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Ex vivo dual perfusion of an isolated human placenta cotyledon: Towards protocol standardization and improved inter-centre comparability

Henning Schneider [a,1], Christiane Albrecht [b,c,1], Mahmoud S. Ahmed [d], Michelle Broekhuizen [e,f], Leonie Aengenheister [g], Tina Buerki-Thurnherr [g], A.H. Jan Danser [f], Sophie Gil [h], Stefan R. Hansson [i], Rick Greupink [j], Rohan M. Lewis [k], Udo R. Markert [l], Line Mathiesen [m], Nicola Powles-Glover [n], Christian Wadsack [o], Paul Brownbill [p,q,*]

[a] *Dept. Obstetrics & Gynecology, Inselspital, Bern University Hospital, Switzerland*
[b] *Institute of Biochemistry & Molecular Medicine, University of Bern, Switzerland*
[c] *Swiss National Centre of Competence in Research (NCCR) TransCure, University of Bern, Switzerland*
[d] *Departments of Obstetrics and Gynecology and Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, TX, USA*
[e] *Division of Neonatology, Department of Pediatrics, Erasmus MC, Rotterdam, the Netherlands*
[f] *Division of Vascular Medicine and Pharmacology, Department of Internal Medicine, Erasmus MC, Rotterdam, the Netherlands*
[g] *Particles-Biology Interactions, Empa, Swiss Federal Laboratories for Materials Science and Technology, St. Gallen, Switzerland*
[h] *University Paris Cité, Placentech®, Paris, F-75014, France*
[i] *Lund University, Department of Obstetrics and Gynecology, Institute of Clinical Sciences Lund, Lund University, Lund, Sweden*
[j] *Department of Pharmacology & Toxicology, Radboud University Medical Center, Nijmegen, the Netherlands*
[k] *University of Southampton, Faculty of Medicine, UK*
[l] *Department of Obstetrics, Placenta Lab, Jena University Hospital, Jena, Germany*
[m] *Department of Public Health, University of Copenhagen, Faculty of Health Sciences, Copenhagen, Denmark*
[n] *AstraZeneca, Regulatory Centre of Excellence, Cambridge, UK*
[o] *Department of Obstetrics and Gynecology, Medical University of Graz, Graz, Austria*
[p] *Maternal and Fetal Health Research Centre, University of Manchester, UK*
[q] *Manchester Academic Health Sciences Centre, UK*

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ABSTRACT

Since the full development of the *ex vivo* dual perfusion model of the human placenta cotyledon, the technique has provided essential insight into how nutrients, lipids, gases, immunoglobulins, endocrine agents, pharmaceuticals, chemicals, nanoparticles, micro-organisms and parasites might traverse the maternofetal barrier. Additionally, the model has been instrumental in gaining a better understanding of the regulation of vascular tone, endocrinology and metabolism within this organ. The human placenta is unique amongst species in its anatomy and transfer modalities. This orthologous diversity therefore requires an appropriate consideration of placental transfer rates of compounds, particles and micro-organisms specific to humans.

Different research centres have adapted this model with a wide variation in perfusion parameters, including in the establishment of perfusion, perfusate composition, gassing regime, cannulation method, flow rates, perfused tissue mass, and also in the application of quality control measures. The requirement to harmonise and standardise perfusion practice between centres is largely driven by the need to obtain consistency in our understanding of placental function, but also in the qualification of the model for acceptance by regulatory agencies in drug and toxicology testing.

* Corresponding author. Maternal and Fetal Health Research Centre, University of Manchester, UK.
E-mail addresses: henning.schneider@hispeed.ch (H. Schneider), christiane.albrecht@ibmm.unibe.ch (C. Albrecht), maahmed@utmb.edu (M.S. Ahmed), m.broekhuizen@erasmusmc.nl (M. Broekhuizen), leonie.aengenheister@lih.lu (L. Aengenheister), tina.buerki@empa.ch (T. Buerki-Thurnherr), a.danser@erasmusmc.nl (A.H.J. Danser), sophie.gil@u-paris.fr (S. Gil), stefan.hansson@med.lu.se (S.R. Hansson), Rick.Greupink@radboudumc.nl (R. Greupink), Rohan.Lewis@soton.ac.uk (R.M. Lewis), markert@med.uni-jena.de (U.R. Markert), lima@sund.ku.dk (L. Mathiesen), Nicola.PowlesGlover@astrazeneca.com (N. Powles-Glover), christian.wadsack@medunigraz.at (C. Wadsack), paul.brownbill@manchester.ac.uk (P. Brownbill).
[1] Joint first authorship.

