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Meta-analysis of data from human ex vivo placental perfusion studies on genotoxic and immunotoxic agents within the integrated European project NewGeneris

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ABSTRACT

In the EU integrated project NewGeneris, we studied placental transport of thirteen immunotoxic and genotoxic agents in three *ex vivo* placental perfusion laboratories. In the present publication, all placental perfusion data have been re-analyzed and normalized to make them directly comparable and rankable. Antipyrine transfer data differed significantly between the studies and laboratories, and therefore normalization of data was necessary. An antipyrine normalization factor was introduced making the variance significantly smaller within and between the studies using the same compound but performed in different laboratories. Non-normalized (regular) and normalized data showed a good correlation. The compounds were ranked according to their transplacental transfer rate using either antipyrine normalized AUC_{120} or transfer index (TI_{120}(%)). Normalization generated a division of compounds in slow, medium and high transfer rate groups. The transfer rate differed slightly depending on the parameter used. However, compounds with passage similar to antipyrine which goes through the placenta by passive diffusion, and good recovery in media (no accumulation in the tissue or adherence to equipment) were highly ranked no matter which parameter was used. Antipyrine normalization resulted in the following ranking order of compounds according to $AUC_{120NORM}$ values: NDMA ≥ EtOH ≥ BPA ≥ IQ ≥AA ≥ GA ≥ PCB180 ≥ PhIP ≥ AFB1 > DON ≥ BP ≥ PCB52 ≥ TCDD. As the variance in all parameters within a study decreased after antipyrine normalization, we conclude that this normalization approach at least partially corrects the bias caused by the small methodological differences between studies.

1. Introduction

The NewGeneris (Newborns and Genotoxic exposure risks) project investigated the role of fetal environmental exposures in childhood disease causation and focused on a series of dietary and environmental chemicals [1]. The *ex vivo* placental perfusion system was used to study placental passage of thirteen test compounds; ^{14}C-benzo[a]pyrene (BP), ^{14}C-2-amino-3-methyl-imidazol [4,5-f]-quinoline (IQ), ^{14}C-2-amino-1-methyl-6-phenylimidazol [4,5-b]pyridine (PhIP), acrylamide (AA), glycidamide (GA), ^{14}C-nitrosodimethylamine (NDMA), aflatoxin B1 (AFB1), deoxynivalenol (DON), ^{14}C-2,20,5,50-Tetrachlorobiphenyl (PCB52), ^{14}C-2,20,3,4,40,5,50-Heptachlorobiphenyl (PCB180), ^{14}C-bisphenol A (BPA), 2,3,7,8-tetrachlorobenzo-p-dioxin (TCDD), and ethanol (EtOH) (Tables 1 and 2). Three placental perfusion laboratories were involved using commonly agreed protocols generated within the European research programs of ReProTect (development of a novel approach in hazard and risk assessment of reproductive toxicity by a combination and application of in vitro, tissue and sensor technologies) and NewGeneris [9].

Abbreviations: CPH, Copenhagen; OUL, Oulu; KUO, Kuopio; AUC, area under the fetal/maternal ratio curve; TI, transfer index; IP, initial permeability rate; FM, fetal/maternal concentration; AP, antipyrine; CW, cotyledon weight; NF, normalization factor; NORM, antipyrine normalized; REG, regular/un-normalized values; hCG, human choriongonadotropin; BP, ^{14}C-benzo[a]pyrene; IQ, ^{14}C-2-amino-3-methyl-imidazol[4,5-f]-quinoline; PhIP, ^{14}C-2-amino-1-methyl-6-phenylimidazol[4,5-b] pyridine; AA, acrylamide; GA, glycidamide; NDMA, ^{14}C-dimethylnitrosamine; AFB1, Aflatoxin B1; DON, deoxynivalenol; PCB52, ^{14}C-2,20,5,50-Tetrachlorobiphenyl; PCB180, ^{14}C-2,20,3,4,40,5,50-Heptachlorobiphenyl; BPA, ^{14}C-bisphenol A; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD); EtOH, ethanol.

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Table 1
The compounds studied using human placental perfusion within NewGeneris project. The selection of compounds was based on common chemical listing generated at the preparatory stages of the NewGeneris project.

Chemical class	Model compound	Sources	Class of toxicity
Polycyclic aromatic hydrocarbons	Benzo[a]pyrene (BP)	Environmental contamination of the food chain	Genotoxic carcinogenesis
		Formation during baking and frying smoking and exposure to environmental tobacco smoke	Immunotoxicity
Heterocyclic amines	2-amino-3-methyl- imidazo[4,5-f]-quinoline (IQ) 2-amino-1-methyl-6- phenylimidazo [4,5-b] pyridine (PhIP)	Formation during baking and broiling	Genotoxic carcinogenesis
Acrylamides	Monoacrylamide	Formation during baking and frying	Genotoxic carcinogenesis
Nitrosamines	Dimethylnitrosamine (NMDA)	Environmental nitrate contamination of the food chain and subsequent endogenous formation	Genotoxic carcinogenesis
Mycotoxins	Aflatoxin B1 Deoxynivalenol (DON)	Environmental contamination of the food chain	Genotoxic carcinogenesis Immunotoxicity
Organochlorinated compounds	2,3,7,8-Tetrachlorodibenzo-p- dioxin (TCDD) PCB	Environmental contamination of the food chain	Cocarcinogenesis Immunotoxicity Endocrine disruption
Alcohols	Ethanol (EtOH)	Life style factor	Cocarcinogenesis Immunotoxicity
Xenoestrogen	Bisphenol A (BPA)	Released from plastics to food chain	Cocarcinogenesis

