregnancy pathologies including gestational diabetes, intrauterine fetal growth restriction, and pre-eclampsia are common and significantly increase the risk of poor pregnancy outcomes. Research to better understand the pathophysiology and improve diagnosis and treatment is therefore crucial. The *ex vivo* placenta perfusion model offers a unique system to study pregnancy pathology without the risk of harm to mother or fetus. The presence of a maternal and fetal circulation and intact villus tree, facilitates investigations into maternal-fetal transfer, altered hemodynamics and vascular reactivity in the human placenta. It also provides a platform to test novel therapeutic agents. Here we review the key studies which have utilized the *ex vivo* placenta perfusion model to study different aspects of such pregnancy pathologies.

1. Introduction

The human placenta has an active role in fetal development and programing. It mediates maternal–fetal communication throughout the pregnancy and is one of the most unique organs in the human body. It regulates the supply of nutrients and oxygen to support fetal growth and removes metabolic waste. Additionally, the placenta contributes to the maternal adaptations to pregnancy, including immunological tolerance and endocrine–metabolic regulation through the release of hormones, growth factors, and cytokines.

A complex interrelationship between maternal nutrition, placental nutrient transport, and fetal needs regulates fetal growth. In turn, an adequate supply of oxygen and nutrients by the placenta depends on the blood flow as well as the structure, morphology, membrane surface area, and transport capacity of the placental unit.

Ex vivo dual perfusion of the placental lobule represents a valuable approach to study the human placenta with unparalleled features. The presence of an intact villous tree and placental barrier enables investigations on the uptake, retention, metabolism, and transfer of nutrients and substances across the placental barrier as well as on vascular reactivity and the effects of altered hemodynamics. This technique was first reported by Panigel et al. [1] and further improved by Schneider et al. [2]. In this approach, referred to as double-sided or dual perfusion, both maternal intervillous and fetal circulations are individually reestablished with pumps, which circulate medium in each circuit. Alternatively, one-sided placenta perfusion, where only the intervillous space is perfused, can be performed for the collection of different factors (e.g., extracellular vesicles) produced by the syncytiotrophoblast cells as well as for the evaluation of compound accumulation in placental tissue [3].

Perfusions can be established in an open- or closed-circuit configuration. In an open-circuit perfusion, fresh perfusate enters the placental lobule on one or both sides and then leaves the tissue as venous effluent that goes to waste. In a closed-circuit arrangement, the venous perfusate is recirculated back through the tissue after re-gassing with appropriate levels of oxygen and carbon dioxide. The configuration used depends on the purpose of the experiment and the parameters being measured. An open circuit enables the addition and washout of drugs and the collection of perfusate at specific timepoints and avoids the need for antibiotics. However, the amount of perfusate required can be a limitation, particularly for longer experiments, and the concentration of some analytes may be too low to detect. Re-circulation of perfusate in closed systems facilities longer experiments and allows the accumulation of

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analytes. This can be beneficial, for example, when measuring extracellular vesicles or transplacental transfer of drugs or microorganisms. However, nonphysiological accumulation of metabolites may affect experimental outcomes. The type of perfusion performed therefore requires careful consideration to ensure the most appropriate conditions for the specific research question.

The flexibility of *ex vivo* dual perfusion represents a significant advantage of the technique and has enabled researchers to model pregnancy complications by changing specific parameters, for example, reduced oxygenation to mimic pre-eclampsia [4]. Perfusion experiments with placentas from pregnancies complicated by diseases, submitted to therapeutic interventions, or exposed to environmental pollutants also allows the acquisition of unique data on placental dynamics and functions (Fig. 1). Here, we review the findings from perfusion studies investigating pregnancy pathologies.

1.0. Pre-eclampsia

The *ex vivo* dual perfusion model of the human placenta has been used in different ways linked to the disease of pre-eclampsia (PE). Several studies have considered the dysregulation of metabolism and cell signaling coupled to this disease and how it affects vascular tone [5,6]. The release of syncytiotrophoblast extracellular vesicles (STBEVs) has also received attention, along with regulation of the immune response and the release of other endogenous substances [7–10]. Low oxygen tension thought to arise in PE has also been investigated using the model.