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A pilot study is proposed, aiming to describe how existing inter-centre variation in perfusion methodology affects placental metabolism, protein synthesis, oxygen consumption, the materno-fetal transfer of key molecular markers, and placental structure.

1. Introduction: what we can learn from previous studies

The human placenta provides a haemomonochorial barrier between the maternal and fetal blood from approximately week 10 of gestation, with the maternal and fetal blood circulation in close juxtaposition at the terminal villi, separated by the fetoplacental endothelium, trophoblast cells and the syncytiotrophoblast. In the *ex vivo* dual perfusion model of the human placenta, the two circulatory systems are simulated with prepared perfusates. The method is usually performed on term placentas from normal pregnancy, with tissue frequently taken from elective Cesarean sections. However, in a different paper within this special edition of Placenta, *ex vivo perfusion of the human placenta to investigate pregnancy pathologies*, the authors describe its application to diseased pregnancy groups, where placentas were perfused from preterm, including vaginally delivered cases. This separate paper highlights the capability of using the model to explore the effects of inflammation, oxidative stress and resulting dysregulated biochemistry and physiology found in pathologies associated with diseases of preeclampsia, fetal growth restriction, diabetes and microbial infection. One complete example of this technique in tissue from normal pregnancy is given by Henning Schneider, although there is variation between laboratories [1]. The *ex vivo* perfusion method is unique in preserving tissue architecture and is the only ethically compliant one providing information on total transplacental transfer of untested medicines and xenobiotics in the human.

Dual circulating perfusates encounter the exchange barrier; a specialisation of the terminal villi called the vasculo-syncytial membrane, where the distance between the two circulatory system is a mere 2–3 μm, with a noticeable absence of single trophoblast cells [2]. There are many modes of transfer that a drug or nutrient might take in crossing the placental barrier. Considering Fick's Law, relating to passive diffusion, pathway shortening at the vasculo-syncytial membrane is key to the efficient transfer of nutrients and xenobiotics, particularly for hydrophilic substances [3].

Mathematical modelling has added to our understanding of the intricacies of flux across the placental barrier, through the derivation of the dimensionless Damköhler number for different compounds [4]. Factors affecting this include: the placental architecture; the solubility coefficient, or solute diffusivity in tissue and also in plasma; and physical dimensions of the terminal villi and intervillous space that influence pressure drops within the two circulations. In considering this further, some parameters of the tissue are preserved between the *in vivo* and *ex vivo* environments, such as the length of terminal villi capillaries; the diffusivity of the solute in tissue; and to some extent, parameters of dimension of the intervillous space, re-inflated by *ex vivo* perfusion [5]. The diffusion coefficient of solute in plasma versus perfusate is also likely similar between these environments. Other deriving factors of the Damköhler number might deviate from the *in vivo* environment with *ex vivo* perfusion, depending on the method of perfusion employed, including network resistance as a whole, which in turn is influenced by the endocrine and paracrine milieu [6]; viscosity [7]; fetoplacental flow rate [8], and how this relates to perfused tissue mass; fetoplacental flow pulsitility (see publication by Martin et al in this special edition of Placenta) [9]; and albumin type and concentration [10]. A limitation of the perfusion model to emulate *in vivo* transfer might therefore be in its deviations of parameters of flow and perfusate composition affecting resistance; and the capacity to supress oxidative stress and inflammation that might alter barrier thickness and resistance to flow. The adequacy of oxygenation in *ex vivo* dual perfusion to support metabolism needed for active transport and efflux mechanisms are other factors that might influence the transfer of some xenobiotics and are considered in detail below.

