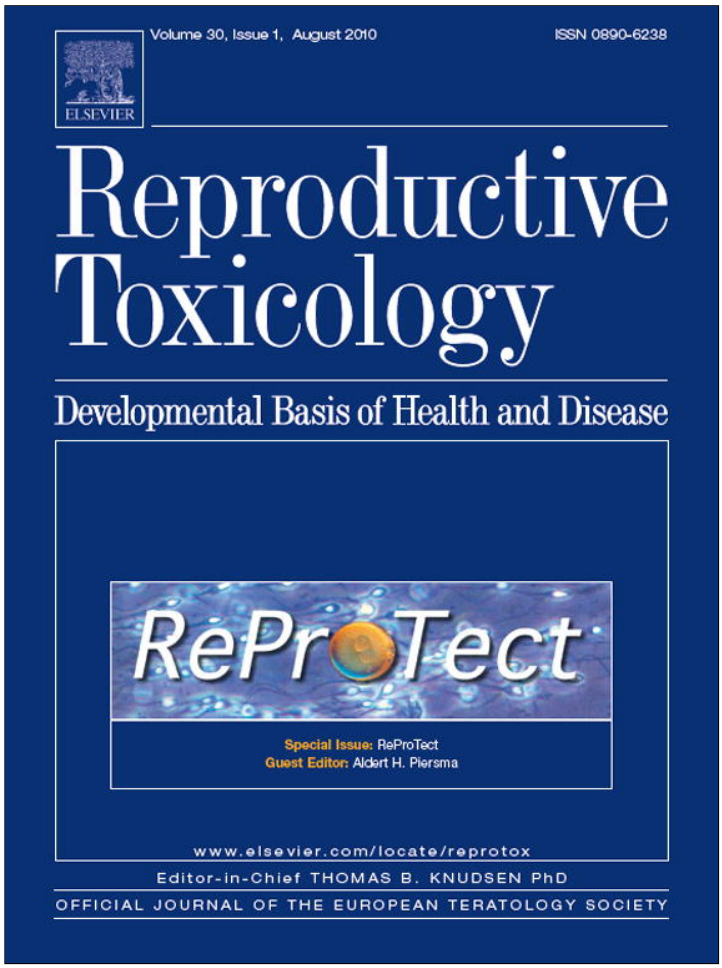

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Preliminary interlaboratory comparison of the ex vivo dual human placental perfusion system

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ABSTRACT

As a part of EU-project ReProTect, a comparison of the dual re-circulating human placental perfusion system was carried out, by two independent research groups. The detailed placental transfer data of model compounds [antipyrine, benzo(a)pyrene, PhIP (2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine) and IQ (2-amino-3-methylimidazo(4,5-f)quinoline] has been/will be published separately. For this project, a comparative re-analysis was done, by curve fitting the data and calculating two endpoints: AUC_{120}, defined as the area under the curve between time 0 and time 120 min and as $t_{0.5}$, defined as the time when the fetal to maternal concentration ratio is expected to be 0.5. The transport of the compounds from maternal to fetal circulation across the perfused placenta could be ranked in the order of antipyrine > IQ > PhIP in terms of both $t_{0.5}$ and AUC_{120} by both partners. For benzo(a)pyrene the curve fitting failed. These prevalidation results give confidence for harmonization of the placental perfusion system to be used as one of the test methods in a panel for reproductive toxicology to model placental transfer in humans.

1. Introduction

The main aim of the EU-project ReProTect has been to develop, apply and initiate prevalidation of alternative methods in reproductive toxicology. When evaluating placental transfer of compounds from the maternal to the fetal circulation, human placental perfusion has appeared useful (see e.g. [1–6]). The transfer processes across human placenta are clearly polarized due to e.g. differential transporter protein expression in apical and basal surfaces of syncytiotrophoblast. One advantage of placental perfusion is that it retains the polarized nature of syncytiotrophoblast. Another aspect is that human placental perfusion represents transfer processes in human tissue which is an advantage in risk assessment of human fetal exposure. On the other hand, only term placentas are perfused and the method therefore does not reflect the situation in earlier stages of the placenta.

The first perfusion of a single placental lobule was accomplished by Panigel [7]. Since then, Schneider et al. [8] further developed the method to maintain a cotyledon of human born placenta viable through artificial circulation. Human placental perfusion has been used extensively during the past 40 years to study placental physiology and transfer of drugs e.g. [5,9–15]. More recently, environmental chemicals have become major target of the method e.g. [12–15] due to the increasing understanding of fetal origin of diseases including toxic syndromes [16].

Even though many studies have been published over the decades using placental perfusion the methodology has never been formally validated as an in vitro test system. As a part of ReProTect, we have compared dual re-circulating human placental perfusions of term placentas carried out by two independent research groups. The approach taken in prevalidation follows the modular approach developed by ECVAM for validation of in vitro test systems [17]. Antipyrine was selected as the first compound for detailed comparison. There is a consensus, that antipyrine passes the placenta by passive diffusion [8,18]. Consequently, antipyrine has been used through the years as a reference compound in placental perfusions for overlap between maternal and fetal cotyledons as well as in comparison when evaluating the transfer mechanism of other compounds. It is thus an excellent compound for interlaboratory study.

The other compounds selected for comparisons are benzo(a)pyrene and two heterocyclic amines, PhIP (2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine) and IQ

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Table 1
Properties of compounds selected for interlaboratory comparisons.

