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An international network (PlaNet) to evaluate a human placental testing platform for chemicals safety testing in pregnancy

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ABSTRACT

The human placenta is a critical life-support system that nourishes and protects a rapidly growing fetus; a unique organ, species specific in structure and function. We consider the pressing challenge of providing additional advice on the safety of prescription medicines and environmental exposures in pregnancy and how *ex vivo* and *in vitro* human placental models might be advanced to reproducible human placental test systems (HPTSs), refining a weight of evidence to the guidance given around compound risk assessment during pregnancy. The placental pharmacokinetics of xenobiotic transfer, dysregulated placental function in pregnancy-related pathologies and influx/efflux transporter polymorphisms are a few caveats that could be addressed by HPTSs, not the specific focus of current mammalian reproductive toxicology systems. An international consortium, "PlaNet", will bridge academia, industry and regulators to consider screen ability and standardisation issues surrounding these models, with proven reproducibility for introduction into industrial and clinical practice.

Abbreviations: PlaNet, placentology network; HPTSs, human placental test systems; FDA, Food and Drug Administration; EMA, European Medicines Agency; PLLR, pregnancy and lactation labelling rule; IVS, intervillous space; HUVEC, human umbilical vein endothelial cell; NET, norepinephrine transporter; EMT, extra-neural monoamine transporter; VMAT2, vesicular monoamine transporter 2; NOAEL, no observed adverse effect level; LOAEL, lowest observable adverse effect level; DART, development and reproductive toxicology testing.

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1. Introduction

The human placenta is a critical life-support system that nourishes and protects a rapidly growing fetus. The structure and function of the human placenta is unique [1]; although rats and rabbits are valid models in the evaluation of potential teratogens, interpretation of their transplacental xenobiotic transfer data is precarious. The fetus is potentially vulnerable to xenobiotics that cross the placental barrier, which either cause direct damage to the fetus, or indirectly affect embryo development by interfering with normal placental function.

Even subtle alterations of the fetal-placental environment may have a lasting effect on health in later life and so create a significant economic burden on healthcare economies. Understanding differences in how xenobiotics are handled by the human placental barrier in the diseased state, compared to normal pregnancy, is also a pressing concern (see below). Hence, there is a need to evaluate evidence for differences and consider how HPTSs might be adapted to meet future niche study demands. The uteroplacental compartmentalisation of biopharmaceuticals is most likely different in humans, since such compounds are often handled by receptor-mediated endocytosis that differs in other mammals; and the relevance of these medicines will expand in the future [2,3]. Below, we outline several HPTSs that could be advanced to standardised applications for use in toxicology testing.

2. Consideration of human placental test systems (HPTSs)

2.1. Added value to mammalian testing

Currently zebrafish larvae and stem cell models are becoming accepted worldwide as a pre-screening tool for embryofetal developmental endpoints. These models are beginning to complement mammalian reproductive toxicology testing, conducted much later in the drug development lifetime [4–6]. However, the zebrafish and stem cells lack a placenta. In order to add a further weight of evidence to the mammalian regulatory studies, there are plans to develop human placental assays. A human placental testing platform may help fill this technology gap (see Fig. 1). The human placenta is a readily available, ethically unproblematic human tissue that could be used to assess placental effects and human transplacental transfer, subject to conforming to national and institutional guidelines and the standards set by the Declaration of Helsinki [7]. This declaration guides physicians and researchers in the ethical principles of handling human subjects, human tissue and associated data, to safeguard the health and interests of people. Professionals must act primarily in the interests of patients. Whilst the declaration accepts that experimentation on humans is an inevitable part in the advancement of medicine, it upholds the interests of the patient as a precedent above advancements of science and society. It defines the usefulness of medical research in the development of prophylactic, diagnostic and therapeutics, as well as in providing an understanding of disease aetiology. Consent must be achieved, but only if the participant is able to make an informed choice without coercion; and where processes are also subject to local statute.

Careful thought is needed to address many caveats in the introduction of a new human testing program. For instance, the responses to toxins are sometimes dependent on genetic predispositions affected by ethnic diversity and environmental conditions [8–10]. Since ethnic make-up and the environment that local populations are exposed to vary enormously within and between nations, this will affect the cohort demographics of the local obstetric clinic and therefore the recruits to associated research centres where the placentas are used. It is unknown how such uncontrolled circumstances might influence variability of placental function, so research development into the utilisation of human placental tissue needs a study design inclusive of a much wider global diversity. Thus, the complexity and multi-faceted nature of toxicological testing in the context of regulatory procedures and industrial practices would require a scale of expertise and cooperation beyond a few national research centres for the evaluation of risk where human placentas are being evaluated. This paper considers the establishment of an international partnership amongst academia, the pharmaceutical industry, standardisation institutes, and the regulators, to (i) review our understanding of human placental transfer processes of xenobiotics; (ii) consider the current state-of-the art in human placental models, emerging bio-physical sensing technology and mathematical modelling; (iii) consider short-term scientific missions between academia, industry and regulators, trialling pilot studies, harmonising practices, writing standardised protocols and training a global network of laboratories in best practice; and (iv) to present outcomes of selected human placental test systems (Fig. 1).

2.2. International placentology network

A network called “PlaNet” (placentology network) is being formed, bringing under one global safety umbrella different facets of chemical effects on fetal survival, growth, function and development, building a critical mass of international cutting-edge expertise in experimental human placental test systems (HPTSs), pharmacology, toxicology, drug delivery and mathematical modelling. This umbrella will additionally cover the global education and training in chemical safety testing with human placenta-based techniques. PlaNet aims to engage academics with the regulatory authorities, reproductive toxicology societies and the pharmaceutical and biotechnology industries, to steer the HPTSs and associated modelling approaches towards standard operating procedures, connected to meaningful screening assays, recognised by the regulators for routine compound testing. As a part of these efforts, the proposed network will also consider innovative methods towards reducing the number of animals used as drug safety models, while improving the reliability of the data.

3. Relevance and timeliness

Standardised HPTSs would provide species relevant data and enable a consideration of placental functional and transfer effects of xenobiotics. They would deliver additional evidence to complement rat and rabbit reproductive toxicology data and newly emergent zebrafish larvae and stem cell functional data used in pharmaceutical testing. With this evidence, medicines labelling could be written on a firmer basis for the informed benefit of obstetricians and patients. Proposed changes in OECD guidelines will soon necessitate a consideration of manufacturers to engage in animal and *in vitro* testing of chemicals. Standardised HPTSs may offer a cost effective means of providing data to assist in safety data sheet writing by the chemical industry.