In developmental toxicology testing by the pharmaceutical and chemical industry, the common approach of assessing the pharmacokinetics of placental transfer is through animal testing, usually employing *in vivo* rodent and rabbit models. *Ex vivo* perfusion of the human placental lobule has the potential to provide more accurate estimates of transfer across the human placenta *in vivo*, and improve risks assessments on fetal exposure levels. It is worth a short comparative assessment of the key attributes affecting transfer between species. Structurally, the notional pore radius of the human placenta is thought to be similar to that of the rabbit and guinea pig, approximately 10 nm [11]; sufficiently wide to permit the passage of most drugs and small non-ionized environmental chemicals, but not for and most (nano)particles, macromolecular biopharmaceuticals, and structurally large pollutants and chemicals. Instead, these seem to be selectively transported by active (endocytotic) uptake mechanisms [12]. Molecular charge may also influence transfer efficiency, as found in the guinea pig [13]. Many active and facilitated transfer modes also exist in the syncytiotrophoblast present as influx- and efflux transporters, but their expression patterns are likely to be quite species-specific. Gases and lipophilic molecules pass unhindered across the phospholipid bilayers in the barrier, with rates greatly influenced by flow [3]. However, different species show differences in flow dynamics across the placental barrier; the rat and rabbit have labyrinthine maternal blood flow arrangements, whereas the human placenta has a less well defined "villous flow" pattern [14–16]; and this difference will have implications on transfer rates of compounds [17]. Aside from primates, the human placenta is unique in its architecture and transfer capacity. Being haemomonochorial, there is only one trophoblast cell layer present at the exchanger site, whereas in rodents there are three. The yolk sac is key to IgG transfer, but this degenerates in the human by the second trimester and the chorioalantoic placenta takes over this role. This is distinct from rodents, where the yolk sac placenta continues to transfer IgGs throughout pregnancy. Therefore, traditional species employed in development and reproductive toxicology testing for placental IgG transfer are of limited value.

The technique of *ex vivo* dual perfusion of an isolated cotyledon of human placenta was first published by Schneider et al. in an International Journal in 1972 [18]. There have since been hundreds perfusion studies published. To date, many centres have collaborated and contributed to the understanding of *ex vivo* placental function and also the advancement of the model, building on the foundation laid down by Professor Schneider. Variation in the human *ex vivo* dual perfusion technique has arisen from a need to adapt it for a variety of study types, investigating: nutrient transfer and modelling [18,19]; pharmacokinetics [20,21]; transfer of other xenobiotics [22–24]; nanoparticle transfer, including a consideration of its size, surface modifications and biocorona [10,25]; endocrine release [26]; placental metabolism [27]; oxygen consumption and oxygen uptake [28–30]; regulation of vascular tone [8,31]; non-infectious maternal and placental pathologies [32–34]; and parasitology and virology [35,36]. It has also been compared with other human placenta models [37]. It is clear that there is an occasional need for bespoke and varied experimental designs in perfusion methodology, especially to address studies linked to mathematical modelling. However, most studies focus on pharmacokinetic and xenobiotic transfer; and it is for this purpose that a standardised approach needs to be achieved if regulatory acceptance is to be gained.

There are limitations in the use of the model, including the short timescale in which tissue architecture preservation can be assured.

Failure to meet important quality controls is an obstacle in all centres as these experiments come with specific challenges: limiting post-delivery ischaemic time and the discarding of preparations when leakiness occurs from the fetal to the maternal circulation through post-partum tears in the villous tissue. Commonly, perfusion of two lobules from the same placenta is not possible, affecting the capacity for paired control perfusions. However, this is overcome by standardising xenobiotic transfer to inert lipophilic and hydrophilic clearance markers included in the maternal perfusate when studying pharmacokinetics; or otherwise, perfusing lobules from different pregnancies as temporal control investigations. The ability to match flow to perfused tissue mass is also a challenge, if attempted at all, and there tends to be an avoidance of perfusion of central lobules due to the need for extensive chorionic plate ligation.

In this paper, we review the key experimental parameters that may vary from centre to centre, before setting an objective to determine which factors are in need of a standardised approach.

Objective: This paper sets out to assess the important physiological parameters that might yield variability in placental tissue responses between centres according to in-house protocols, with a view to piloting a study to test the importance of key variables in how they shape transfer data. This is particularly important for bio/pharmacological studies, but also for traditional medicines and other xenobiotics. We will first appraise some of those variables before suggesting a baseline testing protocol to explore inter-centre variability in: metabolism, protein synthesis, oxygen consumption; maternofetal marker transfer profiles; and post-perfusion placental structure arising from variation in perfusion methods.