1.1. Syncytiotrophoblast extracellular vesicle release

The number of STBEVs released from the placenta in PE is increased, together with altered size and cargos, providing an important focus for research. Tannetta and colleagues have studied the effects of STBEV released from PE placentas on platelet function [11]. STBEVs were collected from maternal perfusate following 3-h closed-circuit dual perfusion and a 20-min wash-out period. Microvillous origin was confirmed by analyzing the expression of placental alkaline phosphatase (PLAP). The STBEVs released were shown to enhance platelet activation, thus providing a potential mechanism to explain increased risk of thromboembolism in PE [11]. Interestingly, aspirin, commonly prescribed in cases of maternal hypertension and PE, ameliorated the effects of the STBEVs on platelet function [11]. STBEVs (100–1000 nm diameter) and syncytiotrophoblast-derived exosomes (STMEX; 20–200 nm diameter) from *ex vivo* dually perfused placentas have both been shown to express endothelial nitric oxide synthase (eNOS). The content and activity of eNOS, however, was reduced when STBEVs and STMEXs were obtained from PE affected pregnancies. Given that nitric oxide (NO) is a potent vasodilator and platelet inhibitor, the reduced capacity of STBEVs and STMEX derived from pre-eclamptic pregnancies to produce NO may contribute to the increased maternal blood pressure and thrombotic propensity observed in pre-eclampsia.

1.2. Adaptations of the perfusion model to mimic conditions of PE

To facilitate investigations into the pathophysiology of PE at The University of Manchester, the *ex vivo* perfusion model has been adapted, altering maternal flow rates and oxygenation of the perfusate. In the first study, two maternal-side flow rates were used: (i) 14 mL/min, representing a healthy placenta and (ii) 45 mL/min, mimicking an increase in shear stress resulting from failed spiral artery remodeling in PE [12], which is supported by mathematical modeling [13]. Maternal perfusate collected from the high flow rate group demonstrated significantly elevated levels of placental alkaline phosphatase, lactate dehydrogenase, human placental lactogen, and the cytokines RANTES and GROα, as compared to the regular flow rate group [8]. The perfusate also reduced cell proliferation, viability, and mitochondrial activity when

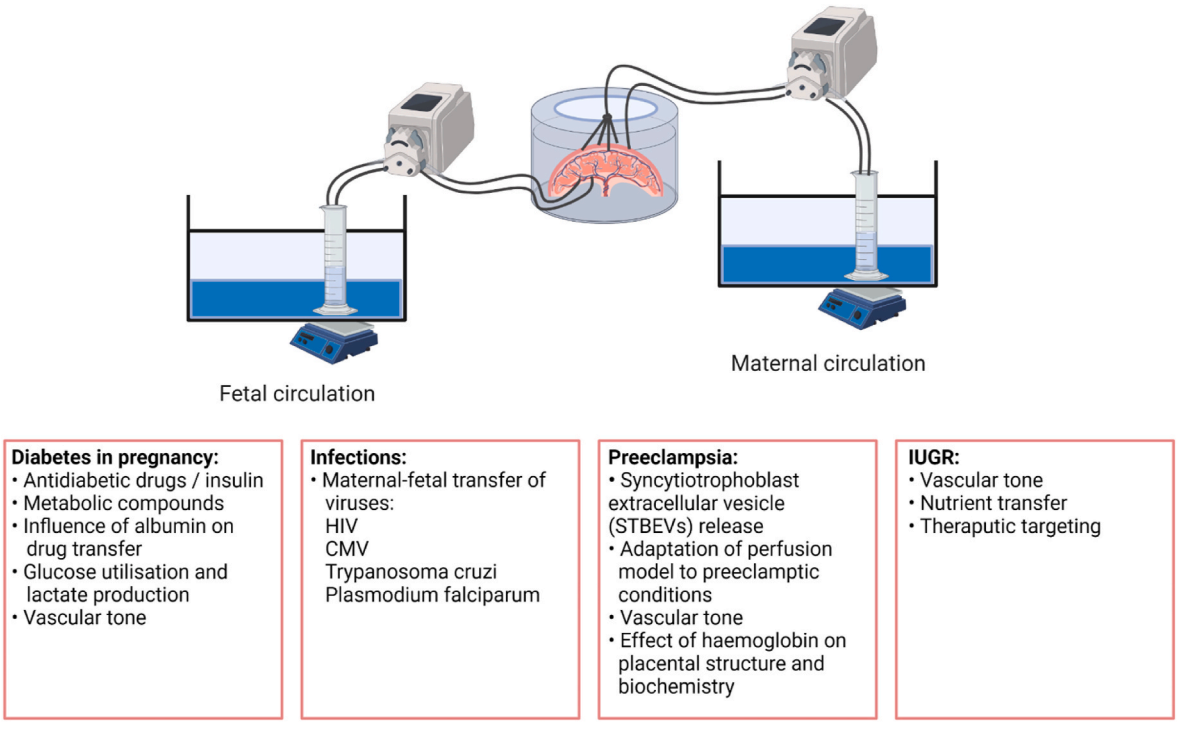

Fig. 1. Utilization of *ex vivo* dual perfusion of the human placenta to investigate pregnancy pathologies. The standard dual perfusion set up is demonstrated with separate reservoirs of perfusate being pumped through the maternal and fetal circulations of the placenta. A closed circuit is shown; however, the outflow canulae can be placed in separate containers allowing collection and subsequent measurement of placental transfer or analytes produced by the fetal vasculature or syncytiotrophoblasts. The boxes highlight the pregnancy pathologies, where *ex vivo* perfusion has been used and the applications of their use. Created with BioRender.com.

added to HUVECs, suggesting that liberated soluble factors might have a deleterious effect on the maternal endothelium [8].