	Antipyrine	IQ	PhIP	B(a)P
Full name	1,2-Dihydro-1,5-dimethyl-2-phenyl-^{3}H-pyrazol-3-one	2-Amino-3-methylimidazo(4,5-f)quinoline	2-Amino-1-methyl-6-phenylimidazo(4,5-b)pyridine	Benzo(a)pyrene
CAS N.	60-80-0	76180-96-6	105650-23-5	50-32-8
Mw (g/mol)	188.2	198.2	224.3	252.3
log P[a]	0.38	1.43	2.23	6.13
pK_a[a]	1.3 (base)	5.1 (base)	5 (base)	–
Concentrations tested	531 μM[c]	0.5 μM (1 μM[b])	(0.2 μM[b]) 2 μM	0.1 μM (1 μM[b])

[a] Values generated using virtual computational chemistry laboratory.
[b] Concentration used only in one of the participating laboratories.
[c] 100 μg/ml.

(2-amino-3-methylimidazo(4,5-f)quinoline), all of which are known chemical carcinogens. The order of lipophilicity of these compounds is benzo(a)pyrene > PhIP > IQ > antipyrine (Table 1). PhIP and IQ have been reported to be substrates for maternally facing efflux transporters [14,19–20] and benzo(a)pyrene is metabolized in placental tissue [21]. Thus, compounds expected to behave differently under perfusion conditions were selected for comparison.

In this paper we report results of within- and between-laboratory comparisons as well as on the transferability of placental perfusion to be used in another laboratory.

2. Methods

2.1. Selection of perfusions for the re-analysis

As part of ReProTect, re-analysis of human placental perfusion data focusing on within- and between-laboratory variations in the placental transfer of antipyrine from two different research groups, the one from Finland functioning in two locations (Oulu (FIN-OUL) and Kuopio (FIN-KUO)) and in Copenhagen (CPH) within the period 10.2.2005–17.6.2009 will be presented. For re-analysis of antipyrine transfer all available placental perfusions were included from CPH, while from FIN-OUL and FIN-KUO only placental perfusions reported as part of ReProTect project were included (Table 2). All included perfusion were primarily carried out to study the transplacental transfer of various environmental compounds with antipyrine added concurrently as a reference compound (Table 2). Antipyrine was used in all of the perfusions at a concentration of 100 μg/ml. Placental transfer data of PhIP from FIN-OUL [14] has been published in detail elsewhere as part of a large perfusion series. Also the detailed data for benzo(a)pyrene from Finland and IQ from FIN-OUL and CPH will be published in detail as parts of larger perfusion series elsewhere [22,23]. Therefore, only a brief interlaboratory comparison and re-analysis of data is presented here as part of the prevalidation of the placental perfusion system (Table 3) and detailed transplacental kinetics are presented in original publications.

Table 3
The compounds tested in more than one of participating laboratories are listed. n = number of perfusions.

Test substance	CPH	FIN-KUO	FIN-OUL
Antipyrine	n = 70	n = 15	n = 41
Benzo(a)pyrene 0.1 μM	n = 3	NA	n = 4
PhIP 2 μM	n = 1	NA	n = 6
PhIP 0.2 μM	n = 2	NA	NA
IQ 0.5 μM	n = 5	NA	n = 6

NA, not available.

2.2. Short description of human placental perfusion

Dual re-circulating placental perfusions of single lobules were performed as described in detail in elsewhere [13–15,22,23] (Table 4, Fig. 1). Briefly, Krebs–Ringer buffer with heparin (25 IU/ml) was injected within 10 min after the birth of the placenta through the vessels of the umbilical cord. An intact peripheral cotyledon with a single chorionic artery and vein was cannulated and the lobe was cut out and placed into the perfusion apparatus. On the maternal side, two cannulae were placed into the intervillous space through the basal plate. Differences in the two groups in the perfusion setup were the heating system (perfusion apparatus in a heated flowbench in CPH; double-walled chambers with water-warming in FIN-KUO and FIN-OUL) and the attachment of perfused lobule into the perfusion chamber during perfusion. In FIN-KUO and FIN-OUL the perfused lobule is clamped into the chamber before removing the surrounding placental tissue while in Denmark, the placenta is not clamped. The perfusion conditions in the different laboratories also showed small variation because standard operating procedure allowed variation e.g. in constituents of perfusion medium (Table 4). In CPH the fetal flow-rate was changed from 3.5 to 3 ml/min to prevent ruptures of the fetal vessels due to pressure. The maternal flow was changed accordingly from 12 to 9 ml/min. The perfusion media used was Krebs–Ringer with supplements for 2.5 h perfusions or RPMI cell culture solution with supplements for longer perfusions to keep the tissue viable (Table 4).

Table 2
Human placental perfusions included in the data analysis.

Laboratory	Primary study compound	Additional study compounds	Number of perfusions	Original reference
CPH	Caffeine		7	[28]
	Glyphosate		7	[28]
	Benzoic acid		5	[28]
	Benzo(a)pyrene		13	[15]
	Bisphenol A		8	[29]
	PhIP		3	Unpublished[b]
	IQ		8	[23][b]
	PBDE (47,99,209)		5	Unpublished[a]
	DON		5	Unpublished[c]
	PCB52		4	Unpublished[a]
	PCB180		5	Unpublished[a]
FIN-OUL	Benzo(a)pyrene	Verapamil	9	[22]
	PhIP	KO143, probenecid	16	[14]
	IQ	KO143, GF120918	16	[23]
FIN-KUO	Acrylamide	–	11	[13]
	Glycidamide	–	4	[13]
Total			126	

[a] Method as in Mose et al. [28] with addition of physiological human serum albumin concentration as done in Mathiesen et al. [15].
[b] Method as in Mathiesen et al. [15] using 2 g/l bovine serum albumin.
[c] Method as in [a] perfusing for 4 h.