Antipyrine was used as the reference compound in all perfusions. Antipyrine is a neutral molecule which passes through the placenta by passive diffusion in a flow dependent manner without binding to protein at physiological pH [14]. Antipyrine data are useful when normalizing data between perfusions to correct for inter-placental/study variations such as perfusion flow, surface exchange area, and matching of the two circulations [15–17]. Most commonly normalization has been performed calculating the transfer index (TI), i.e. the transfer of drug (%) divided by transfer of antipyrine (%). Normalization against the weight of the perfused tissue has been performed resulting, however, in greater variation of data [16].

In this publication, placental perfusion data from all facility sites within the NewGeneris project were re-analyzed, normalized, and ranked according to transplacental transfer rate. Different normalization methods were tested and discussed.

2. Methods

2.1. Data sources

In NewGeneris, placental perfusion data were generated using common protocols in two laboratories in Finland (Kuopio (KUO) and Oulu (OUL)) and one in Denmark (Copenhagen, (CPH)). Some of the study compounds were studied in two of the participating laboratories. The results of the detailed transplacental kinetics, perfusion methodology, perfusion details, and analytical methods are presented separately in the original publications (Table 2). In general, healthy mothers without known pregnancy complications were asked to donate their placentas for placental perfusion studies (Table 3). For data re-analysis, the original data from all successful perfusions except perfusions using multiple compounds (e.g. transporter protein inhibitors) and BP perfusions using non-physiological human serum albumin concentrations [6,18] were included. Generally, the concentrations of study compounds were higher than the ones detected *in vivo* in pregnant women due to the sensitivity of the analytical methods. Data on each compound tested at several concentration levels were pooled because the transfer kinetics did not in any case depend on the concentration level (AA, IQ, AFB1, PhIP, NDMA).

Table 2
Summary of physiochemical properties of study compounds and ex vivo human placental perfusion conditions used for each compound.

Studies, facility	Molecular weight (g/mol)	Solubility in water	Log Kow	Ref	Concentration (μM)	Perfusion media	Sampling times (min)
NDMA, KUO	74.08	Soluble	−0.57	[2]	1 and 5	RPMI + HSA (2 g/L)	0, 30, 60, 90, 120, 180, 240, 300, 360
DON, CPH	296.32	Sparingly soluble	___	[3]	0.34	RPMI + 30/40 g/L HSA	Before, 0,2,15,30,45,60,90,120, 150, 180,240
EtOH, CPH	46.07	Miscible	−0.34	[4]	3425.2 (2‰)	KR + HSA (30/40 or 50/60 g/L)	Before, 0, 2,5,10,15,30,45,60,90, 120,150
BPA, CPH	228.3	Insoluble	3.3	[4]	0.5	KR + HSA (30/40 or 50/60 g/L)	Before, 0, 2,5,10,15,30,45,60,90, 120,150
EtOH, KUO	46.07	Insoluble	1.43	[5]	3425.2 (2‰)	RPMI + HSA (2 g/L)	0,30,60,120
BP, CPH	252.3	Insoluble	6.0	[6]	0.1 and 1.0	RPMI + HSA (30/40 g/L)	Before,0,2,30,60,90,120,150, 180,240,300, 360
IQ, OUL	198.2	Insoluble	1.43	[7]	0.5	RPMI + HSA (2 g/L)	0,30,60,90,120,180,240,300,360
IQ, CPH	198.2	Insoluble	1.43	[7]	0.5 and 1.0	RPMI	Before, 0,2,30,60,90,120,150,180, 240,300, 360
PhIP, OUL	224.3	Insoluble	2.2	[8]	2	RPMI + albumin (2 g/L)	0,30,60,90,120,180,240,300,360
PhIP, CPH	224.3	Insoluble	2.2	[9]	0.2 and 2.0	RPMI	Before, 0,2,30,60,90,120,150,180, 240,300, 360
TCDD, CPH	321.97	Insoluble	6.8	[10]	6.0 pg/mL	RPMI + HSA (30 g/L)	Before, 0,2,30,60,120,240, 360
PCB 52, CPH	291.98	Insoluble	6.09	[11]	1.5	KR + HSA (30/40 g/L)	Before, 0,2,5,10,15,30,45,60,90, 120,150
PCB 180, CPH	395.32	Insoluble	8.27	[11]	1.5	KR + HSA (30/40 g/L)	Before, 0,2,5,10,15,30,45,60,90, 120,150
AA, KUO	70.1	Soluble	−0.67	[12]	70.3 and 140.7	RPMI + HSA (2 g/L)	0,30,60,90,120,180,240
GA, KUO	87.1	Soluble	NA	[12]	57.4	RPMI + HSA (2 g/L)	0,30,60,90,120,180,240
AFB1, KUO	312.3	Insoluble	___	[13]	0.5 and 5.0	RPMI + HSA(2 g/L)	Before,5,30,60,120,180,240 Before,5,30,60,90,120

Soluble>1g/100 mL, sparingly soluble 0.1-1g/100 mL, insoluble <0.1g/100 mL, RPMI = RPMI 1640 cell culture medium, KR = Krebs Ringer Phosphate buffer, HSA = human serum albumin.