1. & 2. Metabolism & protein synthesis: The mode of metabolism and level of protein synthesis in the human placenta depends on oxygen supply. Energy metabolism of the perfused tissue varies depending on the supply of oxygen. In one study we have investigated glucose metabolism using ^{14}C labelled D-glucose [27] (Table 1). When medium in the maternal circuit was equilibrated with 95% O_2 and 5% CO_2, consumption of glucose was 6.96 ± 2.50 μmol/h/g. During severe hypoxia (95% N_2 and 5% CO_2) consumption of glucose almost doubled to 12.50 ± 3.36 μmol/h/g. At the same time, conversion of labelled glucose to lactate dropped only slightly from 77% to 65%, suggesting a compensation in terms of energy generation by a switch from aerobic to anaerobic glycolysis. With medium diluted with fresh human whole blood equilibrated with air and 5% CO_2, consumption of glucose increased to 13.75 ± 3.39 μmol/h/g, whereas conversion of labelled glucose to lactate dropped to 22%. Although there was no collection of radioactive CO_2, the recovery of total radioactivity was 85% with no significant difference for the three groups, suggesting that in the group with medium diluted with fresh human whole blood the major percentage of glucose was neither metabolized aerobically nor anaerobically. In this group, 50% of radioactivity recovered from perfusate and tissue extract was precipitable with perchloric acid. This suggests that with increased supply of oxygen a significantly higher fraction of glucose is channelled into synthesis of proteins. This is consistent with the stimulation of synthesis of human placental lactogen, which had been found with perfusion medium containing whole blood [28] (Table 2). It is reasonable to assume, that with an oxygen consumption close to the *in vivo* situation the overall functional state of the tissue will be a better reflection of what happens *in vivo*. This can only be achieved with the addition of blood to the perfusion medium, but the added technical problems are substantial. One of the main problems stems from haemolysis caused by peristaltic roller pumps, and the release of cell free haemoglobin, which in turn evokes vascular inflammation and the sequestering of nitric oxide, a key paracrine vasodilator in the placenta, therefore causing vasoconstriction [38]. Nonetheless, the use of blood-free perfusates have been found to provide for active physiological processes within the tissue.

Table 1
Production of lactate as a percent of glucose consumption.

Oxygenation	Glucose consumption μmol/h/g	Lactate in %
95% O_2 + 5% CO_2 n = 5	6.96 ± 2.5	77
95% N_2 + 5% CO_2 n = 5	12.50 ± 3.36	65[a]
Air + 5% CO_2 Blood, n = 4	13.75 ± 3.90	22[b]

Table taken from Schneider [27].
[a] Compared to air + 5%CO_2 (blood): $p < 0.05$.
[b] Compared to 95% O_2 + 5% CO_2: $p < 0.05$.

Table 2
Human placental lactogen production in the dually perfused placenta.

Exp.	Control	Phase I	II	III	
1	1.07	1.04	0.95	–	Air + 5% CO_2
2	0.76	0.51	–	–	NCTC/Earle
3	0.89	1.53	1.67	–	
Mean ±	0.91	1.03	–	–	
s.d.	0.11	0.34	–	–	
4	0.73	2.24*	2.54*	–	Air + 5% CO_2
5	0.81	1.43*	1.31	–	
6	3.40	3.90*	5.90*	5.20	
7	1.10	1.90*	1.80	–	
Mean ±	1.5	2.4	3.4	–	
s.d.	0.9	0.8	1.5	–	
Paired *t*-test against control		$P < 0.02$	$P < 0.04$		

Data are expressed in μg/min/g.
Table reproduced from Schneider [54].

3. Oxygen consumption: The consumption and supply of oxygen in the human placenta have been found to be tightly linked. Under *in vivo* conditions human term placental tissue has a very active metabolism consuming high amounts of oxygen, which when normalized for weight, is in the range of other metabolically very active organs such as brain, liver and kidney [39]. For dual *ex vivo* perfusion of an isolated cotyledon of human placenta, plain medium containing only physically dissolved oxygen is commonly used. A linear correlation between oxygen supply and consumption had been shown and only with a minimum supply of 100 mL/min/kg consumption reaches 10.9 mL/min/kg, which is in the range of *in vivo* estimates [40] (Fig. 1). This is only achieved with medium containing whole blood or red cells, whereas with plain medium containing only physically dissolved oxygen, consumption of oxygen is substantially below *in vivo* estimates.

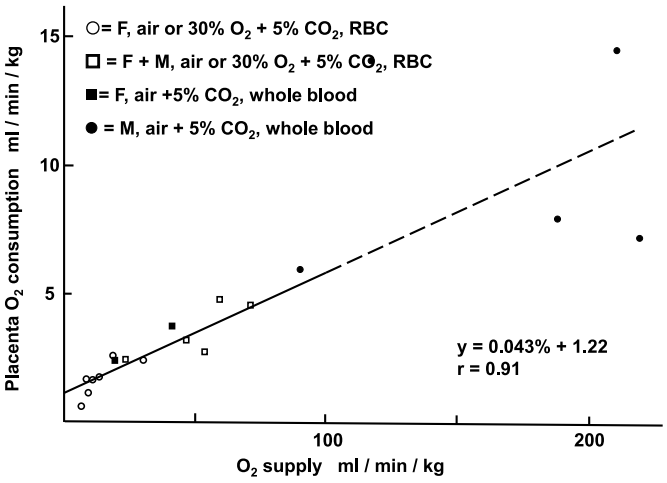

Fig. 1. Oxygen consumption of the human placenta. Oxygen was delivered following equilibration of haemoglobin-containing perfusate with gas mixture. RBC, washed red blood cells suspended in buffer; F, fetal circulation; M, maternal circulation. Image taken from Challier et al., 1976 [40].