Table 4
Comparison of used experimental conditions in different laboratories.

	CPH	FIN-KUO	FIN-OUL
Delivery	Caesarean section, vaginal delivery	Caesarean section, vaginal delivery	Caesarean section, vaginal delivery
Flow rates			
Maternal (ml/min)	9–12	9	9
Fetal (ml/min)	2.6–3.8	3	2.5–3
Oxygenation			
Maternal	95% O_2, 5% CO_2	95% O_2, 5% CO_2	95% O_2, 5% CO_2
Fetal	95% N_2, 5% CO_2	95% N_2, 5% CO_2	95% N_2, 5% CO_2
Blood gas analysis (pH, PO_2, PCO_2)	Maternal reservoir Fetal vein Fetal reservoir	Maternal artery Maternal vein Fetal reservoir	Maternal artery Maternal vein Fetal reservoir
Perfusate			
Base	RPMI[a]/KRB[b]	RPMI	RPMI
Albumin fetal (g/l)	0–40	2	2
Albumin maternal (g/l)	0–30	2	2
Dextran fetal (g/l)	0–30	2	2
Dextran maternal (g/l)	0–8.4	2	2
Other constituents[c]	Heparin L-Glutamine Penicillin–streptomycin	Heparin Non-essential amino acids L-Glutamine Sodium pyruvate Penicillin–streptomycin	Heparin Non-essential amino acids L-Glutamine Sodium pyruvate[c] Penicillin–streptomycin[c]
Analytical methodology for antipyrine	HPLC with internal standard	LC/MS	HPLC with or without internal standard

[a] RPMI, RPMI 1640 with or without phenol red.
[b] KRB, Krebs–Ringer Phosphate buffer.
[c] Sodium pyruvate was not used in IQ or PhIP perfusions and Penicillin–streptomycin in IQ perfusions.

A re-circulating pre-perfusion, to stabilize the conditions of the placenta and to reverse hypoxia, was carried out for 30–60 min similarly in all participating laboratories after which the reference compound antipyrine (100 μg/ml) was added to all perfusions simultaneously with the actual study compound. The concentrations of other compounds used for within- and between-laboratory comparisons are presented in Table 3. If necessary, pH values were adjusted using either hydrochloric acid or sodium hydroxide during the perfusions by both groups. Still the pH values were slightly lower in CPH compared to FIN-OUL and FIN-KUO in fetal reservoir (Table 5). Furthermore, a small variation was seen in pH values between FIN-OUL and FIN-KUO although perfusion systems were identical (Table 3).

2.3. Calculations and statistical analyses

The placental perfusions had duration of 2.5–6 h after addition of antipyrine to maternal reservoir. Samples were collected from both circulations during the perfusions although the sampling intervals showed some variation depending on the study setup. [Both maternal and fetal antipyrine concentration available: 0 min (n = 80), 2 min (n = 65), 5 min (n = 33), 10 min (n = 34), 15 min (n = 56), 30 min (n = 125), 45 min (n = 38), 1 h (n = 125), 1.5 h (n = 118), 2 h (n = 122), 2.5 h (n = 74), 3 h (n = 89), 4 h (n = 87), 5 h (n = 45) and 6 h (n = 46).] For each perfusion a number of samples was sufficient for generating a model using a non-linear function and therefore the variation in sampling intervals or length of perfusions did not inter-

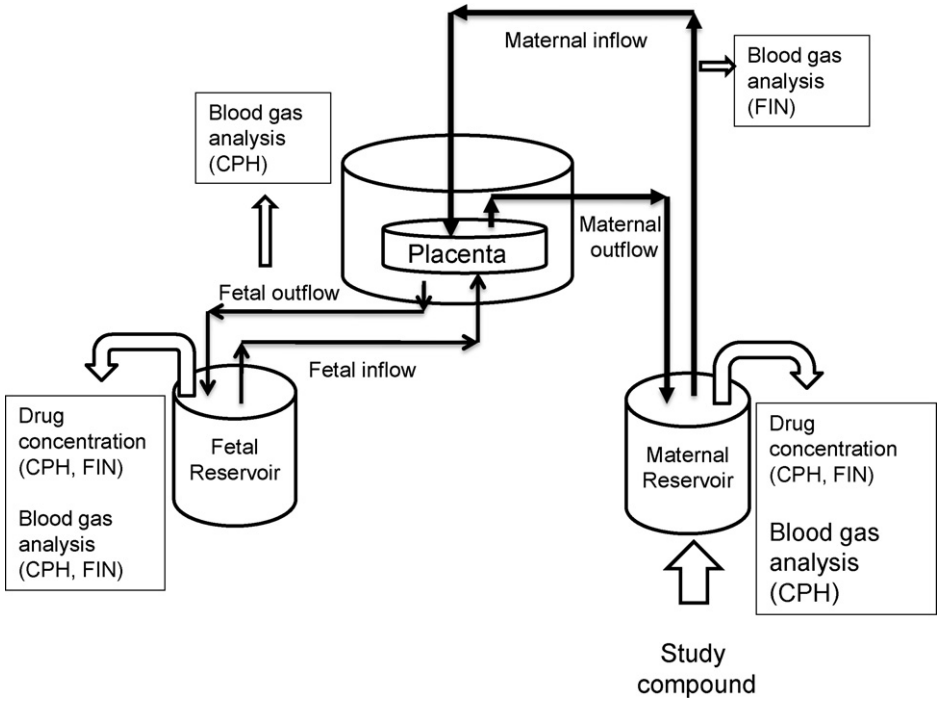

Fig. 1. A schematic presentation of placental perfusion system. Sampling sites in each laboratory are indicated (CPH: Copenhagen, Denmark; FIN-KUO: Kuopio, Finland; FIN-OUL: Oulu, Finland).