3.1. Reproductive toxicology testing in the pharmaceutical industry

Reproductive toxicity refers to the adverse effects of a substance on any aspect of the reproductive cycle. Due to the complexity of the mammalian reproductive cycle, it is hard to model the whole cycle in a single *in vitro* system in order to detect chemical effects on mammalian reproduction [11]. Development and reproductive toxicology testing (DART) studies in rats and rabbits form the main basis for regulatory assessment of the potential effects of pharmaceuticals on the developing fetus. Current approaches assessing the

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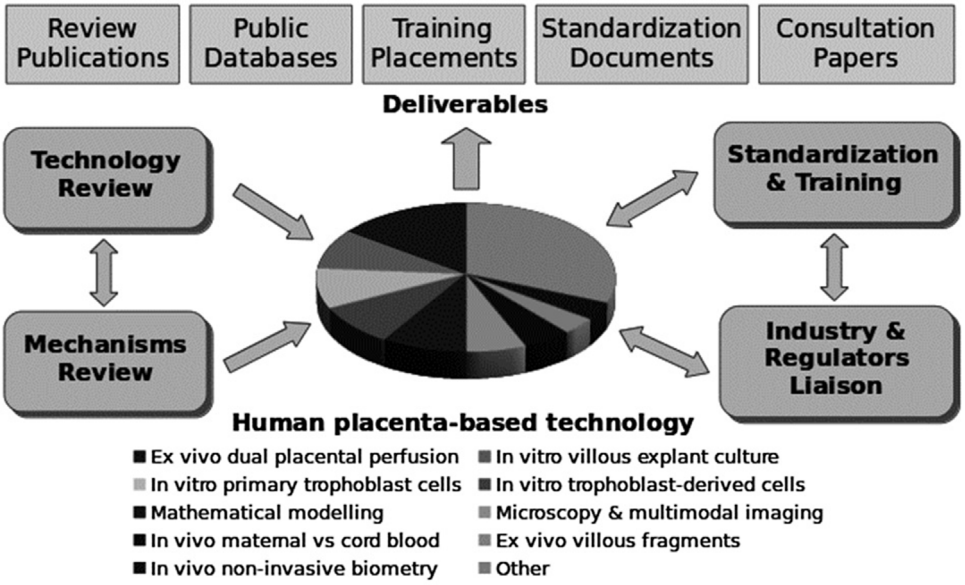

Fig. 1. Demonstrating the academic capacity for human placental test systems amongst the current PlaNet (placentology network) research centres and how this will be exploited for the purpose of advancing new methods in industrial reproductive toxicology testing.

safety of pharmaceuticals in women of child bearing potential are conducted in animals using highly regulated agreed predictive procedures following regional or international guidelines to assess the risk of a compound on the developing fetus. There has been a recent decision by the US Food and Drug Administration (FDA) to increase awareness of the effects of prescription medicines on women during pregnancy [12]. However, all toxicity testing (*in vivo*, *in vitro* and *in silico*) has limitations when extrapolating from animals to the human or even from cell to whole organism. More approaches are being investigated, both *in vitro* and *in silico* to improve the weight of evidence of data in order to build a bigger picture for risk assessment for a compound. Safety testing is of worldwide concern and therefore international acceptance of new approaches and models is an important element in addressing the challenge. A key issue is to increase the screen ability of test compounds within new test systems as well as to prove the reproducibility of the models.

3.2. Environmental exposure to chemicals in pregnancy

Aside from pharmaceuticals, a wider spectrum of environmental and consumer chemicals may affect fetal development and have been associated with adverse reproductive, neurobehavioral and immunological effects later in the child's life. Improved assessment of the human health risk posed by chemicals therefore requires additional information about placental transfer and hazardous effects of substances [13]. The collective expertise of PlaNet should help to identify appropriate techniques and procedures which can protect the mother and the fetus from health risks arising from their exposure to environmental and consumer chemicals. The proposal to progress human placental systems from a pure academic interest to recognised regulated test systems will be extremely innovative and will necessitate close scrutiny of their usefulness along with tight validation.

The toxicology of industrial chemical testing is guided by OECD regulations relating to agrochemicals, with OECD TG 421 covering reproduction and developmental toxicity screening testing [14]. These recommendations have recently changed, requiring an assessment of "internal dose" in hazard assessments, leading to the internal dose metric studies in animals. With a heightened requirement for toxicokinetic analysis, there are triggers in this regulation for the requirement to study endocrine disruption, covering some of the sensitive periods of development, including the pre-natal period [15,16]. Only short duration *in vivo* animal studies are expected to be undertaken; and increasingly, *in vitro* methods are being used in this field, including the use of mouse and human stem cells. Since the human placenta is a key endocrine organ in pregnancy, the future use of HPTSs could bring added benefit in the elucidation of any disruptive effects on placental endocrine function. The European Chemicals Agency has accepted the OECD guidelines, with a principled view by the European Union of banning the production and import of endocrine disruptors [15]. A new EU draft guidance suggests that every registered industrial chemical produced at levels of 10 t/annum or more must undergo tests for potential effects on development and reproduction. The document states that the level of testing, including considerations on the number of species tested and the recommendations and requirements for extended one-generation reproductive toxicity studies (EOGRTS) should be dependent on the thresholds reached for tonnage of production. These practices relate to the EU marketplace; elsewhere global expectations of the chemical industry vary.