As standard in *ex vivo* perfusion, attempts should be made to mimic the partial pressure of oxygen (pO_2). In blood, or perfusion medium within the intervillous space and fetoplacental vasculature, pO_2 reflects physically dissolved oxygen, which is the balance between supply of oxygen and tissue consumption. The pO_2 found under physiological conditions is called "physioxia", which varies in different organs. For *ex vivo* studies of pathological deviations in a particular organ, in a *control* phase a partial pressure close to physioxia should be achieved and in the following *experimental* phase different variables might be tested [41]. In the maternal circulation of the placenta, physioxia changes in the course of pregnancy with a pO_2 around 30 mmHg in early pregnancy, which rises with the start of maternal blood circulation inside the intervillous space (IVS) at the end of the first trimester to around 50 mmHg, continuing to the end of pregnancy [42,43] (Fig. 2). In order to mimic *in vivo* conditions, for *ex vivo* perfusion of tissue from term placentae the target value for pO_2 of the IVS in the control phase should therefore be ideally average between 50 and 60 mmHg.

4. Maternofetal transfer profiles. The surface area available for exchange and flow rates together affect the transfer of inert lipophilic markers such as antipyrine. Whereas the rate of transfer of small hydrophilic markers relate to the surface area for exchange; for larger hydrophilic markers in the low kilo-Dalton range (kD), their transfer rates are linked to the tightness of the placental pores. Creatinine (molecular weight = 113 g/mol) is not likely to be sterically hindered by pore size in its transfer across the human placenta barrier and so its clearance is likely to reflect the extent of contact with the villous tree during perfusion.

5. Placental structure. Post perfusion assessments of endothelial intactness and vacuolisation at the syncytiotrophoblast basement membrane are key considerations in the quality control of placental perfusions. A loosening of the endothelium will likely associate with enhanced transfer of hydrophilic-biased xenobiotics that might otherwise become restricted and could affect vascular resistance to flow. Excessive positive gradients in fetomaternal transmural hydrostatic pressure will likely increase the length of pathway, which according to Fick's law could hinder the maternofetal transfer of hydrophilic-biased xenobiotics, thus under-representing their transfer. However, trophoblast vacuolisation is also significantly associated with fetomaternal leakage, which is expected to lead to rejection of the perfused lobule preparation [44].

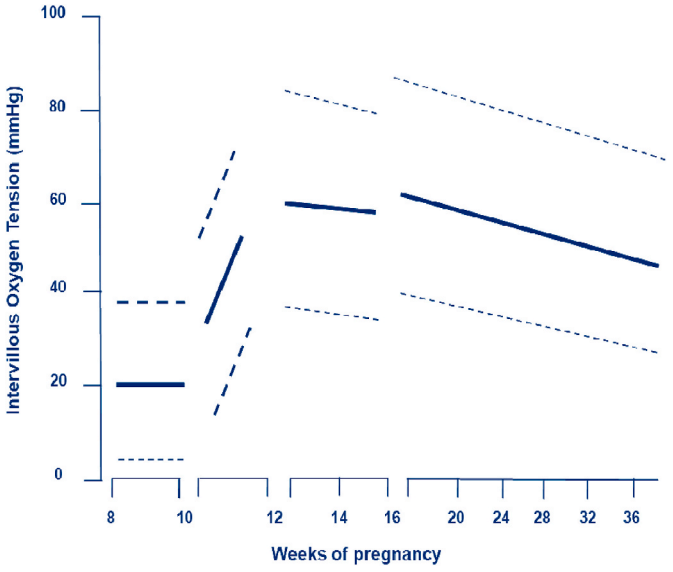

Fig. 2. From S. Zamudio et al., 2011 [42]. The means and 95% confidence intervals of oxygen tension throughout gestation in the intervillous space were taken from: Rodesch et al., 1992; Soothill et al., 1986 & 1987; Jauniaux et al., 1999, 2000 & 2001 [43,55–59].