Adaptation of the perfusion model to mirror the hypoxic condition of the term placenta in PE was performed by Soydemir et al. [4] who reduced maternal perfusate oxygenation to <3%, (hypoxia) and compared this to 5–7%, the oxygenation levels experienced by the placenta in healthy pregnancy [14]. To counteract the reduced oxygen-carrying capacity of perfusate compared to blood and achieve a similar level of metabolism to that found in traditional super-oxygenation *ex vivo* perfusate conditions (95%), the number of maternal cannulae was increased from 5 to 22 [4]. This was a pertinent step given the apparent steep oxygen gradients found in the intervillus space (IVS) [4]. Glycolysis was reduced in the hypoxia model as compared to that in normoxic perfusion, and there was a greater release of the hypoxic-sensitive marker, macrophage inflammatory protein-1α, during low oxygen perfusion. There was also an increase in the proinflammatory cytokines, namely interleukin-6, interleukin-8, tumor necrotic factor-α, and interferon-ϒ, in the hypoxic perfusion group [4,7], which potentially contributes to dysregulation of the systemic maternal endothelium. Endothelin-1 and 8-isoprostane levels were also higher, in keeping with expected trends implicated in the pre-eclamptic inflammatory response and lipid peroxidation in this disease. This perfusion model adaptation, at a low oxygen tension mimicking PE, appears to have a biochemical signature in agreement with this disease. It could therefore be used to examine a potential reversal of signaling by potential therapeutic compounds.

1.3. Investigations of effects on fetoplacental vascular tone

Human placental vessels lack autonomic innervation; therefore, vascular tone is exclusively regulated by endocrine or mechanical stimuli [15]. Low vascular resistance is maintained by vasodilators including NO, prostacyclin (PGI_2), and endothelial-derived hyperpolarizing factor (EDHF) [16], while constriction is stimulated by vasoactive mediators, including thromboxane A2, endothelin-1 [17], and adrenergic agonists [18]. The dual-perfusion model has enabled researchers to investigate vascular reactivity and intravascular pressure in healthy and complicated pregnancies. This was achieved by monitoring fetal-side inflow hydrostatic pressure (FIHP) also referred to as fetal arterial perfusion pressure (FAP), thus providing an indication of vascular resistance in the fetoplacental circulation. Dual placental perfusion has been used in such studies since the early 1980s to characterize the role of a plethora of vasopressors and dilators in placental circulation of healthy pregnancies [19,20]. More recently, the validity of *ex vivo* perfusions and FIHP as an accurate indicator of *in vivo* vascular resistance was demonstrated in a study by Jones et al. The study showed significant correlation between umbilical artery Doppler pulsatility and resistance indices (RI and PI) and *ex vivo* FIHP measurements from the same placenta following delivery [21]. In this study, the maternal and fetal circulation of cotyledons were perfused in an open-circuit configuration.

To assess the NO axis in the fetoplacental circulation in PE, Bisseling and colleagues perfused placentas from normal pregnancy and those complicated with PE [22] in the presence of eNOS inhibitor L-NAME whilst measuring FIHP. NO production was impaired in the pre-eclamptic group compared to the healthy control group. However, the antioxidant, N-acetylcysteine, enhanced NO production in both groups, with greater effect in the PE group. Offering N-acetylcysteine as a potential anti-oxidant therapy, may therefore improve fetoplacental blood flow in PE, which could be particularly beneficial in later pregnancy when the capacity for oxidative stress progressively increases [23].

A further study using *ex vivo* dual perfusion has demonstrated increased vasodilation in response to exogenous vascular endothelial growth factor ($VEGF\text{-}A_{165}$), in cotyledons from pre-eclamptic pregnancies as compared to that in healthy pregnancies [24]. Endogenous secretion of $VEGF\text{-}A_{165\&121}$ into the fetoplacental circulation of *ex vivo* perfused tissue was, however, lower in the PE group. This is in agreement with reduced $VEGF\text{-}A_{165}$ sera levels observed in the cord blood of the PE group [24]. These latter observations may therefore offset the enhanced vasodilator response in fetoplacental circulation of placentas from pregnancies complicated by PE.