Table 3
Background characteristics for placentas studied.

	CPH	OUL	KUO
Method of delivery			
Vaginal	27.9%	41.7%	52.9%
C-section (no labor)	72.1%	52.3%	47.1%
C-section (with labor)	0%	0%	0%
Length of gestation	>37 weeks	>36 weeks	>36 weeks
Smoking			
Non smokers	80%	NA[a]	NA[a]
Smoking cessation during first trimester	9.5%		
Smokers	7.1%		
Not known	2.4%		
Medications	No medications (59.5%) Clotrimazole (7.1%) Antibiotics (7.1%) Ondansetron (2.4%) Morfin (2.4%) Paracetamol (2.4%) Prednisolon (2.4%) Heparin (2.4%) Nasal corticosteroid (2.4%) Lamotrigine (2.4%)	NA[a]	NA[a]

[a] Healthy mothers with no medications or pregnancy complications reporting themselves as non-smokers were asked to donate their placentas for human placental *ex vivo* perfusions.

2.2. Placental perfusion method

In short, a chorionic artery-vein pair was cannulated. The perfused lobe was isolated by clamping or by cutting it to fit into the perfusion chamber with the basal plate facing upwards. Two cannulae, representing maternal arteries were placed in the intervillous space through the basal plate. The fetal flow was approximately 3 mL/min and the maternal flow 9 mL/min except in the IQ study from OUL and the BPA/EtOH study from CPH where the fetal flow was 2.5 or 3.5 mL/min, respectively. In both maternal and fetal circuits a recycling system was used. Recycling was initiated after preperfusion (30–60 min). After preperfusion, the study compound was added to maternal circulation simultaneously with antipyrine (100 μg/ml).

The quality markers originally included: leak from the fetal compartment (<3 mL/h except for AFB1 <4 mL/h was accepted), decrease in fetal flow rate or pressure in fetal circulation, antipyrine transfer kinetics (FM_{150}>0.7), oxygen transfer/consumption, physiological pH and glucose consumption. The perfusion methods were refined during the study period (2005–2010), and additional viability markers were included: lactate production and hCG production. The results for quality markers have been presented in detail in the original publications (Table 2).

2.3. Calculations and statistical analysis

The fetal/maternal concentration ratio (FM ratio), the indicative permeability coefficient (IP_{30}), and area under the curve of fetomaternal concentration ratio within 120 min of perfusion (AUC_{120}) were calculated for all compounds (Fig. 1).

FM ratio was calculated as the concentration in fetal perfusion medium (C_{fetal}) divided by the concentration in maternal perfusion medium ($C_{maternal}$) at a defined time point during perfusion.

$$FM = C_{fetal}/C_{maternal}$$

IP_{30} was defined as the slope of the FM ratio curve between time 0 and 30 min (ΔX) (Fig. 1)

$$IP_{30} = \int_{0}^{30} \Delta FM/\Delta X$$

AUC_{120} is defined as the area under the FM ratio curve between the time points 0 and 120 min (Fig. 1). AUC was calculated using the trapez algorithm dividing the area under the curve up to 120 min into smaller time intervals (ΔX) depending on sampling time points and the FM values.

$$AUC = \int_{0}^{120} 1/2(FM_1 + FM_2)*\Delta X$$

FM_{120} and AUC_{120} uses data obtained at or up to 120 min, where equilibrium of antipyrine is established. After 120 min antipyrine is close to equilibrium and therefore sampling around this time point provides good information on the perfusion quality and flow rates. Although the majority of perfusions lasted more than 2 h it is not feasible to normalize the transfer data with antipyrine after that period of time. After approximately 2-3 h antipyrine equilibrium has been reached in most perfusions and the concentration ratio no longer represents the dynamic phase.

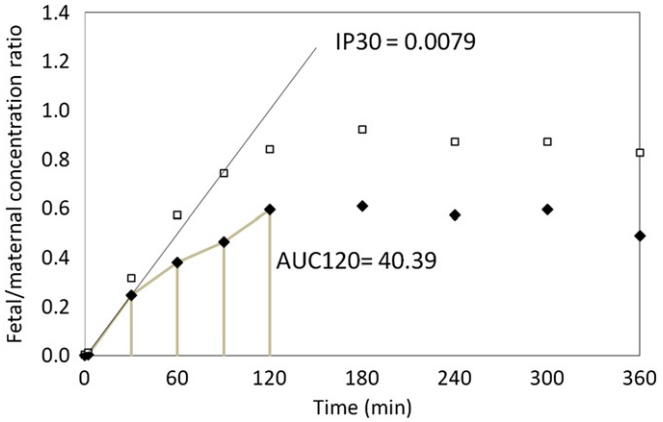

Fig. 1. An example illustrating the method for calculating IP_{30} and AUC_{120} values. Fetal to maternal concentration ratio curves were used to calculate these parameters for each perfusion using non-normalized and antipyrine normalized values separately. Black diamonds = normalized average fetal to maternal concentration ratio for PhIP; white squares = the total mean fetal to maternal concentration ratio for antipyrine.