3.3. Information permitting informed patient decisions

To optimise safe drug administration in Europe, the European Medicines Agency (EMA) has suggested that new rules for content and format of labelling for drugs in pregnancy are required to include "narrative sections on risk summary, clinical consideration, and a data section including more detailed information" [17]. Under EMAs Pregnancy and Lactation Labelling Rule (PLLR) this has already led to a reconfiguration of safety data information, based upon existing *in vivo* mammalian data; but new formats will remain devoid of any potential further functional data until regulatory acceptance of zebrafish, stem cell and human placenta models becomes a full reality. With the help of leading international academic and clinical research centres, medicines' regulators and representatives of the pharmaceutical industry, PlaNet could enhance the quality of information leading to such labelling by providing additional information on human placenta and effects and human-specific maternofetal transfer pharmacokinetics. Likewise, The FDA has recognised the limitations of the current drug labelling system and safety testing practices that potentially compromise clinical care of patients and their children and is also recently

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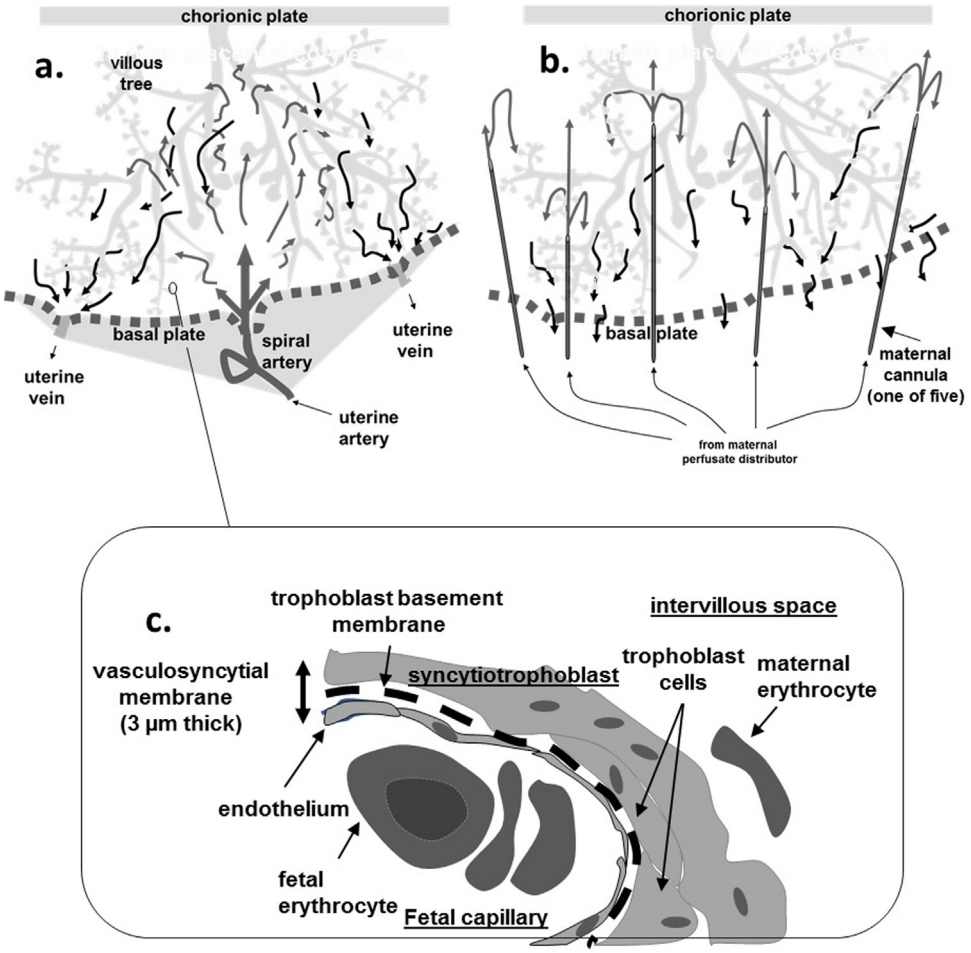

Fig. 2. Cross sectional diagrammatic representations of the anatomy of the term human placenta. (a) Shows the juxtaposition of maternal blood flow to the villous tree, which emanates into the intervillous space from spiral arteries, thought to empty into the centre of a cotyledon, followed by percolation through the spaces between multiple villi (light shaded arrows), before returning for uterine vein drainage (dark arrows). (b) Demonstrates the probable maternal-side intervillous space flow geometry in the *ex vivo* dually perfused human placental lobule, as physiological buffer emanates from multiple maternal cannulae into the intervillous space at a considerable depth (circa 1 cm) into the tissue. (c) Illustrates the terminal capillary bud of the villous tissue, highlighting the vasculosyncytial membrane, a thinning of the syncytium and capillary endothelium that separates maternal and fetal blood, enabling a more effective diffusion, which is particularly important to hydrophilic solute transfer processes.

guided on the need for the integration of animal and human testing data from reproductive toxicology testing [18]. A European guideline (EMA; Guideline on risk assessment of medicinal products in human reproduction and lactation – from data to labelling; adopted by CHMP 2008) also recommends reporting data on pharmacokinetics of placental transfer of the medicinal product and/or its metabolites [17].

3.4. Filling the knowledge gap

Pharmaceuticals are in use by over 60% of women of childbearing potential in the developed world [19]. Often they, are taken after conception, but before pregnancy has been detected. All medications on the market will have undergone a package of reproductive toxicology testing, including effects on F1 generation development to sexual maturity and mating and behavioural development, in mammalian models. However, many pharmaceutical risk assessments air on the side of caution in their guidance for administration of medicines during pregnancy and breast feeding. With the exception of medicines required to prevent serious maternal morbidity and survival, most pharmaceuticals taken to treat chronic medical conditions are withdrawn on the guidance of the obstetrician once pregnancy is confirmed. This is based on the labelling advice such as "not tested in pregnant women – avoid in pregnancy", found in information sheets for the sheer majority of prescribed items and over-the-counter products. This happens regardless of the fact that regulated mammalian reproductive toxicology testing has been performed. Therefore, one ultimate key goal of PlaNet is to bring into discussion a framework to help make available placental risk-assessments, that provide more meaningful information to clinicians and patients, permitting more informed choices regarding the continued taking of specific medications [20].

4. Placental anatomy

4.1. Placental circulations at the maternofetal interface

Blood circulates between fetus and placenta *via* the umbilical cord which houses two arteries and one vein. The umbilical arteries carry blood from the fetus to the placenta, firstly branching as two divergent patterns across the chorionic plate, then dividing extensively to generate the sub-chorial placental villous trees [21]. The placental microcirculation is present within these villi [22]. Stem arterioles within stem villi branch to form arterioles within mature intermediate villi, dividing further to from sinusoidal capillaries within terminal villi [22]. The villi are the nutrient exchange units of the placenta and their outermost cell layer in contact with maternal blood is the syncytiotrophoblast, a