Table 5
Comparison of leak and pH values in different laboratories in the perfusions considered successful. Values are mean ± SD.

	CPH	FIN-OUL	FIN-KUO
Number of perfusions (% of total)	70 (55.5%)	41 (32.5%)	15 (12%)
Leak (ml/h)	1.66 ± 1.01	1.53 ± 0.66	2.33 ± 0.85$^{**,\#\#}$
pH fetal reservoir[a]			
2 h	7.327 ± 0.098(n = 70)	7.433 ± 0.065*** (n = 34)	7.465 ± 0.085*** (n = 14)
4 h	7.299 ± 0.092(n = 34)	7.414 ± 0.061*** (n = 34)	7.482 ± 0.059$^{***,\#\#}$(n = 14)
6 h	7.293 ± 0.108(n = 24)	7.406 ± 0.085** (n = 18)	NA
pH fetal vein[a]			
2 h	7.291 ± 0.103(n = 70)	NA	NA
4 h	7.243 ± 0.103(n = 34)	NA	NA
6 h	7.198 ± 0.091(n = 24)	NA	NA
pH maternal artery/reservoir[a]			
2 h	7.170 ± 0.187(n = 70)	7.490 ± 0.063*** (n = 33)	7.530 ± 0.094*** (n = 15)
4 h	7.195 ± 0.129(n = 34)	7.469 ± 0.0758***(n = 34)	7.539 ± 0.116$^{***,\#}$ (n = 15)
6 h	7.169 ± 0.168(n = 24)	7.457 ± 0.104*** (n = 18)	NA

NA, not available.
[a] Independent samples t-test was used to compare pH values between laboratories when values were available from more than one laboratory.
** Copenhagen vs. Oulu $p < 0.01$.
*** Copenhagen vs. Oulu or Copenhagen vs. Kuopio $p < 0.001$.
$^{\#}$ Oulu vs. Kuopio $p < 0.05$.
$^{\#\#}$ Oulu vs. Kuopio $p < 0.01$.

fere with the data analyses. To minimize the effect of differences in the analysis of study compounds between different laboratories fetal to maternal concentration ratios (FM-ratios) were used for within- and between-laboratory comparisons.

Statistical analysis was done using the statistical software R [R Development Core Team (2009). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org]. For each experiment, FM-ratio vs. time t (in minutes) was modeled using the non-linear function

$$FM(t) = \text{theta1} \times (1 - \exp(-\text{theta2} \times t)).$$

The model was fitted using R function nls. From the fitted model, two derived endpoints were calculated:

- AUC_{120}, defined as the area under the curve between time 0 and time 120 min
- $t_{0.5}$, defined as the time when the FM-ratio is expected to be 0.5.

Modeling responses AUC_{120} and $t_{0.5}$ with linear predictors was done using R function lm. Testing of predictors was explorative. The parameters selected for modeling are sensitive in detecting variation in the initial slope of transfer (Fig. 2). Thus, even if all experiments would suggest significant transfer the modeling parameters will reveal whether the transfer rate is variable.

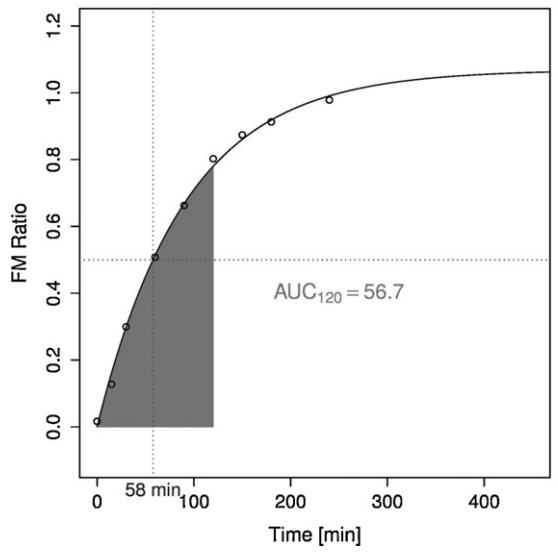

Fig. 2. An example of statistical analyses performed for each individual perfusion.

2.4. Ethical aspects

The official Ethics Committees of the Municipalities of Copenhagen and Frederiksberg (KF 01-145/03 + KF(11) 260063), the Danish Data Protection Agency, the Northern Savo Hospital District and The Northern Ostrobothnia Hospital District, Finland had approved the study protocols for placental perfusions. All mothers gave a written informed consent for the use of their term born placentas in the studies. The participation in placental perfusion studies did not affect the management of the delivery in any way.

3. Results

3.1. Within- and between-laboratory variations in antipyrine transfer

In the CPH laboratory, antipyrine was clearly detectable in fetal circulation 5 min after addition of the study compound in maternal reservoir in most of the perfusions, with the average concentration being 5.94 ± 2.54 μg/ml (n = 34). After 30-min the average fetal concentration of antipyrine was 28.82 ± 20.28 μg/ml (n = 69) and the FM-ratio was 0.42 ± 0.13 (n = 69). After 4-h in perfusion the maternal and fetal concentrations of antipyrine were equal, suggesting significant placental transfer (Table 6, Fig. 3).