Antipyrine normalization was defined as an internal standardization procedure to correct every perfusion for inter-placental and inter-laboratory variations minimizing the data variation. Antipyrine normalization was performed using either transfer index (TI_{120} (%)) or normalization factors ($FM_{120NORM}$):

$$TI(\%) = (F120/M0)_{test\ compound}/(F120/M0)_{antipyrine}$$

$$FM_{NORM}: \sum (FM120_{antipyrine})_{all}/(FM120_{antipyrine})_{perfusion} \times (F_{120}/M_{120})_{test\ compound}$$

The transfer index (TI_{120}(%)) was chosen as a parameter capable of relating to the amount of test compound transferred to the amount of compound initially added to maternal circulation (1). FM_{NORM} values were calculated at all sampling points to get a normalized FM curve to enable calculation of IP_{30NORM} and $AUC_{120NORM}$ in every perfusion using the same method as described previously.

Cotyledon weight (CW) normalization was defined as a standardization method to normalize data according to cotyledon size representing the fetal-maternal exchange area. It was performed using a ratio of the mean weight of cotyledons in a study using the same study compound in relation to the cotyledon weight in a specific perfusion within the same study (FM_{120CW}). However, CW normalization increased the variation supporting earlier findings by Schneider [16] indicating that cotyledon weight is a poor indicator of the perfused surface area. The CW normalization data is thus not presented or discussed further.

$$FM_{CW}: \sum (CW)_{study}/CW_{perfusion} \times (F_{120}/M_{120})_{test\ compound}$$

Statistical analyses were performed using IBM SPSS Statistics 19 software. Comparisons between multiple groups were done using Oneway ANOVA and Tukey as a posthoc test. If there were only two groups to compare, comparisons were done using the *t*-test for independent samples. The Levene's test for equality of variances was conducted before pair-wise comparisons. The Pearson's correlation was used to evaluate the correlation between non-normalized and normalized parameters. *P*-values less than 0.05 were considered statistically significant.

2.4. Ethics

The official Ethics Committees of the Municipalities of Copenhagen and Frederiksberg (KF 01-145/03 + KF(11) 260063), the Northern Savo Central Hospital District (Kuopio, Finland) and the Northern Ostrobothnia Central Hospital District (Oulu, Finland) had approved the placental perfusion study protocols of each individual perfusion study (Table 2). All mothers gave a written informed consent for the use of their term born placentas. The participation in the placental perfusion study did not affect the management of the delivery in any way and the placentas were anonymized.

3. Results

3.1. The transfer of antipyrine

Ninety three perfusions were included in this re-analysis; 12 from OUL, 38 from KUO, and 43 from CPH (Table 2). Antipyrine transfer kinetics measured as FM_{120} values were significantly

different between the studies ($p = 0.002$) and between the laboratories ($p = 0.01$; Oneway ANOVA). Especially, antipyrine FM_{120} values were significantly different between CPH and KUO ($p = 0.013$; Tukey's posthoc analysis). The differences in antipyrine passage between studies suggest that there are some differences in perfusion and flow conditions. Antipyrine normalization was therefore needed to compare data between the studies. The method of delivery (vaginal birth vs. C-section without labor) did not affect antipyrine FM_{120} values ($p = 0.96$; independent samples *T*-test).

3.2. The transfer of ethanol before and after normalization

EtOH perfusions were carried out in CPH and KUO. The antipyrine FM_{120} in CPH was 0.99 ± 0.064 and in KUO 0.92 ± 0.14 ($p = 0.01$, independent samples *t*-test). Thus, the antipyrine FM_{120} showed a small but statistically significant difference in antipyrine transfer between the laboratories. Therefore, EtOH transfer data from KUO ($n = 4$) and CPH ($n = 5$) were compared before and after antipyrine normalization. Similarly to antipyrine, the parameters calculated before normalization describing EtOH transfer, FM_{120REG}, AUC_{120REG} and IP_{30REG}, were significantly different between the laboratories. In fact, the between laboratory difference in EtOH transfer was higher than in antipyrine transfer (Table 4). However, antipyrine normalization of EtOH data resulted in $FM_{120NORM}$, $AUC_{120NORM}$ and IP_{30NORM} values that were no longer significantly different between the laboratories (Fig. 2). Antipyrine normalized data of EtOH from both laboratories were therefore pooled for further analyses.

3.3. The transfer of IQ and PhIP before and after normalization

IQ data from CPH ($n = 7$) and OUL ($n = 6$) did not show significant differences in FM_{120REG}, AUC_{120REG} and IP_{30REG} values before antipyrine normalization most likely due to similar antipyrine transfer (FM120) between the laboratories ($p = 0.857$; independent samples *t*-test). After normalization of IQ data, $AUC_{120NORM}$ and $FM_{120NORM}$ values were not statistically different, either. However, IP_{30NORM} showed a significant difference between the laboratories mainly due to few sample points before 30 min and missing antipyrine values at 30 min thereby generating poor normalized data from CPH at these sampling times.