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transporting epithelium that exchanges solutes between maternal blood in the intervillous space and the vascularised fetal blood. Where the fetal capillaries are most proximal to maternal blood, at the "vasculosyncytial membrane" (Fig. 2c), the capillary endothelium and the syncytiotrophoblast are the only cell layers forming a barrier [23]. Here, trophoblast cells are displaced, as are the organelles of the syncytiotrophoblast. Hence, the length of the pathway for transfer between the two circulations is minimised, ensuring transplacental diffusion is particularly efficient for hydrophilic solutes (Fig. 2c) [24]. Conversely, maternal blood is not vascularised in the human placenta. The functional exchange unit of the human placenta is the cotyledon, comprising of an entire villous tree. Blood enters the intervillous space (IVS) and surrounds the frond-like villi from transformed spiral arteries [25]. Maternal oxygenated blood, replete in nutrients, enters the IVS from a transformed spiral artery into the centre of each cotyledon, encountering many terminal villi during its percolation towards the decidual veins, where it drains from the IVS (Fig. 2a). Within the villous tree, fetal blood returns to the fetus within a vascularised venous system, draining blood from the sinusoidal capillaries, which flows through venules in the villous tree and then through subchorial veins, taking with it dissolved solute acquired from the maternal circulation. On the chorionic plate, fetal placental veins converge and increase in diameter, extending across the chorionic plate, ultimately converging into the single large vein of the umbilical cord which returns oxygen and nutrient rich blood to the fetus [26].

4.2. Human placental pharmacokinetics

EMA notes that it "..is important to consider the transfer of substances entering the embryo-foetal compartment, and that to do so, separate studies are needed to assess placental transfer" [27]. Therefore understanding the pharmacokinetics of human placental transfer is of key importance to appreciating fetal exposure in the human. Placental transfer efficacy varies between species, attributable to variation in placental structures, pore size and haemodynamic factors [1]. In the human placenta, as a rough guide, if a hydrophilic molecule exceeds 5 KDa (a molecular radius equivalent to circa 1–2 nm), placental transfer by simple diffusion in the maternofetal direction is effectively zero, dictated through steric hindrance at the paracellular route, which has a mean notional pore radius of approximately 10 nm (Fig. 3) [28–30].

In contrast, lipophilic molecules transfer across the placenta more like gases. Their transfer is less constrained by species differences in placental barrier morphology, but instead highly influenced by the factors associated with placental blood flow, which acts to negate their transmembrane equilibrium [31]. For these lipophilic molecules, (i) the rate of fetoplacental flow; (ii) the maternal and fetal blood flow geometries and (iii) the occurrence of villous blood shunts are the main drivers of transfer efficacy [32]. Different flow geometries confer upon the placenta differences in transfer efficiency between species, which are unrelated to the specific histological nature of the barrier [33].

For monoclonal antibodies with a molecular weight in the order of 150KDa, the Fc (FcRn) receptor is a specific transport mechanism; its expression widely varies between species, making the human *ex vivo* placental perfusion the model a vital choice in studying biopharmaceutical maternofetal pharmacokinetics [34]. Furthermore, for non-human primates and humans, IgG placental transfer is low in the first trimester, increasing in the early second trimester, reaching highest levels late in the third trimester [35]. This contrasts markedly with rodents, where the yolk sac placenta constitutes a possible earlier pathway in IgG maternofetal transfer by FcRn transport mechanisms [36,37]. Thornburg and Faber demonstrated that guinea pig yolk sac vessel ligation prevents gamma globulin transfer to the fetus [38]. However, the human yolk sac is only present within the first trimester, becoming markedly degenerate by week 9 [39]; a period in which human fetal antibody accrual is in any case very low [35,37].

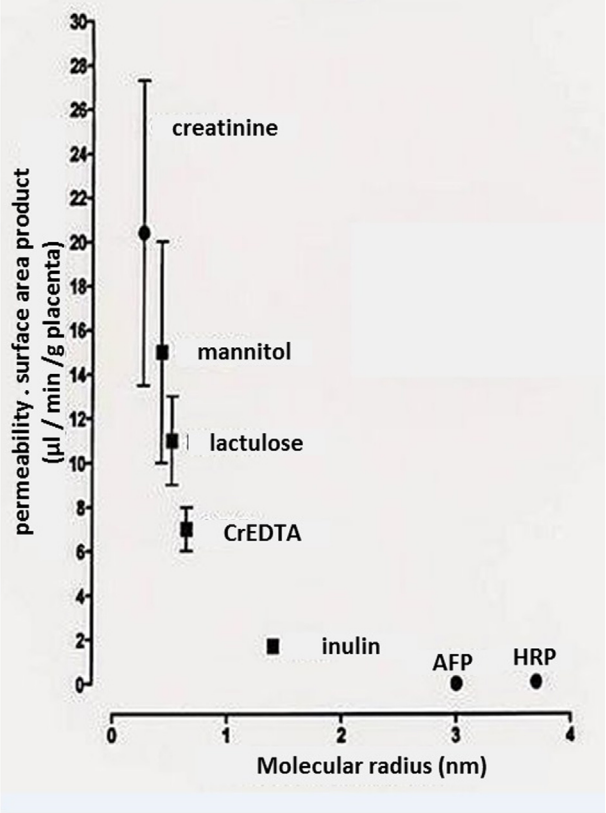

Fig. 3. Maternofetal permeability of the human placenta to inert hydrophilic solutes by diffusional transfer. Reproduced with the kind permission of Prof C.P. Sibley.

4.3. Current regulatory embryo and fetal development assessment guidance

In relation to pharmaceuticals, the FDA has outlined non-clinical data needed for the integrated assessment of in human development and reproductive risk, citing general toxicology, reproductive and developmental toxicology, and pharmacokinetic studies [18]. There is some flexibility in reporting risk relating to the type and extent of available toxicology data, depending on the product's biological actions, the test systems available for studying the compound and the positivity of the data [18].

HPTSs, therefore have the potential to be coupled to assays that might add value to risk assessments by providing new relevant and validated studies, with outputs that could feed into the decision tree process. To reach this goal, their predictive *in vitro* mechanistic relevance must be proven to link to an *in vivo* effect and the relationship between the *in vivo* outcome and the *in vitro* test result must be explained [40]. In a recent concept paper, EMA has highlighted that laboratories that participate in large collaborative studies are in a great position to publish methods in regulatory texts, as they will gain more valuable experience with the method as a whole. The agency encourages facilitation towards gaining laboratory and product specific validation and the implementation of methods in other laboratories [41].