Similarly, antipyrine crossed placenta rapidly both in FIN-OUL and FIN-KUO (Fig. 3). In FIN-OUL the average fetal concentration was 21.92 ± 9.23 μg/ml (n = 41) after 30 min and the FM-ratio was 0.30 ± 0.12 (n = 41). After 4-h perfusion the maternal and fetal concentrations were equal (Fig. 3, Table 6). In FIN-KUO at 30 min the average antipyrine concentration in fetal reservoir was 21.48 ± 8.55 μg/ml (n = 15) and FM-ratio was 0.26 ± 0.1 (n = 15). Similarly to CPH and FIN-OUL after 4-h perfusion, the maternal and fetal antipyrine concentrations were equal (Fig. 3, Table 6).

The average $t_{0.5}$ varied in participating laboratories from 39.9 to 61.0 min (Table 6). The range of variation in all laboratories was rather similar (Table 6, Fig. 3). In the FIN-OUL data set, 1 perfusion showed clearly slower antipyrine transfer compared to the other perfusions (Fig. 3F). The most probable reason for this is suboptimal placing of the maternal cannulae leading to decreased surface area for transfer. To overcome this problem antipyrine (or alternatively some other compound such as creatinine) is regularly used as a reference compound in all perfusions and perfusions with irregular antipyrine transfer can be excluded from the final data analyses.

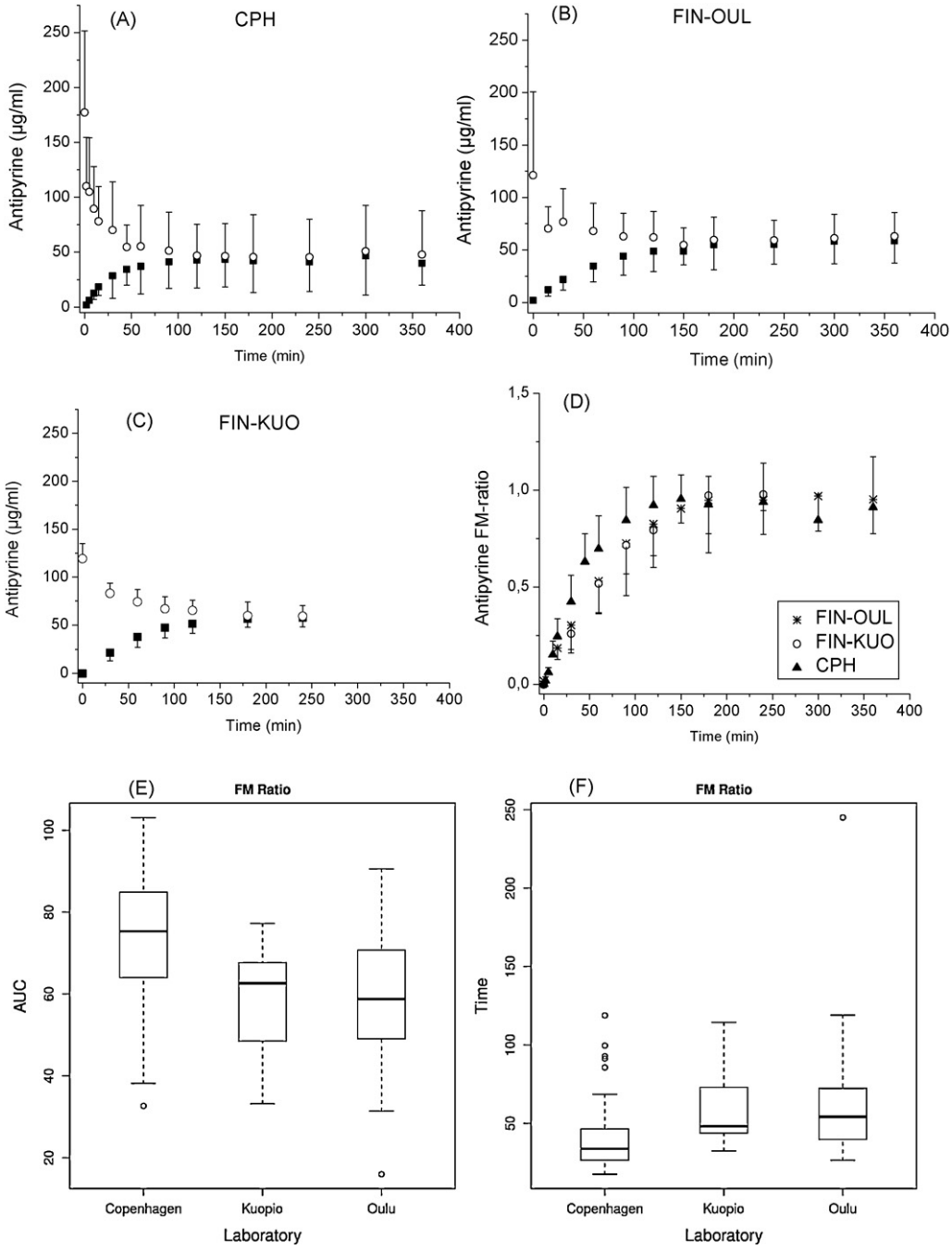

Fig. 3. Placental transfer of antipyrine in different participating laboratories and results from statistical analyses. Antipyrine concentrations in maternal (open circles) and fetal (solid squares) circulations in (A) CPH (35 perfusions run for 2.5 h, 5 run for 4 h, and 25 for 6 h) (B), FIN-OUL (25 perfusions lasting 4 h and 16 for 6 h) and (C) FIN-KUO (15 perfusions lasting 4 h). (D) FM-ratios at different laboratories. (E) AUC_{120} in different laboratories. (*F*) $t_{0.5}$ in different laboratories. Values are mean ± SD.