Similarly to IQ, PhIP data from CPH ($n = 3$) and OUL ($n = 6$) did not show any significant differences in FM_{120REG}, AUC_{120REG} and IP_{30REG} values before antipyrine normalization (independent samples *t*-test). After normalization of PhIP data, $AUC_{120NORM}$ and $FM_{120NORM}$ values were not significantly different, either. Therefore, data from IQ studies and PhIP studies performed in CPH and OUL were pooled for further analyses.

3.4. Transfer of other compounds before and after normalization

Other compounds were studied in a single research site. For all studied compounds a good correlation ($r = 0.811$, Pearson's correlation) was found between the regular/non-normalized (AUC_{120REG}) and normalized ($AUC_{120NORM}$) values (Fig. 3). Similar correlations were found between the other regular and normalized parameters ($FM1_{120REG}$ vs. $FM_{120NORM}$ $r = 0.714$ and IP_{30REG} vs. IP_{30NORM}, $r = 0.468$). As expected, antipyrine normalization of the data generally elevated the transfer rate of the compounds with slow antipyrine transfer rates (Fig. 3).

3.5. Ranking of compounds according to their AUC_{120} value and TI_{120}(%)

The non-normalized AUC_{120REG} values of all compounds were not directly comparable due to the difference in the transplacental transfer of antipyrine between the studies and the laboratories. However, the results indicate that BPA and EtOH belong to the group with highest AUC_{120REG} values, AA, NDMA, IQ, and PCB180 in the medium group, GA, AFB1, PhIP, and DON in the low group and BP, PCB52, and TCDD in the group with very low AUC_{120REG} values (Fig. 4a).

Antipyrine normalization results in the following ranking order of compounds according to the $AUC_{120NORM}$ values: NDMA (68)≥ EtOH (65)≥ BPA (65) ≥ IQ (62) ≥AA (61) ≥ GA (56) ≥ PCB180 (47) ≥ PhIP (42) ≥ AFB1 (40) > DON (22) ≥ BP (22) ≥ PCB52 (17) ≥ TCDD (6). Normalization resulted in an evident division of the compounds into slow and fast transported compounds with a statistically significant difference between these groups (Oneway ANOVA followed by Tukey's posthoc test; Fig. 4b). Compounds with

Table 4
Summary of the results before and after normalization using different normalization methodologies. Data for compounds studied in more than one laboratory (EtoH, IQ and PhIP) is pooled only after normalization.

Compound	Number of perfusions	Antipyrine FM_{120}	$FM_{120NORM}$	FM_{120REG}	$AUC_{120NORM}$	AUC_{120REG}	IP_{30NORM}	IP_{30REG}	TI_{120}(%)
NDMA	11	0.81 ± 0.11	0.92 ± 0.14	0.87 ± 0.13	68.3 ± 6.8	58.6 ± 9.12	0.011 ± 0.0017	0.0088 ± 0.0021	1.0 ± 0.13
EtOH	5[a]	0.99 ± 0.006[a]	0.87 ± 0.081	0.99 ± 0.09[a]	65.4 ± 8.2	85.7 ± 10.7[a]	0.010 ± 0.0023	0.018 ± 0.002[a]	0.80 ± 0.10
	4[c]	0.92 ± 0.14[c]		0.82 ± 0.11[c]		51.9 ± 10.0[c]		0.0069 ± 0.0019[c]	
GA	4	0.67 ± 0.15	0.86 ± 0.22	0.66 ± 0.12	56.4 ± 10.1	38.1 ± 7.5	0.0132 ± 0.007	0.0048 ± 0.002	0.78 ± 0.21
AA	11	0.84 ± 0.10	0.82 ± 0.11	0.82 ± 0.14	60.8 ± 7.9	59.4 ± 10.9	0.0109 ± 0.0033	0.0095 ± 0.0028	0.91 ± 0.18
IQ	7[a]	0.79 ± 0.27[a]	0.89 ± 0.38	0.73 ± 0.15[a]	61.6 ± 14.1	55.1 ± 23.4[a]	0.011 ± 0.0057	0.0046 ± 0.010[a]	0.95 ± 0.32
	6[b]	0.87 ± 0.1[b]		0.85 ± 0.13[b]		59.6 ± 9.7[b]		0.011 ± 0.003[b]	
BPA	6	1.04 ± 0.11	0.75 ± 0.15	0.92 ± 0.18	65.0 ± 4.5	83.2 ± 14.9	0.0117 ± 0.002	0.017 ± 0.0053	0.61 ± 0.13
AFB1	8	0.78 ± 0.17	0.64 ± 0.15	0.58 ± 0.12	40.1 ± 5.6	35.2 ± 14.0	0.0093 ± 0.0028	0.0077 ± 0.0026	0.47 ± 0.11
PCB 180	5	0.93 ± 0.16	0.63 ± 0.085	0.70 ± 0.17	47.4 ± 5.0	54.1 ± 17.6	0.0089 ± 0.0021	0.013 ± 0.0070	0.49 ± 0.16
PhIP	3[a]	0.69 ± 0.18[a]	0.60 ± 0.17	0.46 ± 0.16[a]	41.6 ± 10.0	34.8 ± 11[a]	0.0084 ± 0.0038	0.0061 ± 0.0020[a]	0.57 ± 0.23
	6[b]	0.71 ± 0.21[b]		0.50 ± 0.11[b]		32.0 ± 6.4[b]		0.0046 ± 0.0019[b]	
DON	5	1.02 ± 0.022	0.37 ± 0.06	0.41 ± 0.06	21.6 ± 4.4	27.4 ± 4.7	0.0025 ± 0.001	0.0036 ± 0.006	0.67 ± 0.15
BP	5	0.77 ± 0.24	0.34 ± 0.24	0.38 ± 0.28	20.2 ± 16.2	22.3 ± 18.3	0.0014 ± 0.0009	0.0016 ± 0.0011	0.38 ± 0.32
PCB 52	4	0.93 ± 0.08	0.21 ± 0.06	0.23 ± 0.070	17.0 ± 7.2	16.8 ± 4.8	0.0029 ± 0.001	0.0033 ± 0.0008	0.27 ± 0.14
TCDD	3	0.89 ± 0.31	0.058 ± 0.038	0.068 ± 0.066	6.1 ± 5.0	6.1 ± 5.8	0.00086 ± 0.00038	0.0062 ± 0.00021	0.081 ± 0.026