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4.4. Network objectives

The highly interdisciplinary network will critically assess existing HPTSs (see below) for development, through the implementation of best practice in standards with proven reproducibility, acceptable for systematic safety testing of pharmaceutical and consumer products used during pregnancy. PlaNet aims to capacity build internationally, with training for new investigators in the use of techniques, once harmonised. Another objective is to complement existing mammalian toxicology databases with a publicly accessible database containing human placental data, linked to potential toxicology mechanisms and associated testing technology. There is also a need for the promotion of integration of clinical, experimental and computational *in silico* tools for understanding chemical transfer and effects on placental function in healthy and compromised pregnancies. The network plans to publish "off-the-peg" protocols for use by industry in toxicology testing, although some of the non-cell line techniques will need to be performed within academic research centres, where access to fresh human placental tissue is possible *via* associated obstetric departments. In the latter case, trained academic centres with regulatory laboratory accreditation will be able to act as contracting consultancies for testing panels of compounds, as required by industry.

A wide range of HPTSs are already in use by the academic community on a variety of research themes, including xenobiotics transfer and pharmacokinetics [42]; blood flow [43], nutrient and ion transfer [44,45]; oxygenation effects and osmolality regulation [46,47]; endothelial signalling [48], angiogenesis and vasculogenesis [49]; inflammation and immunology [50,51]; placental trophoblast cell turnover, multi-nucleation, differentiation and regeneration [52]; DNA damage and repair, genotoxicity and carcinogenesis [53]. Below we give an overview of the state-of-the-art in the current employment of HPTSs.

4.5. Ex vivo *dual placental perfusion of the human placenta*

Currently, this is the only HPTS capable of providing reliable transfer data of xenobiotics across the human placenta. The level of organisation of the tissue with this model is far more likely to approximate to *in vivo* performance than working with oversimplified tissue slices, trophoblast cells, or isolated membrane vesicles [31]. The disadvantages of the model are that (i) its use is prone to a high preparation rejection rate, through intra- and post-partum breakages occurring randomly throughout the lobule and if perfusion is not established within a reasonable timeframe; (ii) it is a relatively expensive model to employ; (iii) only one lobule can practically be perfused at any one time; (iv) it only lends itself reliably to acute perfusion time periods of no more than 6–7 h; (v) validated perfusion of second trimester placentas has not yet been achieved. The schematic representation of one particular design of an *ex vivo* human placental perfusion rig is given in Fig. 4. Permeability measurement using one or more cotyledons is an integration of the effects of transfer into all the perfused fetal vessels [54–56]. Whilst preventing any insight into specific barrier characteristics at different foci of the villus tree, or across the endothelium of different fetal vessels, the permeability estimates obtained through the *ex vivo* perfusion are nonetheless representative of the intact *in vivo* macro placental structure.

There have been several attempts to devise an acceptable method of *ex vivo* human placental perfusion across many decades in the twentieth century. Internationally, we have come to adopt the description of this model given by Schneider, Dancis and Panigel published in the early 1970's, where the authors describe a mastered adaptation of intervillous space perfusion, complementing the already established method for fetoplacental villous perfusion, using several cannulae which simply pierced the decidual plate to mimic spiral artery blood flow irrigating the outside villous tree [57,58]. A depiction of what this probably means to IVS flow is given in Fig. 2b and how this compares to *in vivo* IVS flow (Fig. 2a).

Although the placenta has a remarkable capacity to enter into anaerobic respiration and "partial metabolic arrest" during ischaemia and low oxygenations [59], several attempts have been made to address the imperfection of super-oxygenation given to the tissue during *ex vivo* perfusion, which is employed to compensate for a poor oxygen carrying capacity in blood-free conditions [60]. The ideal methodology of oxygenation is still undetermined and varies enormously between research centres [61,62]. As mentioned above, the cotyledon is the functional unit of the placenta described from the fetal villous aspect, but the number of these included on *ex vivo* perfusion will vary. In addition the juxtaposition of maternal cannulae within the IVS (Fig. 2b) means that maternal and fetal flow could mismatch, with variation between preparations. Hence, in order to standardise placental transfer data for a compound, transfer rates of the xenobiotic is standardised against that of an inert lipophilic marker, antipyrine [63], or potentially against test compound enantiomers. Due to protein binding of some drugs, the presence of albumin concentration in the maternal circulation and the species of origin might also affect the pharmacokinetics of transfer [64].

It has been possible to model nutrient transfer across microvillous (apical) and basal membranes of the syncytiotrophoblast, with the knowledge of activities of transporters from microvillous and basal microvessicle experiments [65–67]. Likewise, eventually this should also be possible for all known drug uptake and efflux transporters present in the human placenta. This information could enable an integrated evaluation of complex drug–drug and drug-transporter interactions and physiologically based pharmacokinetics through *in silico* modelling to predict fetal safety, related to maternal pharmaceutical dosing. Real transfer data from *ex vivo* perfusions could then be used to test the accuracy of this modelling.

In addition to studying compound transfer, the *ex vivo* dual perfusion model is useful in elucidating effects on placental endocrinology, inflammation, metabolism, placental immunology; and perhaps even fetal vascular effects, since the fetoplacental vasculature is in continuity with the fetal vasculature and shares the same milieu [59,68–72]. A key challenge of the network is in devising and disseminating a unified protocol for *ex vivo* perfusion that best suits industrial and regulatory requirements for chemicals safety testing.

4.6. Villous explant culture

This is a more chronic tissue culture model allowing a study into effects of a compound on the regenerative capacity of the trophoblast to form a syncytiotrophoblast, or the effect of a xenobiotic on the steady state endocrinology, structure and function of the trophoblast layer. Tiny fragments of dissected villous tissue, approximately 3 mm in length are cultured in suspension on porous mesh support inserts, at the meniscus of culture medium, held within a twelve well culture plate. The culture medium is replaced daily, and at 24 h a surge in hCG release, followed by a very low level of release at day 2, coincides with entire syncytiotrophoblast shedding. From days 2–4 a new syncytiotrophoblast forms and during this time basal levels of hCG become released into the tissue culture medium. By day 5, hCG release is normal and plateaus thereafter. Tissue histology is assessed to ensure regeneration of the syncytiotrophoblast shell, or effects on a known regenerated structure. Lactate dehydrogenase release levels report on disturbances in trophoblast architecture. Functional studies might include effects on amino acid, or other nutrient uptake; culture-conditioned medium can be assayed for the release of paracrine and inflammatory mediators [73]. Optimisation and standardisation of protocols to