Boxplot (Fig. 4) suggests that AUC_{120} tends to be larger in CPH. However, when CPH experiments are grouped by perfusion medium the larger AUC_{120} seems to be linked to medium 'K' which is Krebs–Ringer buffer. The distribution of AUC_{120} of CPH experiments with medium 'R' (RPMI 1640) is similar to AUC_{120} distributions at FIN-OUL and FIN-KUO, where medium 'R' was used in all experiments (Fig. 4). Prior to statistical analysis of AUC_{120}, four experiments were excluded from the dataset: Three experiments because of missing leak information and one experiment because of failed curve fitting.

Since medium 'K' was only used in CPH no general statement about the effect of medium can be made with regard to other laboratories. For the statistical analysis of putative predictors of AUC_{120} in CPH a linear model was used with response AUC_{120} and predictor terms medium (factor), leak (continuous), and flow ratio (continuous) (model without interactions). The conditional *t*-test was only significant for the factor medium ($p < 0.001$) suggesting that in CPH AUC_{120} depends on which of the two media is used.

For the statistical analysis of putative predictors of AUC_{120} across different laboratories only experiments with medium 'R' were used. In a linear model with response AUC_{120} and predictor terms laboratory (factor), leak (continuous), and flow ratio (continuous) none of the predictors was significant (*F*-test, $p = 0.61$, model without interactions). Statistical analysis of $\log_{10}(t_{0.5})$ was done as for endpoint AUC_{120}. There was a significant effect of factor medium

Table 6
Summary of results obtained from different laboratories. Data collected from different original publications. Values are mean ± SD.

Compound	Parameters based on model			Non-model based parameters			Reference
	$t_{0.5}$ mean ± SD (min, max)	AUC_{120} mean ± SD (min, max)		Fetal conc. at 4 h mean ± SD (μg/ml) (*n*)	Maternal conc. at 4 h mean ± SD (μg/ml) (*n*)	FM (4 h) mean ± SD (*n*)	
Antipyrine							
CPH	39.9 ± 20.0 (17.5; 118.8)	73.5 ± 14.9	(32.6; 103.1)	41.28 ± 27.46 (*n* = 29)	45.53 ± 35.20 (*n* = 34)	0.94 ± 0.20 (*n* = 30)	See Table 2
FIN-KUO	59.6 ± 23.0 (32.3; 114.3)	58.4 ± 12.9	(33.1; 77.2)	57.64 ± 9.62 (*n* = 15)	59.29 ± 10.98 (*n* = 15)	0.97 ± 0.08 (*n* = 15)	See Table 2
FIN-OUL	61.0 ± 38.0 (26.4; 245.1)	59.7 ± 16.3	(16.0; 90.6)	55.34 ± 17.5 (*n* = 41)	59.25 ± 17.85 (*n* = 41)	0.95 ± 0.18 (*n* = 41)	See Table 2
PhIP							
CPH	152.2[A] (83.3; 221.2)	33.16[A]	(21.9; 44.5)	0.379[B] 0.057[C]	0.761[B] 0.086[C]	0.50 0.65[C]	Unpublished
FIN-OUL	136.7 ± 32.4 (80.7; 166.2)	32.6 ± 6.7	(25.8; 45.42)	0.137 ± 0.03	0.208 ± 0.03	0.66 ± 0.07	[15]
IQ							
CPH	78.1 ± 55.4 (30.1; 165.3)	51.7 ± 20.2	(22.8; 73.6)	0.10 ± 0.01	0.13 ± 0.01	0.77 ± 0.06	[23]
FIN-OUL	53.8 ± 17.1 (40.7; 83.7)	61.0 ± 10.3	(44.0; 69.1)	0.04 ± 0.01	0.04 ± 0.01	0.97 ± 0.11	[23]
B(a)P							
CPH	NA	NA		0.0023 ± 0.0027	0.0131 ± 0.0114	0.17 ± 0.12	Unpublished
FIN-OUL	NA	NA		0.0077 ± 0.0044	0.0195 ± 0.0126	0.49 ± 0.30	[22]

[A] Perfusion with PhIP 0.2 and 2 μM included.
[B] PhIP 2 μM.
[C] PhIP 0.2 μM.

Fig. 4. Variation in placental transfer of antipyrine. (A) The effect of perfusion medium on AUC_{120}. R, RPMI; K, KRB, Krebs–Ringer buffer. Number of perfusions in each group is indicated at upper panel. (B) Placental transfer of antipyrine in FIN-OUL in perfusions with different primary study compounds.

in CPH (conditional *t*-test, $p = 0.003$). In the interlaboratory analysis none of the predictors was significant (*F*-test, $p = 0.44$).

In the data set from FIN-OUL laboratory there seemed to be a small variation in antipyrine results depending on the primary study compound (Fig. 4B). In benzo(a)pyrene study, the transporter inhibitor used has vasodilatory effects and probably contributed to a slightly slower average antipyrine transfer and a higher 'perfusion to perfusion' variation. The main difference between PhIP and IQ perfusions is the smaller variation in FM-ratio of antipyrine in IQ perfusions, which most likely reflects the inclusion of internal standard in antipyrine analytical methodology in IQ study and not actual changes in antipyrine transfer.