Variability of approaches to ex vivo perfusion: Having reviewed many papers based on this technique one is amazed by the variety of modifications, which have been developed over the years. Descriptions of frequently used modifications of the technique have been reported [26,37,45,46]. In most of these studies using "normal" term placentae, under control conditions a high variability for basic parameters such as consumption of oxygen and glucose, production of lactate and various placental proteins is found. To find significant differences between "normals" and specimens from common obstetric diseases such as pre-eclampsia, gestational diabetes, fetal growth restriction, pre- or post-mature deliveries etc., a substantial number of experiments would be needed. Therefore, most investigators compare a control with an experimental phase in normal term placentae. Since it is difficult to obtain funding for investigations focussing on comparative evaluation of different modifications of the technique, there are only few respective studies and there still is no widely accepted standard for the quality control phase [47,48].

Moreover, metabolites, hormones and other substances produced by the placenta are found in both the maternal and fetal compartment [46,49,50]. Their release and fetal to maternal ratios are affected by different perfusion conditions such as flow [8] and factors such as alcohol consumption in pregnancy, which might continue to have effects in the *ex vivo* perfused tissue [51]. For example, Burd et al. have summarised the effects of alcohol on placental lobule function, including rapid vasoconstriction, likely through: enhanced thromboxane release; elevated nitric oxide production that fails to compensate for resistance increases in the fetoplacental circulation; and reduced L-arginine transport across the placental barrier [51]. Consequences of elevated villous vascular resistance include a reduction in oxygen transfer efficiency. Therefore, inclusion criteria for accepting placental tissue for perfusion can potentially influence *ex vivo* placental physiology.

The nature of perfusion medium and the circuit (open, semi-closed or closed circuit) must be taken in consideration in the released levels of endocrine and paracine agents, as well as metabolites. An example of a perfusion rig is given in Fig. 3. When both circuits are in open circuit, perfusate flowing through each of the fetal- and maternal circulations leaves the tissue and runs to waste. Closed circuit perfusion implies that the perfusate is recycled from a drip-return reservoir in both circulatory systems (fully), or only in one (semi). Open circuit means that salts and glucose levels are maintained at a constant level; whereas glucose levels slowly reduce in the closed-circuit system through metabolism, whilst lactate levels rise. This represents a gradual shift in glycolysis mode from aerobic to anaerobic metabolism, and occurs if there is excessive recycling from a small reservoir volume and/or if perfusion continues for too long.

Hence, the advantages of open-circuit perfusion are obvious, but closed-circuit perfusions allow a better comparative assessment of transfer rates of test substance in a ratio to inert transfer markers. Commonly a transfer index is provided at a set point into closed circuit perfusion, whilst also auto-correcting for differences in tissue mass between preparations and possible differences in reservoir volumes between research centres [52]. The use of disappearance and appearance curves from respective maternal and fetal reservoirs also provides a means of assessing potential tissue retention of compounds in transfer studies.

The use of albumin in studying pharmacokinetics: Pharmaceuticals are in use by over 60% of pregnant women in the developed world [53]. The maternal to fetal transfer of many medicines and their effects on the fetal circulation are not well understood. The *ex vivo* placenta perfusion model provides a unique opportunity to study the transfer of medications under conditions comparable to the *in vivo* situation. A paper has been published in this special edition of *Placenta* journal, "Placental transfer and vascular effects of pharmaceutical drugs in the human placenta *ex vivo*: a review", appraising many placental pharmacokinetic studies across recent decades. In many perfusion studies, bovine or human serum albumin is added to the perfusion medium to mimic