$_{NORM}$ = antipyrine normalized, $_{REG}$ = un-normalized/regular data.
[a] CPH.
[b] OUL.
[c] KUO.

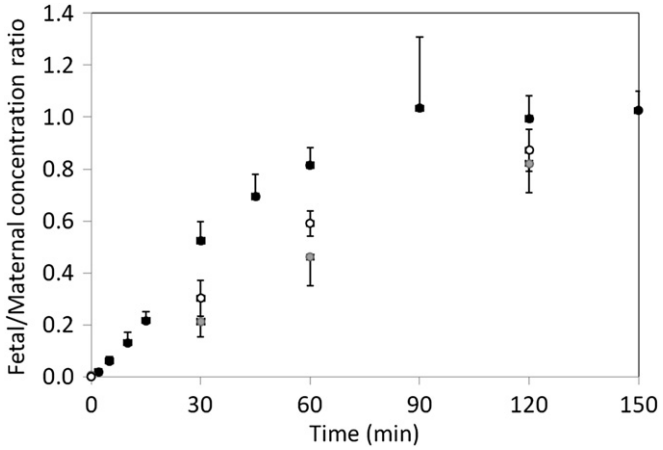

Fig. 2. EtOH transfer illustrated as Fetal/Maternal (FM) concentration ratios during 2-2½ hours of perfusion in Copenhagen (black, $n = 5$) and Kuopio (Grey, $n = 4$). After antipyrine normalization, data from Copenhagen and Kuopio is no longer statistically different and therefore the FM_{NORM} values from the two laboratories are pooled (white, $n = 9$).

an $AUC_{120NORM} \leq 22$ (DON, BP, PCB52, and TCDD) were significantly different from the compounds with intermediate (over 40) $AUC_{120NORM}$ values (PCB180, PhIP, and AFB1) or high (over 60) $AUC_{120NORM}$ values (NDMA, BPA, AA, GA, IQ and EtOH).

$TI_{120}(\%)$ normalization produced the following ranking order: NDMA(1) ≥ IQ (0.91) ≥AA (0.78) ≥ GA (0.78) ≥ EtOH (0.72) ≥DON (0.67) ≥ BPA (0.61) ≥ PhIP (0.5) ≥ PCB180 (0.49) ≥AFB1 (0.47) ≥BP (0.41) ≥ PCB52 (0.0.27) > TCDD (0.08) (Fig. 4c). Compounds with placental transfer kinetics similar to passive diffusion having $TI_{120}(\%)$ values close to one (NDMA, EtOH, IQ, AA) correlate well with $FM_{120NORM}$ and $AUC_{120NORM}$ (data not shown). However, the compounds that disappear from maternal circulation without appearing in fetal circulation (e.g. binding to tissue and perfusion system) generated low $TI_{120}(\%)$ values which did not correlate well with their respective $FM_{120NORM}$ values.

The method of delivery did not affect the parameters used to rank compounds, $AUC_{120NORM}$ or $TI_{120}(\%)$ for EtoH, PhIP or IQ ($p > 0.29$; independent samples t-test). For other compounds the number of perfusions either in the C-section or vaginal birth group was too low (<4) to allow statistical evaluation.