Sun and others studied the expression and release of components of the renin angiotensin system (RAS) in healthy and pre-eclamptic placentas. Angiotensin II is known to upregulate placental levels of soluble Flt-1, an anti-angiogenic factor which is elevated in maternal systemic circulation in PE. The authors used the *ex vivo* perfusion model to determine whether components of the RAS, including renin, angiotensin converting enzyme (ACE), and angiotensinogen, were derived in the placenta or originated from the maternal circulation to affect s-Flt production. The authors assessed the temporal pattern of release and perceived wash-out of substances into the maternal venous perfusate [25]. This work was coupled with PCR analysis of expression of pro-renin, renin, and angiotensinogen. The authors concluded that renin and pro-renin were indeed expressed and released into the maternal perfusate, but angiotensinogen was not. This is somewhat controversial, because other researchers have found angiotensinogen expression in the placenta [26]. Whilst the monitoring of venous perfusates for the release of substances can be of use, it is known that some compounds exhibit a significant drop, which is partly explained by an accumulation of substances during the ischemic period prior to *ex vivo* perfusion, as found for $VEGF\text{-}A_{165/121}$ [27]. Furthermore, a "partial metabolic arrest" reducing protein synthesis from *in vivo* levels has also been studied in this model, where oxygen supply is compromised in the absence of a cooperative supply from erythrocytes [28].

Zardoya-Laguardia and colleagues investigated the effect of L-tryptophan (L-Trp) on inflow hydrostatic pressure in the fetoplacental circulation of cotyledons from healthy pregnancies [29]. The authors demonstrated a significant vasodilatory response to 8 mM L-Trp in both the absence and presence of pre-constriction with the thromboxane A2 mimetic U-46619. Vasodilation was shown to be dependent on indoleamine 2,3-dioxygenase-1 (IDO-1), a cytoplasmic enzyme that metabolizes L-Trp via the kynurenine pathway. Expression of IDO-1 was found to be reduced in the fetoplacental microcirculation of placentas from pregnancies complicated by PE and fetal growth restriction (FGR), leading the authors to speculate that reduced IDO-1 levels may contribute to the pathogenesis of PE and IUGR. However, the study did not perform *ex vivo* perfusion experiments using placentas from pregnancies complicated by PE or FGR to demonstrate reduced vasodilation in response to L-Trp.

1.4. Elevations in free fetal hemoglobin in pre-eclampsia

Elevations in free hemoglobin (fHb) have been found in the maternal circulation of pregnancies complicated by PE. Using human placental perfusion, May and colleagues [30] examined the structural and biochemical effects of fHb on the placenta. They found enlarged and damaged mitochondria and a dysmorphic endoplasmic reticulum in the syncytiotrophoblast as well as elevated fetal-side inflow hydrostatic pressure [30]. This was later characterized by Brook and colleagues as the consequence of NO sequestration [31]. Gene arrays from the perfused tissue suggested that perfusion with fHb results in oxidative stress, apoptosis, and tissue damage, supporting the morphological changes observed [30]. These observations corresponded with pre-eclamptic injuries found in the placenta and were prevented by co-perfusion with α1-microglobulin [30]. A further investigation by Cronqvist demonstrated that *ex vivo* placental perfusion with fHb altered the micro-RNA cargo of STVEBs [32].

2.0. Intrauterine (fetal) growth restriction

Fetal growth is determined by genetics, nutrient availability, and

environmental factors. It is also heavily influenced by the ability of the placenta to adequately transfer oxygen and nutrients from mother to fetus and the regulation of this by the endocrine system [33,34]. Aberrant fetal growth and the development of FGR can occur due to maternal factors (nutrient deficiency or hypertensive disorders), fetal factors (chromosomal abnormalities) or more commonly, placental dysfunction, leading to insufficient oxygen and nutrient transfer to the fetus [34,35]. Very few studies have used dual *ex vivo* perfusion to investigate FGR. These studies can be categorized into those investigating vascular tone, nutrient transfer, or therapeutic targeting.

2.1. Vascular tone and reactivity in FGR

In severe cases of FGR, vascular adaptations that occur during gestation to promote a high flow, low resistance circulation are perturbed, resulting in elevated fetoplacental resistance [36]. Clinically, this can be observed using Doppler ultrasound velocimetry of the umbilical artery as increased PI and RI [37], with the severity of fetal compromise correlating with the degree of abnormality of the Doppler waveform [38,39]. Structural abnormalities of the placental vasculature are thought to contribute to the elevated vascular resistance observed in FGR; however, more transient influences such as vascular tone are also likely to contribute [40].