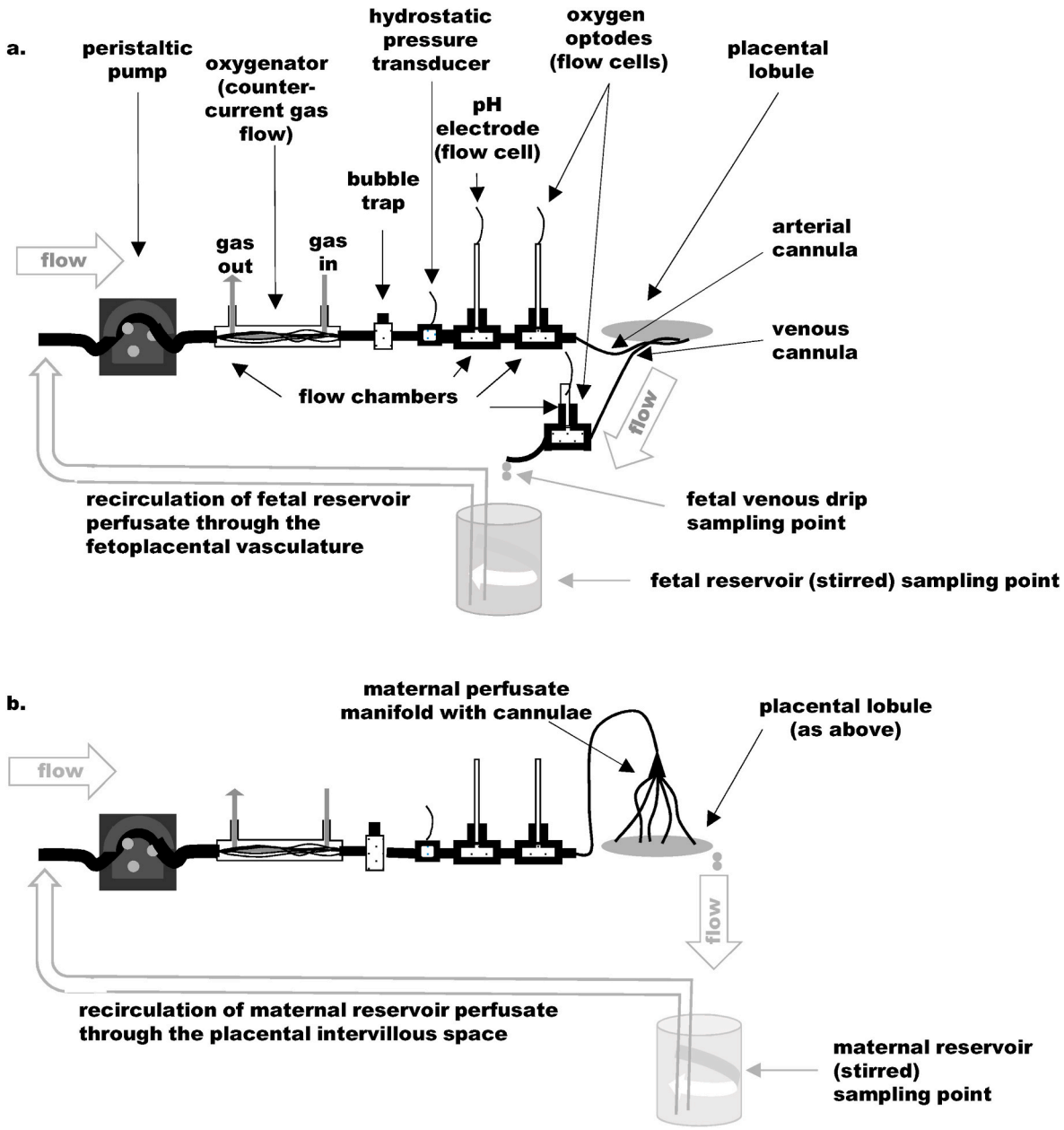

Fig. 3. Example schematic of a rig for the e*x vivo* dual perfusion of the human placental lobule depicting fetal- (**a**) and maternal-side (**b**) perfusion; the capacity to measure real-time in-flow hydrostatic pressure as a measure of resistance to flow; pH; and pO_2 in the fetal and maternal inflow perfusate and the fetal venous perfusate, permitting a measure of tissue oxygen consumption. Options are available to recirculate perfusate in closed-circuit perfusion with reservoir sampling, or send to waste in the open-circuit method with direct sampling.

physiological conditions. This may or may not affect the transfer rates of the xenobiotic [22,23]. However, many medicines have a high affinity for albumin, often reaching >90% binding (not rarely >99%). Consequently, albumin *ex vivo* placenta perfusions can function as a "drug trap". The transfer of high albumin affinity compounds is difficult to observe with accuracy for the free drug, and obviously measurements have to be corrected for free and albumin-bound drug. At free drug levels being <10% (or even <1%) of the applied levels, detecting free-drug transfer may become problematic. This was exemplified by the study of the endothelin receptor antagonist sitaxentan, for which transfer was only detectable in the absence of albumin (Fig. 4). With further complexity, differences in albumin concentration either side of the placental barrier has the potential to influence free drug transfer rates, with acceptor-side albumin acting as a sink for the free drug and thereby influencing the equilibrium of its transfer. On the other hand, for rapidly metabolized drugs, by binding to albumin within the feto-placental circuit, drugs might be preserved in structure, allowing detection. Nonetheless, caution must be given to the clinical interpretation of drug transfer rates, since quantifiable levels are sometimes heavily dependent on albumin dynamics, which might differ from the human *in vivo* circulations, where only human serum albumin is present at physiological levels.