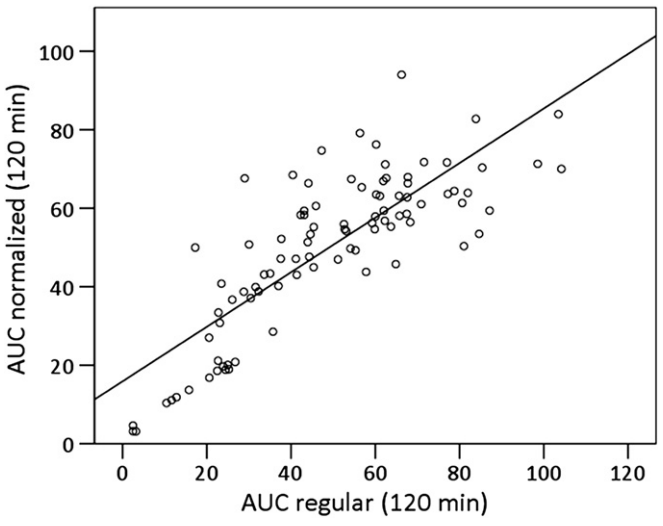

Fig. 3. Correlation between regular (AUC_{120REG}) and normalized ($AUC_{120NORM}$) AUC values for all compounds ($r = 0.811$, Pearson's correlation).

4. Discussion

The purpose of performing placental perfusion studies is to generate data that can contribute to the evaluation of fetal exposure and thereby fetal risk assessment of hazardous compounds. However, comparing data originating from different experimental conditions, even following the same general protocol, may be challenging. Normalization or standardization of perfusion data using the transfer kinetics of antipyrine in the same perfusion is a concept often mentioned in the scientific papers or methodological reviews within this research field [19–23]. However, in most cases it has not been studied or described in detail [16]. In our previous studies pooling data from several research sites or laboratories antipyrine data were comparable and antipyrine normalization was therefore considered unnecessary [8,9,24]. In the present meta-analysis normalization was important due to the differences in the transplacental transfer rate of antipyrine.

We tested several methods for the normalization of perfusion data and found both $TI_{120}(\%)$ and normalization factor based calculations useful. Using EtOH as an example, normalization factor based calculations enabled us to make perfusion data comparable among different study sites providing the opportunity to rank the compounds regardless of their origin. Also, the data variance was smaller suggesting a reduction of at least some of the perfusion to perfusion variation e.g. due to differential flow conditions.

For transfer by passive diffusion permeability of the placental barrier and binding of the compound to placental tissue are the two major determinants. $AUC_{120NORM}$ is based on the FM ratio and reflects uptake from the maternal circulation together with transfer across the placenta with release into the fetal circulation. $TI_{120}(\%)$, on the other hand, is more useful when evaluating the exposure risk of the fetus as it relates directly to the net transfer of compound into fetal circulation. In our data BPA was highly ranked in $AUC_{120NORM}$ values compared to DON. In $TI_{120}(\%)$ ranking the opposite was true for BPA. The difference between the two calculated parameters may arise from binding of the compound to the tissue and potential metabolism. The recovery of BPA in the perfusates was low due to the high tissue binding which generates a low $TI_{120}(\%)$ value and explains the finding. In general, compounds with high tissue binding generate low transfer indexes [17].

$TI_{120}(\%)$ is easier to calculate than the other tested normalization methods because there is no need to pool all the available data for calculations. Thus, appending data sets is much easier. However, in contrast to $AUC_{120NORM}$, $TI_{120}(\%)$ relies on a single sampling point which emphasizes the use of good quality antipyrine data. The variances for $TI_{120}(\%)$ values are larger than the variances in the respective FM_{120REG} or antipyrine FM_{120} values which is not desirable when normalizing against an internal standard (Table 4). The quantitative analysis of antipyrine was not included in the protocol developed within ReProTect and NewGeneris, which caused some problems during this meta-analysis. The problems in antipyrine data affected the parameters relying on data at single time points more than AUC_{120} values.

A limitation of antipyrine normalization is that it is not useful after 2-3 h when antipyrine equilibrium is reached. Normalization using additional perfusion markers may improve the normalization of perfusion results for compounds that have a slow and/or limited transplacental transfer. Creatinine is a suitable diffusion marker passing through the placenta more slowly and in a flow independent manner compared to antipyrine [25]. Instead, creatinine diffusion is related to the fetal membrane surface area exposed to maternal perfusion medium. In the case of highly permeable