In FGR, elevated vascular tone in the fetoplacental circulation has been in part attributed to alterations in vasoactive mediators, including reduced NO synthesis [41], increased endothelin fetal plasma levels [42,43], and elevated angiotensin II concentration [44]. To date, there have only been a handful of studies published that have used dual placental perfusion to investigate vascular tone in FGR. An early but important study that perfused placentae obtained from pregnancies complicated by FGR or elevated UA Dopplers was published by Read and colleagues [45]. They investigated the reduction in FIHP of pre-constricted vessels (PGF2-alpha) in response to the tissue-independent NO donor sodium nitroprusside (SNP). No difference was observed in response to SNP in basal or pre-constricted vessels when compared with controls, indicating that smooth muscle sensitivity to NO and the subsequent vasodilatory response was not altered. However, pre-treatment of vessels with an eNOS inhibitor enhanced the vasodilation mediated by SNP [45] consistent with an upregulation of guanylate cyclase/cGMP in the vascular smooth muscle to compensate for reduced endogenous NO levels [45].

More recently, altered sensitivity of placental vessels to prostaglandin E2 (PGE_2) has been demonstrated in FGR. PGE_2 can mediate vasodilation or constriction of vessels depending on the receptors that it binds to. In uterine vessels, PGE_2 induces vasodilation, whereas constriction is observed in placental vessels. Luria et al. investigated the reactivity of placental vessels in response to PGE_2 in FGR as compared to that in uncomplicated pregnancies [46]. Perfusion of the placentas revealed no significant difference in baseline resistance between the normal control and FGR groups. However, a bolus injection of PGE_2 stimulated a blunted contraction in the FGR group and accelerated relaxation [46]. Histological analysis of the placentas revealed increased prevalence of vascular lesions in the FGR as compared to control placentas (80% versus 25%, respectively, $P = 0.047$), which the authors suggest could explain the altered vascular supply in FGR.

A benefit of the dual-perfused placenta is the ability to collect perfusate from the fetal circulation, thus allowing the quantification of mediators released by the fetal vasculature, for example inflammatory, thrombotic, or vasoactive regulators, in parallel to measuring vascular resistance. This facilitates investigations on differences between placentas from healthy and FGR pregnancies at baseline or in response to insult or intervention. A study by Holcberg et al. used this approach to investigate whether TNF-alpha secretion by the placental vasculature was altered in FGR or by exposure to angiotensin II (AII) [47]. Isolated placental cotyledons from healthy and growth restricted pregnancies (defined as <5th centile) were dually perfused, and TNF-alpha levels in the perfusate measured by ELISA [47]. There was over a 10-fold increase in the amount of TNF-alpha measured in the perfusate of FGR placentae as compared to normal placentae after 120 min of perfusion. The study also demonstrated a dose-dependent increase in TNF-alpha levels, concurrent with an increase in vascular resistance, following bolus injection of AII (1nM-10μM) in healthy placentas. The profound effects of TNF-alpha on the vascular system have been well characterized over the past 20 years, with reduced NO bioavailability [48] and increased COX-dependent vasoconstrictors [49,50] thought to mediate TNF-α induced increase in vascular resistance. Results from the dual perfusion study by Holcberg et al. [51] may therefore in part explain the increased placental vascular resistance associated with FGR.

Mechanical stimuli generated by hemodynamic forces is an important regulator of vascular tone. Throughout gestation, placental blood flow is increased, which requires adaptive dilation on the blood vessels to prevent elevated vascular resistance. In a study by Jones et al., dual placental perfusion was used to investigate flow mediated vasodilation (FMVD) in the fetoplacental circulation to determine whether it was compromised in pregnancies complicated by FGR [21]. In the study, FIHP was continuously measured, whilst the flow rate was increased incrementally, enabling the dilation response to be measured. The results clearly demonstrated an inability of placental vessels from FGR pregnancies to dilate in response to increased flow, particularly in cases where there was no- or reversed end diastolic flow. Investigations on the NO vasodilator response revealed that, consistent with other studies [52,53], it was the predominant regulator of FMVD. In FGR placentas, eNOS was shown to be upregulated [21], thus suggesting a failed attempt to rescue low resistance.

FMVD has further been studied in the dual perfused *ex vivo* placenta by Hitzerd et al., who investigated the influence of placental volumetric parameters in the first trimester of pregnancy on fetoplacental vascular function at term [54]. In this study, placental volume (PV) and uteroplacental volume (uPVV) were measured at 7-, 9-, and 11-weeks gestational age (GA) by using ultrasound and specialist Virtual Organ Analysis and Virtual Reality software [54]. These measurements were then correlated with FMVD. The results from the study demonstrated that PV in the first trimester was negatively correlated with pressure suggesting an association between larger placental volumes and lower intravascular pressure. This was likely due to improved FMVD, which positively correlated with PV. Taken together, the data from this study indicate that larger placentas may have more capacity to adapt to changes in blood flow and pressure, ultimately improving pregnancy outcome.

2.2. Transport and transfer in FGR

Dual placental perfusion has long been used to study the transplacental transfer of molecules from the maternal circulation to the fetal circulation *ex vivo*, including oxygen and nutrients [4,55] as well as maternal antibodies, alcohol and therapeutic and recreational drugs [56]. It is the only *in vitro* experimental model to encompass the maternal and placental circulation, with the integrity of the tissue intact. A systematic review in 2011, demonstrated good correlation between the perfusion model and *in vivo* drug transfer in healthy pregnancies [57].