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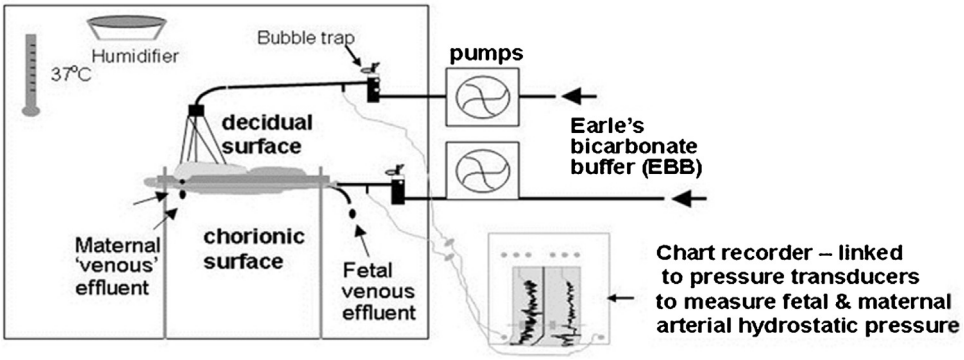

Fig. 4. One example of an equipment design for *ex vivo* human placental dual perfusion. Here the placental lobule is mounted within a double ring structure, with part of the chorionic plate cannulated at the artery and vein (downward facing), corresponding to an identified intact lobule (indicated by light shading; upward facing). There is currently international variability in perfusion rig design, the number of maternal cannulae employed, the mean tissue mass perfused, the buffer composition, the oxygenation regimen, the flow rates used and the adoption of open-, or closed-circuit perfusion (venous perfusate goes to waste, or becomes recycled, respectively).

maintain syncytial trophoblast layer integrity is needed for successful translation. This model can be applied to human placental tissue from all available gestational ages.

4.7. Chorionic plate artery & vein wire myography

Fetoplacental vascular resistance is normally maintained at very low levels, particularly as gestation progresses, ensuring adequate blood flow to the developing fetus [74]. This technique permits an acute or semi-acute assessment of xenobiotics on fetoplacental microvascular function, either through direct exposure of the isolated fetoplacental blood vessels to the compound and assessment of substance effects on vascular tone; or through overnight vessel culture with the test agent, followed by vascular tone assessment the next day. Since the human placenta is not innervated, the functional properties of isolated vessels reflect the endocrine milieu imposed on the test system, as well as the autocrine and paracrine effects maintained within and between cells. This is an important functional assessment, because any constriction effects of xenobiotics on fetoplacental vascular tone relate to the impedance of blood flow between the placenta and the fetus, thereby restricting oxygen and nutrient delivery and impinging on fetal growth and development. Using existing data in a paired analysis, PlaNet could explore the capacity of chorionic plate resistance vessels to correlate with the *in vivo* efficacy of placental blood flow through the use of clinical indices of umbilical arterial Doppler velocimetry and fetal growth outcome data. In this HPTS, blood vessels (100–500 μm diameter) are representative of the stem villous microcirculation and are cut into 2 mm lengths; two steel wires are inserted into the lumen and wire ends secured to steel jaws; one wire is attached to a force transducer and the other to a movable micrometer. The wired vessel sits within a warmed bath containing gassed physiological buffer. The diameter of the vessel lumen can be manipulated using the micrometer and tension generated by constriction/stretch of the vessel monitored *via* the force transducer. Active pressure of the vessels is measured in response to agonists/xenobiotics and relates to their contractility. Dose responses are easily acquired within the preparation and arteries and veins can be evaluated separately. Vasoconstriction and vasodilation measurements can be assessed and the potency of compounds to cause changes from temporal vehicle control or inhibitor effects in a neighbouring bath are reported [75,76]. Standardisation of placental myography protocols, with mutually agreed baseline and rejection thresholds, among over variables, is needed for toxicology testing applications. The model does not represent vascular resistance within the full villous tree.

4.8. Cell culture

A variety of cell types can be isolated from the human placenta, including primary trophoblast cells [77,78], placental microvascular endothelial cells [79] chorionic plate artery and vein endothelial cells [80], fibroblasts [81] and myocytes [82], to assess effects of xenobiotics on their survival, division rates, apoptosis, endocrinology, transporter activity and intracellular and second message signalling. Dual cell culture systems, *e.g.* trophoblasts with fibroblasts [83], or trophoblasts with endothelial cells [84,85] bring a level of complexity enabling a study of effects of an agonist at the primary cell surface to evoke changes in cell–cell signalling and DNA damage, affecting the second cell layer. This level of complexity is considered further (see Section 4.11, below). Adoption of this technique depends on the development of harmonised protocols that address cell line variability, particularly for primary trophoblast cells. It should be noted that the behaviour of a single cell line may change when isolated from the placenta, since it loses the paracrine influence from other cell types. For instance, in most laboratories trophoblast cells will only exhibit limited multinucleation.

4.9. Trophoblast spheroid cultures

The trophectoderm of the blastocyst differentiates into several trophoblast subsets in order to create the placenta in the first trimester of pregnancy. Of these subsets, the cytotrophoblast is considered a putative "progenitor cell," which replenishes the outer layer of the villous (syncytiotrophoblast). The extravillous trophoblast necessarily invades the decidua in a cancer-like manner to ensure that pregnancy is successful. Propagation of trophoblast cells into spheroid aggregates under stem cell culturing conditions confirms the maintenance of the invading/progenitor status of this cell type, proving its capacity for self-renewal. Cells with self-renewing potential can be disaggregated from the spheroids and passaged multiple times with retention of spheroid-forming ability. This provides a potentially useful test in the safety testing of xenobiotics, since interference with this spheroid renewal process would infer dysregulation of the placental barrier and invasion process. In the "hanging drop" protocol, tens of thousands of cells can be amassed into a spheroid, recovered into single cells and re-aggregated into spheroid cultures, or fixed and immune-stained for the existence of "stemness-" associated transcription factors OCT4, NANOG, and SOX2; and for the trophoblast lineage, *e.g.* cytokeratin positivity. Early unpublished work indicates that the trophoblast phenotype (*e.g.* hCG) is maintained in spheroid cultures, which can be dysregulated by transcription and mitosis inhibitors. Spheroids stained with tracer dye can also be assessed for their capacity to

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invade fresh placental explants within the hanging drop, providing functional data related to their "stemness" [86].