2. Description of a prospective future pilot project

Since variations do exist between research centres in *ex vivo* dual perfusion methodology, some differences may have a bearing on permeability and transfer processes across the placenta barrier, whilst others might not. The main objective of the planned multi-centre project is to find out which modifications of the "control" phase are closest to *in*

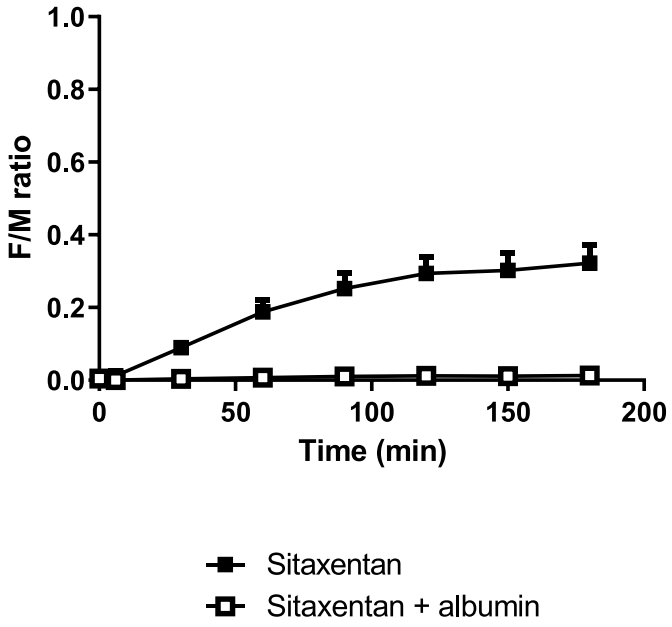

Fig. 4. *Ex vivo* placenta perfusions with albumin in the maternal and fetal circulations prevented the transfer of sitaxentan to the fetal circulation. Data is provided by E Hitzerd, M Broekhuizen, and AHJ Danser. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). The study received exemption for approval from the local institutional Medical Ethics Committee according to the Dutch medical Research with Human Subjects Law (MEC-2016-418 and MEC-2017-418), and all patients gave written consent prior to donating their placenta.

vivo performance of placental tissue and therefore could be recommended as standard procedures.

Key endpoint indicators will be: the prevention of a significant fetomaternal leak; oxygen and glucose consumption; lactate and human placental lactogen production; efficient maternofetal marker clearance profiles for the transcellular and paracelullar markers, antipyrine and creatinine, respectively; stable and low resistance to perfusate flow; stable pH within a physiological range; and intact tissue integrity, specifically endothelial tightness and lack of vacuolisation at the trophoblast layer. This is particularly important in the future guidance for transplacental transfer and endocrine study designs, where the closed-circuit perfusion is being used. The degree of reproducibility of data between laboratories, even where conditions might vary, will be another important endpoint of this study. Groups, who currently are working with the technique of *ex vivo* dual perfusion of an isolated cotyledon of human placenta are invited to join this project and share their data of the "control" phase and further details are provided in the Appendices. The "experimental" phase as described relates to ongoing personal projects will remain unaffected. Co-authorship of the resulting publication will be offered to each participating group.

3. Developmental toxicology applications for the model

European reproductive toxicology testing guidance have recently been modified by regulators (ICH S5 (R3), 2020). Under new European Medicines Agency guidance, there are recommendations for the use of alternative systems to minimise *in vivo* animal studies, as drug discovery screens for evaluating adverse effects on embryo and fetal development [60]. Placental endocrine disruption is to be avoided for non-pharmaceutical products in line with European Chemical Agency guidelines [61]. Hence, there is potential for a renaissance in the use of the human *ex vivo* dual placental perfusion model in assisting "dose level selection", appropriate to the fetal circulating milieu concentration of compounds, as essential prerequisite information in the design of a wide portfolio of *ex vivo* and *in vitro* alternative fetal test systems. With further development, the model could also be used to generate baseline placental metabolic, endocrine and vascular data that might additionally indicate subsequent impacts on fetal morphology and growth.

The importance of this work is in the application of this model to better define human maternofetal pharmacokinetics, particularly of small, large and different modality molecule pharmaceuticals, vaccines, most nanoparticles and macromolecular pollutants, where orthologous diversity in transfer processes present a problem in interpreting *in vivo* animal data; or if used in isolation as one of a battery of tests to add weight of evidence when using alternative assays, instead of a second *in vivo* species. Adding to the value of *in vitro* and *ex vivo* data when using alternatives addresses the 3R's initiative, as well as being quicker and compound/cost saving, rather than running studies in a second *in vivo* species as the previous ICH S5 (R2) guidance version recommended.

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Declaration of competing interest

None of the authors have anything to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2022.05.003.

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