It is important, however, to study the transplacental transfer in pregnancies complicated by FGR to account for any alterations in the expression and function of membrane transporters or disrupted barrier function of the fetoplacental endothelium or syncytiotrophoblasts. Bisseling and colleagues used *ex vivo* dual perfusion to investigate whether impaired transport of folate was associated with FGR [58]. They studied the transport of 5-methyltetrahydrofolate (5MTF) from maternal perfusate to the fetal perfusate in healthy and FGR placentae. The authors demonstrated that transport did not saturate over a concentration range of 50–500 nM, and there was no significant difference in transport between placentae complicated with FGR and uncomplicated

pregnancies [58].

While currently there are no treatments available for FGR, recent advances have been made, including the development of a VEGF165 containing adenovirus. The adenovirus has been shown to provide a sustained (4 week) increase in uterine blood flow [59] and improved placental efficiency and fetal growth [60] when injected into uterine arteries in an over-nourished adolescent sheep model of FGR. To assess the transfer and toxicity of the adenovirus (*Ad.VEGF-D*$^{\Delta N\Delta C}$) across human placentae, Desforges et al. dual perfused normal and FGR placentae with perfusate on the maternal side containing 5×10^{10} vp/mL *Ad. VEGF-D*$^{\Delta N\Delta C}$ or vehicle control [61]. The study revealed no effect on placental fetoplacental vascular resistance or permeability with minimal transfer of the virus to the fetal circulation. Endocrine function was preserved, and except for a minor elevation in lactate dehydrogenase (LDH) on the maternal side, there was no evidence of toxicity to the human placenta [62].

Another experimental treatment to improve uteroplacental blood flow and reduce fetoplacental resistance in FGR is based on the use of Sildenafil. Using the dual placenta perfusion model, it was shown that in healthy placentas, where fetal vessels were pre-constricted with thromboxane A2 analogue U46619, Sildenafil (but not Tadalafil), in maternal perfusate, led to dilation of the fetoplacental vessels and a reduction in vascular resistance [63]. However, thus far, the effects of Sildenafil have not been tested using *ex vivo* perfusion on placentas from pregnancies complicated by FGR; therefore, it remains unclear whether the same beneficial effects would be observed.

3.0. Diabetes

Diabetes is one of the most common complications affecting pregnancy. It is commonly divided into two main groups: preexisting diabetes (type 1 and type 2 diabetes) and gestational diabetes (GDM) [64]. The incidence of GDM worldwide varies between 5% and 20%, whereas around 0.5% of all pregnancies are of women with diabetes diagnosed prior to conception [65]. It is well established that hyperglycemia during pregnancy can lead to spontaneous abortion, macrosomia, neonatal hypoglycemia, and birth defects [66]. However, the effect of diabetes on placental functions such as nutrient and drug transfer are poorly understood. This is an issue that can be addressed through placental perfusion. Changes in hormone and nutrient levels, blood volume, and pharmacokinetics [67] may affect different properties. Type 1 diabetes is treated mainly with insulin, whereas type 2 diabetes prior to pregnancy can usually be controlled with dietary changes and oral antidiabetic drugs. While the impact of these drugs in nonpregnant subjects has been well studied through clinical trials, the influence on placentas of pregnant women and their unborn fetus remains to be further characterized.

3.1. Perfusion of diabetic placentas

In pregnant women, the transport and metabolism of different compounds can be altered due to various prevailing pathologies, including diabetes. Osmond et al. showed a lower glucose uptake, transfer, and utilization as well as a decreased lactate production in GDM placentas as compared to that in normoglycemic ones [68]. Further analysis of the same group evaluated the impact of different GDM treatments (diet and insulin) on placental glucose transfer and lactate production [69]. A higher glucose consumption was reported in the control group than in both GDM groups. Additionally, lactate production was decreased in the fetal compartment in GDM placentae from both groups and in the maternal compartment of the GDM-diet group. It was finally concluded that GDM women treated with insulin had a higher placental maternal-fetal transfer of glucose compared with those treated with diet only.

Lipids play a key role during pregnancy in the development of a fetus [70,71]. During early- and mid-pregnancy, the maternal body accumulates fat deposits as a source of energy [71]. Additionally, lipids such as essential fatty acids and long-chain polyunsaturated fatty acids are fundamental for organogenesis and fetal tissue development. Arachidonic acid (AA) is an omega-6 fatty acid found in different tissues in the human body, including the brain, liver, and muscle. It is involved in intercellular communication. Kuhn et al. investigated the uptake, transfer, and lipid distribution of AA in both normal and insulin-dependent diabetes mellitus (IDDM) [72]. The authors reported an increased placental uptake and transfer of AA in diabetic pregnancies and a reduction in incorporation of AA into phosphotriglycerides in placental tissue. Additionally, it was shown that in IDDM pregnancies, there is an increase in incorporation into triglycerides in placental tissue and fetal and maternal effluents.