4.10. Syncytiotrophoblast microvillous membranes

The maternal-blood facing microvillous membrane of the syncytiotrophoblast can be isolated from human placental homogenate following processing with magnesium precipitation, differential centrifugation and characterisation of purity and reconstituted morphology [87]. In conjunction with Mechaelis-Menton kinetics, they are particularly useful at characterising the presence and relative abundance of specific membrane transporters and the sodium dependency of transfer [88]. However, data on regulatory potential from placental autocrine and paracrine influence is not possible, because of the loss of intracellular organelles and signalling machinery.

4.11. Placenta on a chip

An alliance of placental physiologists, obstetricians, bioengineers, computational and mathematical modellers would provide a novel opportunity to begin to integrate tissue and cell culture systems on a micro-scale with real-time bioanalytical read-outs to recapitulate physiological responses of interest in reproductive toxicology testing; however, the development of a micro-engineered placenta is still in its infancy. Preliminary attempts to micro-fabricate the cellular environment of the placenta, either in a multi-layered system to replicate placental barrier function, or as an organoid culture have shown promising potential, but these models require further characterisation with respect to toxicology testing and need to be better coupled to real-time analysis [84–86]. Advances in this field would facilitate xenobiotic testing, inclusive of more simplified placental transfer experiments and toxicology studies looking at effects on cell survival, homeostatic regulation and endocrinology. Similar concepts have already been advanced for other organ systems including the lung, where bio-mechanics to simulate breathing have been developed, enabling studies on the exposure to nanoparticle stressors, with the evaluation of endothelial pro-inflammatory adhesion molecules expression and intracellular production of reactive oxygen species [89].

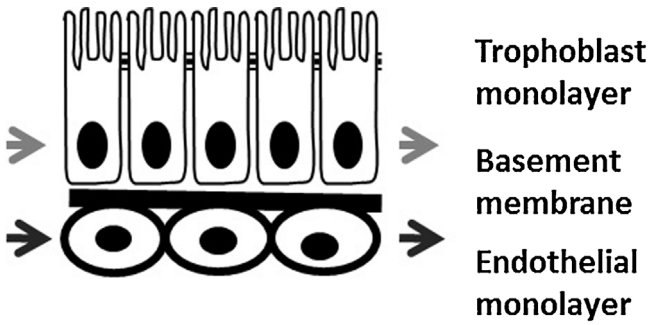

Fig. 5. Diagrammatic model of "placenta on a chip". Trophoblast cells and fetal endothelial cells are grown on an amniotic membrane support in a chamber that allows two independent systems of medium flow (arrows). The 3-D model allows uptake studies from either compartment or transfer across the trilayer, as well as endocrine sampling. The endothelial layer can be replaced by other cell types, as can the chosen basement membrane.

Hamilton and others have simulated the placental barrier, under flow, using retinal epithelial pigment cells, to represent the placental trophoblast layer, and human umbilical vein endothelial cells (HUVECs), replicating the placental microcirculation endothelium (Fig. 5) [84]. They studied the pharmacokinetics of transfer of a 4KDa dextran tracer molecule, with a molecular radius approximating that which is sterically hindered by the placental barrier [28,30]. It was discovered that the inclusion of human placental amnion to form a third middle layer had the effect of supporting junctional integrity [84]. This study demonstrates how a slightly more complex system is supportive of paracrine interactions needed to regulate cell junction interactions in micro-chip models. Indeed this has been shown to be important in other organ-chip systems, including studies on the blood-brain barrier [90]. In a follow up study to the earlier Hamilton work, an *in vitro* biological model of the placental barrier was constructed in another co-culture, this time using the more tissue-specific immortalised human placental trophoblasts and HUVECs on opposite sides of a denuded amniotic membrane [85]. In a further multi-layer cell barrier construct, involving exposing BeWo cells to nanoparticles, other investigators have been able to demonstrate gap-junctional signalling leading to

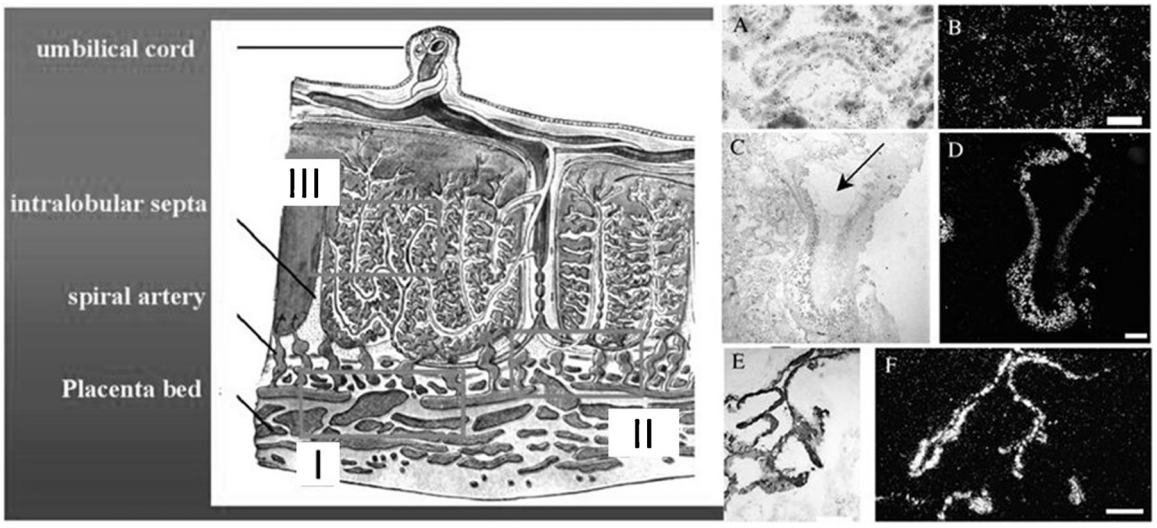

Fig. 6. Cross sectional diagram of the human term placenta modified from Bottalico et al. [101]. A hypothesised three line defence (I–III) against circulating excessive levels of monoamines in the fetal and maternal circulations. (I) Within the spiral arteries *in-situ* hybridisation showed expression of VMAT 2 mRNA in darkfield image (F) in cytokeratin positive cells (E), (II) surrounding the incoming spiral arteries (arrow) *in-situ* hybridisation showed expression of NET mRNA in brightfield (C) and darkfield (D). (III) In trophoblast cells of chorionic villi from the central part of the placenta *in-situ* hybridisation showed expression of SERT in brightfield (A) and darkfield (B). Scale bars: B = 50um D = 120 um, E,F = 220 um.

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inflammatory cytokine release and DNA damage within the second barrier layer [83].