3.2. Within- and between-laboratory comparisons of other perfused compounds

Placental transfer data of PhIP from FIN-OUL [14], has been published in detail elsewhere. Also the detailed data for benzo(a)pyrene from Finland and IQ from FIN-OUL and CPH will be published in detail as parts of larger perfusion series elsewhere [22,23]. Therefore, only a brief re-analysis of results and between- and within-laboratory comparisons are presented. For more details see the original publications.

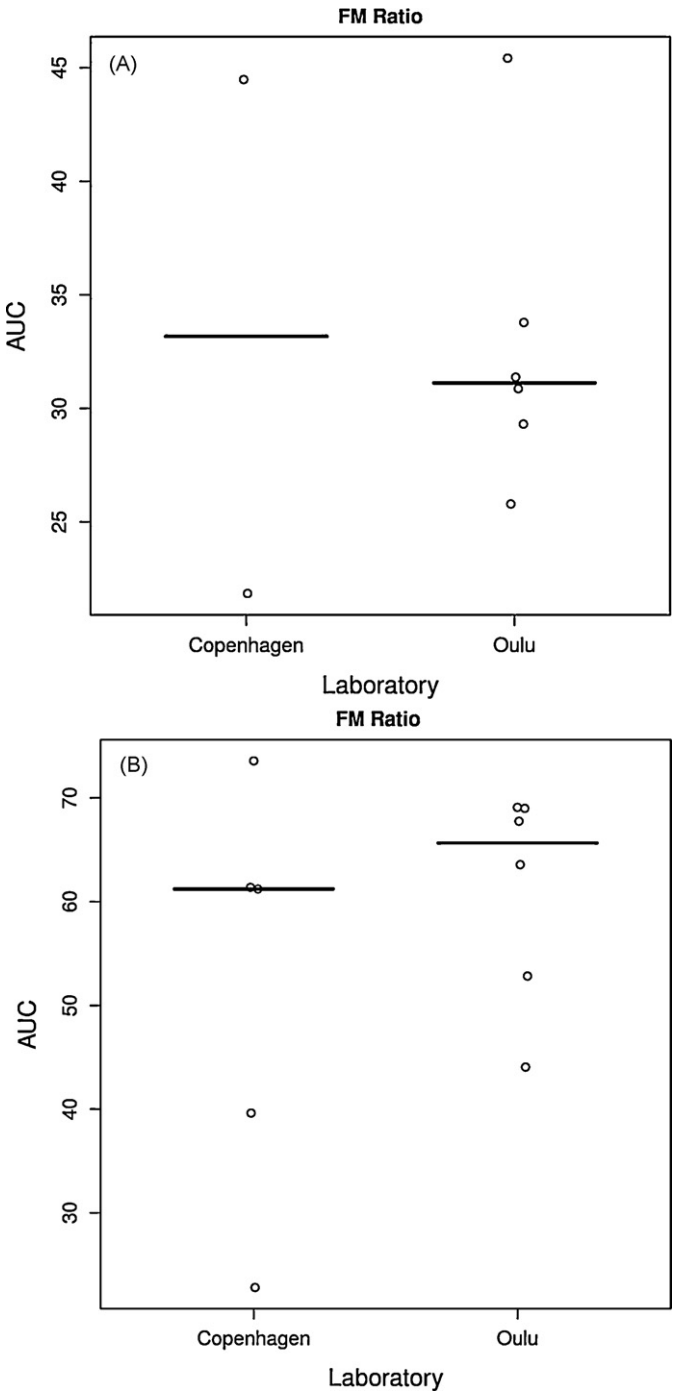

Fig. 5. Placental transfer of PhIP and IQ. (A) AUC_{120} values for placental transfer of PhIP. (B) AUC_{120} values for placental transfer of IQ.

3.2.1. IQ

IQ data from altogether 11 perfusions were available for within- and between-laboratory comparisons. All the perfusions used for comparisons were done using 0.5 μM ^{14}C-IQ. Similarly to antipyrine, IQ crossed placenta easily from maternal to fetal circulation in all experiments in the laboratories by both partners (Fig. 5). In both laboratories the initial transfer of IQ was slightly slower than that of antipyrine (Table 6). The mean $t_{0.5}$ and AUC_{120} were rather similar in both laboratories (Fig. 5, Table 6). Also, in both laboratories the range was similar (Fig. 5, Table 6).

3.2.2. PhIP

PhIP data from 9 perfusions, all of which suggested significant transfer from maternal to fetal circulation, were available for statistical analyses. The perfusions were done using two different concentrations. Because curve fitting failed for 1 perfusion AUC_{120} and $t_{0.5}$ values were available only from 5 perfusions with 2 μM PhIP from FIN-OUL and 2 perfusions with 0.2 μM PhIP from CPH (Fig. 5). The average transfer rates as well as the range of variation evaluated by $t_{0.5}$ and AUC_{120} were rather similar in both laboratories. The transfer rate based on AUC_{120} and $t_{0.5}$ was slower than that of antipyrine or IQ in both laboratories. However, the AUC_{120} and $t_{0.5}$ results are not directly comparable between laboratories because perfusions were done using different concentrations in FIN-OUL and CPH. PhIP is an ABCG2 substrate and therefore different substrate concentrations may affect both AUC_{120} and $t_{0.5}$.