An aspect that may influence drug transport and accumulation in the placenta is circulating proteins levels. Associated with an increase in the blood volume along human gestation, there is a decrease in serum albumin (SA) concentration [73]. SA is an important binding protein that can influence drug transport. The influence of SA on glyburide transfer and accumulation through the human placenta in healthy and GDM pregnancies was explored using *ex vivo* dual placental perfusion [74]. Data showed that these processes are SA concentration-dependent in both study groups. On the other hand, uptake and transfer of free/unbound glyburide (independent of SA) by the placenta was not different in GDM samples [74].

Type 1 diabetic women exhibit changes in placental vascularity that may result in altered maternal–fetal transport of different nutrients and oxygen [75]. The effects of NO on the vascular tone baseline in the fetal placental circulation in type 1 diabetic placentas was evaluated using the double-sided placenta perfusion model [76]. In this study, the effect of different concentrations of eNOS inhibitor N^G-nitro-arginine-methylester ($_L$-NAME) on the NO pathway and the influence of insulin on this process were assessed. Perfused placentas from type 1 diabetic women had an increased baseline fetal arterial pressure along with a higher net $_L$-NAME-induced increase in fetal arterial pressure when compared with controls. Insulin did not influence this parameter. $_L$-NAME addition in contrast resulted in an increased maximum perfusion pressure in the diabetic group.

Ex vivo placental dual perfusion has also been applied to assess the transfer of antidiabetic drugs across the human placenta and to determine the influence of diabetic state on this process. The transfer of metformin in normoglycemic and GDM placentas [77] revealed no differences between the groups. Other studies evaluated the transfer of different antidiabetic drugs [78–83] and insulins [84–86] only in placentas from uncomplicated pregnancies. Such analysis should also be performed in pathological conditions as there are morphological, functional, and metabolic changes in these placentas that could impair drug transfer.

4.0. Placenta perfusion and microbial infection

Antenatal fetal microbial infections are still a major cause of perinatal morbidity and mortality. The transmission of pathogens from an infected mother to her fetus, known as vertical transmission, can lead to devastating perinatal and neonatal complications including miscarriage and perinatal death [87]. The placenta serves as a physical barrier to prevent vertical transmission of infections from mother to fetus. Consequently, the syncytiotrophoblast layer, is reasonably resistant to most pathogens, in contrast to the cytotrophoblast and other cells within the chorionic villus that are highly susceptible to infections [87]. However, although the syncytial layer forms a robust barrier, vertical transmission still occurs, and molecular mechanisms are still not fully elucidated. *Ex vivo* placental perfusion has been used to investigate some of the mechanisms involved.

4.1. Pathogens crossing the placenta

Vertical transmission of bacterial species from the mother to the fetus, particularly in relation to the microbiome, is currently an area of contention. The concept of the "sterile womb" [88] and the acquisition of the fetal microbiome during and following birth is widely accepted [89]. Recent studies, however, have challenged this paradigm, suggesting that colonization of the human gastrointestinal tract starts in utero [90,91]. Evidence has suggested that the placenta has its own distinct microbiome, which may ascent from the vaginal tract, the maternal intestine, or even the oral cavity [90–92]. However, ascending bacterial infection during pregnancy is more likely to occur where the placental barrier is not involved [92,93]. Remarkably, the only bacterium where relevant vertical transmission with consecutive fetal damage is described is *Treponema pallidum* where transmission can occur at any gestational age [94].

In contrast, a rising number of clinically relevant viruses are vertically transmitted to the fetus following maternal infection, thereby causing different patterns of fetal alteration. Relevant vertically transmitted infections include HIV, hepatitis B virus, hepatitis C virus, varicella zoster virus, rubella virus, parvovirus B19, cytomegalovirus, and more recently, the Zika virus [95,96]. However, clinical, and experimental data suggest that the placenta is a relatively effective barrier to many of these viruses, resulting in diverse transmission rates occurring in different trimesters (Table 1). Despite the apparently high number of pathogens associated with in utero fetal disease following vertical transmission, the molecular mechanisms of transplacental vertical transmission of maternal harmful microorganisms to the fetus remain unclear.

Table 1
Transmission rates and time of transmission for microorganisms known to cross the human placenta.