Across many physiological systems, there are increasing efforts in building multi-scale computational models that go hand-in-hand with refined experimental models. Indeed, there has been a recent surge in theoretical studies of placental morphology [91–93] and its relation to haemodynamics and transfer function [65,67,94–98]. We now have the opportunity to combine the synergy of detailed inspection of placental barrier properties *ex vivo* and *in vitro* with the power of testing key mechanisms *in silico* that should accelerate the development of placenta-on-a-chip and its translation to industrial and regulatory use.

The innovative aim of the network is to consider the design of a multicellular placental system that could be used to derive real-time data on the effects of xenobiotics on placental function, since placenta health itself is vital in the support of fetal growth, development and survival. In studying placental spheroids, or exploring the role of microfluidics on anchored placental cells, it should be possible to obtain live bioanalytical data acquired by coupling biosensors with the culture system. Raman spectroscopy, has so far applied to microbial detection, but perhaps is also adaptable to *in situ* metabolic analysis [99]. The challenge is that outcome measures should be indicative of dysregulation of complex human placental physiology whilst permitting an efficient appraisal of large compound libraries [100]. However, it should be borne in mind that such "organ-on-a-chip" technology cannot easily represent of off-target effects of xenobiotics on other organ systems.

5. Human placental drug handling in pregnancy pathologies

Placental barrier handling of xenobiotics, either by transporter or efflux protein activity, could alter fetal exposures to drugs consequential of pregnancy pathologies such as preeclampsia and or fetal growth restriction (FGR). Deleterious effects on the fetus could partly be explained by the failure of the placenta to clear both endogenous hormone/transmitter molecules or pharmaceuticals and other xenobiotic substances from the circulating milieu. An example of this relates to the trophoblast and stromal expression of the monoamine transporters: norepinephrine transporter (NET) and extra-neural monoamine transporter (EMT; or organic cation transporter 3–OCT3) [101]. Monoamines play important roles as physiological paracrine mediators in early pregnancy, including in decidualisation, implantation and immune modulation, but dysregulated levels in early pregnancy risk miscarriage [102,103]. Unlike many other transmitter substances, monoamines are recycled rather than enzymatically inactivated. In neuronal tissues, regulation of their extracellular milieu normally involves their compartmentalisation within neuronal synapses. Whilst the placenta is not innervated, NET, EMT and vesicular monoamine transporter 2 (VMAT2) transporter types appear to serve to clear monoamines from the maternal and fetal circulations. Bottalico discovered that both NET and EMT mRNA expression was reduced in the central parts of human placentas delivered from preeclamptic pregnancies, compared to those from normal pregnancy [104]. Specifically, reductions in the expression of these transporters in myofibroblasts associated with large villous vessels and placenta septa might infer a loss of protection from circulating levels of vasoactive monoamines which become elevated in this disease. These loci normally represent the third line of defence against excessive circulating monoamine levels, after NET and VMAT2 function in trophoblasts (that line the spiral arteries) and high levels of NET expression in trophoblasts lining spiral artery entrances (Fig. 6). In the non-innervated placenta, pseudo sympathetic hyperactivity, consequential of poor NET and EMT expression in preeclampsia, might change placental haemodynamics by affecting maternal and fetal blood flow around and within placental villi and could contribute towards the general elevation of blood pressure within the fetal and maternal circulation [104]. From the perspective of exploiting the placenta as a test model it should be noted that, within the *in vitro* and *ex vivo* human placental tissue, only the septa and villous vessel expression will remain (the third line of monoamine defence), as the post-partum placenta is devoid of spiral artery material.

Other extrinsic pathology factors affecting human placental drug transporter expression and activity include intrahepatic cholestasis of pregnancy where the fetomaternal clearance of toxic bile acids are reversed, leading to fetal accumulation of these substances [105,106]; and the down-regulation of apical transporters and concurrent up-regulation of basal transporters during inflammatory conditions leads to decreased protection of the fetus from toxic effects of xenobiotics [107,108]. It is therefore an essential part of drug risk assessment in reproductive toxicology to understand the effects of dysregulated drug transport on human placenta accumulation and transport of compounds in the diseased state.

6. Innovation and future challenges in reproductive toxicology testing

The outlined European placentology network, PlaNet, aims to catalyse a concerted approach to testing the safety of chemicals for the fetus as part of a group of assessments, using the most relevant human placenta-based tools and procedures. This will only be possible by crossing specialism boundaries and directly engaging with end-users, such as the pharmaceutical industry and regulatory authorities. The synergy and capacity within PlaNet will also allow for a consideration of reproductive toxicology testing, accounting for human variability in placental blood-barrier transfer dynamics, as expected for (i) compromised pregnancies, such as fetal growth restriction, preeclampsia, diabetes, intrahepatic cholestasis of pregnancy and other inflammation related conditions; (ii) cohorts with an unusual exposome, including high exposure to environmental pollutants, that might have already conditioned the placental phenotype; and (iii) genetic differences, including studying cohorts of ethnic diversity and placental polymorphisms; where placental handling of drugs and their derivatives and therefore susceptibility of the fetus to maternal medication might differ.

In addition to understanding placental transfer and using HPTSs to complement current teratology methods, some HPTSs might be suitable for high-throughput pre-reproductive toxicology triaging testing of compounds, perhaps also reducing animal usage to a degree in the longer term. However, developing appropriate models and assays will be difficult, requiring multi-disciplinary collaboration, ensuring techniques are harmonised across many centres and with the need for a proven reproducibility prior to standards being approved by regulatory authorities, including the EMA and the FDA.

In the long term, such endeavours of the network will help contribute to the data on safety in pregnancy labelling. From the pharmaceutical industry's perspective, the critical data that we will be able to facilitate on the pharmacokinetics of human placental transfer will assist in more accurate "dosage margin setting" for maternal medications, in conjunction with other data on "no- and lowest observed-adverse-effect levels" (NOAELs/LOAELs) obtained from animal testing [109]. This is pertinent to chemical compound risk assessment reliability due to differences in placental compartmentalisation between species [2] and other aforementioned extrinsic human factors. In the long term, it could be that early stage triage testing using the reviewed HPTSs may lead to the refinement of animal use in toxicology testing. Liaison with organisations such

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as the European Centre for the Validation of Alternative Methods could advance our test systems towards endorsement by international regulators.

Conflict of interest

None.

Transparency document

The Transparency document associated with this article can be found in the online version.

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