3.2.3. Benzo(a)pyrene

The curve fitting using the non-linear function was not successful for benzo(a)pyrene transfer mainly due to the slow transfer rate leading to a small slope (Table 6). Thus, comparison of results from the two laboratories cannot be made using the same parameters as for the other compounds.

3.3. Transferability of method

The perfusion setup was transferred from FIN-OUL to FIN-KUO both laboratories having identical perfusion equipments and being part of the same research group. Antipyrine transfer rate indicated by fetal to maternal concentration ratio of antipyrine was very similar in these two locations indicating good transferability (Fig. 3).

The perfusion setup in CPH is further detailed in Mathiesen et al. [24]. The researchers from Copenhagen visited 3 times in Finland to get familiar with the technique and exchange experiences when setting up the methodology and one meeting was organized in Copenhagen. In addition the group members have met in several meetings and have had regular e-mail correspondence. The initial results from Finnish and Copenhagen teams indicate good transferability of the methodology.

4. Discussion

As expected, antipyrine crossed placenta easily in both participating research groups. Because antipyrine crosses the placenta by passive diffusion, theoretically the main parameter affecting the transfer is the flow of perfusion medium, which, indeed, has been confirmed earlier [25]. However, in this study, the variation in flow ratios was too small for any significant changes in antipyrine transfer to be detected. There was also variation in perfusion mediums used. Standard operating procedure used in this prevalidation experiment allowed the selection of medium 'K' for short perfusions while longer perfusions were made using medium 'R'. Unexpectedly, selection of perfusate had a small influence on the transfer rate of antipyrine. Also, the placement of maternal cannulae may affect the transfer efficiency of antipyrine due to the variation in the overlap of maternal and fetal circulations and thus it may be a source for within- and between-laboratory variations.

In addition to antipyrine the placental transfer of other compounds has been compared using placental perfusion methodology within the ReProTect project. The compounds' transport across perfused placenta from maternal to fetal circulation can be ranked in the order of antipyrine > IQ > PhIP in terms of both $t_{0.5}$ and AUC_{120} both of which measure transfer during the initial slope in the transfer. The PhIP concentrations were different in both laboratories which may have affected the results. However, results from both laboratories indicate that PhIP is transferred more slowly

than antipyrine or IQ. Both PhIP and IQ transfers showed within-laboratory variation in FM-ratios. In the placental perfusion human tissue is utilized and thus it is expected that placental transfer shows variation due to interindividual differences. In fact, for PhIP it has previously been shown that FM-ratios from FIN-OUL data correlate with ABCG2 protein expression [14]. Therefore the variation seen in the modeling parameters at least partially reflect true person to person variation in transplacental kinetics and are not due to experimental conditions.

For benzo(a)pyrene the curve fitting failed because of a low transfer rate in comparative perfusions and therefore a meaningful comparison could not be done. Benzo(a)pyrene also behaved problematic in other aspects. The placental transfer of benzo(a)pyrene is greatly affected by perfusion conditions, as shown previously by the CPH team [15]. Benzo(a)pyrene transfer is highly dependent on albumin concentration and the type of albumin used [15], suggesting that selected perfusion conditions may significantly contribute to estimates on fetal exposure in some cases. In the comparative perfusions the FIN-OUL and FIN-KUO teams used human albumin while the CPH team used bovine albumin in some of the perfusions, which probably contributed to the observed differences in FM-ratios. Furthermore, the Copenhagen team used ^{14}C-labeled benzo(a)pyrene while the Finnish team used ^{3}H-labeled substances. Substances that are ^{3}H-labeled may exchange with water. Further, in vitro experiments by the Finnish team suggest that binding of benzo(a)pyrene to the tubes of the perfusion apparatus was significant and may have affected the results (unpublished results).

Ex vivo perfusion of human placental cotyledon offers several advantages compared to other methods pursuing transplacental transfer of compounds. The major advantage is that results represent transfer in human tissue. Furthermore, the method utilizes born placenta and thus causes no safety concerns for mother and child. The preliminary data analysis also indicates relatively good interlaboratory comparison and transferability of the methodology. However, in the case of some lipid soluble compounds such as benzo(a)pyrene or diazepam [15,24], prediction of placental transfer in vivo may be more problematic. Problems with poor recovery of the studied compound have been described earlier in the case of diazepam [26]. Therefore, for each study the recovery of the study compound from experimental apparatus should be tested before starting the actual experimentation. In addition, more studies, especially about lipid soluble compounds and the possibility by modifying the perfusion medium for better recovery of such compounds are needed.

Overall ex vivo placenta perfusion has limited potential within a large scale chemical screening and testing because the method is very time- and labor consuming. However, based on our results it may have use within the final stages of a tiered approach of testing for reproductive toxicity. Interestingly, a recent study by the CPH team comparing the BeWo cell line and ex vivo placental perfusions showed similar results in ranking the compounds according to their transfer rate [27]. A step-wise approach might therefore be feasible. The in vitro BeWo cell transport or other cell based experiments might be employed as an initial screening tool, because these experiments are not as time consuming and still show high relevance and predictability towards the data that would be collected by the ex vivo dually perfused placenta experiments. If the results from BeWo cell experiments are consistent with expectations based on physical and chemical characteristics and comparison to analogous compounds, appropriate data classification from these in vitro experiments may be sufficient. Divergent results, the need for more complete information, or a desire to more closely approach to the in vivo state would warrant further experimentation with the more sophisticated ex vivo dually perfused human placenta. Further research and evaluation, however, are needed to validate this step-wise approach.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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