Microorganism	Transmission rate	Time of transmission	Reference
Viruses			
HIV	25% in mothers who do not receive antiviral therapy 2% when viral load is reduced by medication	With onset of labor	[103,104]
Varicella zoster	2%		[105,106]
Cytomegaly	5.5%	Preconception period	
	66.2%	Third trimester	
Zika virus	47%	First trimester	[107]
	28%	Second trimester	
	25%	Third trimester	
Rubella virus	Not evaluated		[108]
Parvovirus B19	30–50%	any	
Herpes simplex	30–50%	any	
Chikungunya virus		any	
Chicken pox	None		
Measles	None		
Protozoan			
Toxoplasma gondii	14%	First trimester	[109–111]
	29%	Second trimester	
	59%	Third trimester	
Plasmodium falciparum	30% and more derived from detection of parasites in cord blood less than 0.5% derived from studies of newborn peripheral blood	any	
Trypanosoma cruzi	<4%	any	

4.2. Placental perfusion to study pathogen transmission

Syncytiotrophoblasts are continuously differentiating from underlying mononuclear cytotrophoblasts. A basement membrane separates these trophoblastic cells from a connective tissue that contains fetal capillaries. Several pathogens are known to invade the placenta and cause trophoblast and placental infection, but few are known to actively cross the placental barrier and disseminate to the fetus [87,97]. While molecular mechanisms of trophoblast infection can be investigated using cultured trophoblast cells or placental explants, investigation of mechanisms of barrier crossing is far more complex, with *ex vivo* dual-sided placental perfusion being the only method capable of studying this.

Most important for pregnancies occurring in developed regions are the infections caused by "TORCH" pathogens, which includes *Toxoplasma gondii*, other (*Treponema pallidum*, parvovirus, HIV, varicella zoster virus, amongst others), Rubella, Cytomegalovirus (CMV), and Herpesviruses (HSV) 1 and 2). In the United States, maternal infections with TORCH pathogens are associated with significant fetal disease, as 400–4000 infants are affected with congenital toxoplasmosis annually, and approximately 8000 children require complex medical care due to congenital CMV infection [87].

In 1994, Bawdon and colleagues published the first study on the transplacental passage of cell-free HIV-1. They used an *ex vivo* placental perfusion setting to determine HIV transfer from the maternal to the fetal circulation. Infectious HIV-1 was not detected in any of the fetal perfusate samples taken periodically during experiments [98]. Although these results were in line with clinical observations where transmission of HIV only occurred following onset of labor, the study was limited by a maximum perfusion time of 6 h [98]. These methodological aspects were addressed in 1996 by Polliotti and colleagues [99]. The authors performed long-term perfusion (12–24 h) of both maternal and fetal circulations in isolated human placental lobules, allowing the study of more prolonged biological and physiological events. The study by Polliotti and colleagues is the first to describe a placental perfusion model modified to enable perfusion under biohazard conditions to investigate the uptake and transfer of infectious agents, in particular, HIV [99].

In 1995, Mühlemann and colleagues used placental perfusion to investigate the transition of CMV [100]. Interestingly, in this study, CMV 169 CE did not cross the placenta, even with exposure to high virus titers. Perfusion time in these experiments were up to 9.5 h. The absence of infection of placental cells was confirmed by immunocytochemistry [100]. These results suggest that trophoblast infection with and placental transfer of CMV may require contact with cell-bound virus, e.g., CMV-infected white blood cells. Furthermore, CMV were isolated from three perfused placental tissues, which indicates at least adherence to the placental surface [100].

More recently, Shippey and colleagues perfused trypomastigotes of *Trypanosoma cruzi* as a bolus into the maternal circulation using the dual placental perfusion model. T cruzi DNA was identified in all post-inoculation maternal perfusate samples and post-inoculation placental tissue specimens; pre-inoculation and fetal effluent specimens remained negative for virus DNA [101].

Most recently, Pehrson et al. used the *ex vivo* placental perfusion system to study adhesion of erythrocytes infected with *Plasmodium falciparum*. The authors reported that infected erythrocytes expressing VAR2CSA accumulated in perfused placental tissue, whereas transgenic parasite did not. Furthermore, they demonstrated that soluble CSA and antibodies specific against VAR2CSA inhibited binding of infected erythrocytes [102]. These studies demonstrate the usefulness of *ex vivo* placental perfusion to investigate the mechanisms involved in pathogen transmission, across such a complex structure like the feto-maternal barrier.

5. Conclusion

Pregnancy pathologies such as infection, pre-eclampsia, FGR, and GDM are associated with increased risk of morbidity and mortality, both in the perinatal period and adulthood. To improve understanding of mechanisms which underpin the pathophysiology of these complications and develop better treatment strategies, the *ex vivo* perfusion model offers a remarkable tool, enabling mechanistic investigations into the human placenta and communication between the maternal and fetal circulation without compromising pregnancy or causing harm to mother or fetus.

Declaration of competing interest

The authors have no conflict of interests to declare.

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