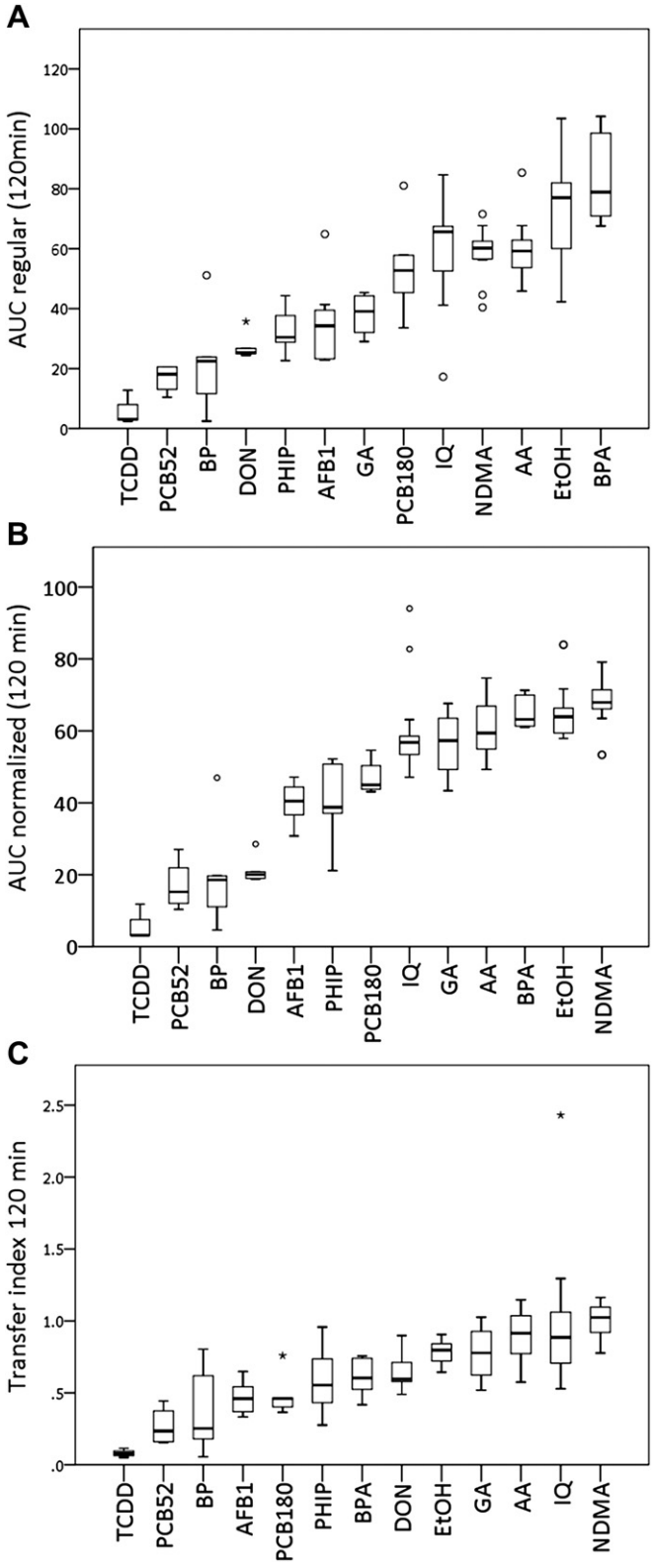

Fig. 4. Box-plot illustrating the ranking order and variation. A) AUC_{120REG}. BPA is significantly different from all except EtOH, AA and IQ. EtOH statistically differs from GA and downwards. AA, NDMA and IQ differ from AFB1 and downwards. PCB180 differs from PCB52 and downwards. Compounds below PCB180 do not differ significantly from each other. B) $AUC_{120NORM}$. NDMA and EtOH statistically differ from PCB180 and downwards. AA, BPA, and IQ differ statistically significantlyfrom PhIP and downwards. GA, PCB180 and PhIP significantly differ from DON. AFB1 differs from all except PCB180, GA and PhIP. DON differs significantly from all compounds above and none below. C) TI_{120}(%). NDMA and IQ are statistically different from PhIP and downwards. AA is statistically different from AFB1 and downwards. GA and EtOH are only statistically different from TCDD. Compounds below EtOH are not statistically different from each other. DON and BPA are not statistically different from any compounds. All the comparisons were made using Oneway ANOVA followed by Tukey's posthoc test.

compounds, the rapid-pair tracer dilution method has been used to study transport and the mechanisms of transfer [26,27]. In this method the study compound is analyzed in the non-circulating perfusion setup against an impermeable extracellular marker such as L-glucose which allows the semi-quantification of transfer and provides data on efflux.

Normalization generated a clear division of the studied compounds into high, medium and slow transfer groups. Compounds with a small molecular mass (500 g/mol), low polarity, moderate lipid solubility, and low protein binding capacity pass through the placenta by passive diffusion [28,29]. The partition coefficient between octanol and water (log Kow) and the dissociation pH (pKa) may also predict transplacental behavior provided the compound crosses the placenta by passive diffusion. The passage of hydrophilic highly ionized compounds is limited by the lipid membranes of the placenta [28,30]. Binding to plasma proteins can limit the transplacental transfer rate, but the opposite may also be true, as shown for some compounds, e.g., BP [6] and BPA [4]. Also, the differences in binding capacity of maternal and fetal albumin [31] may affect the *in vivo* placental transfer. All investigated compounds have molecular masses below 500 g/mol and are non-ionized at physiological pH. However, the placental transfer ranked from fast and easy (e.g. NDMA) to very limited transfer (TCDD). The compounds having transfer rates very similar to simple diffusion are NDMA, EtOH, BPA, AA, GA and IQ, of which NDMA, EtOH, AA, and GA have small molecular masses and are soluble in water. IQ and BPA have moderate hydrophobic properties and molecular masses at approximately 200 g/mol and also diffuse passively.

PhIP, PCB180, and AFB1 are insoluble in water and have moderate to high hydrophobic properties with a rate limited transfer across the placenta reaching an FM ratio between 0.6 and 0.8 after 2 h of perfusion. PhIP and AFB1 are substrates for the ABCG2 transporter (also known as BCRP or breast cancer resistance protein), an efflux transporter. It is located in syncytiothrophoblast facing the maternal circulation and partly preventing entry of its substrates into fetal compartment [8] which could explain the lower transfer rate. Interestingly, IQ is also a substrate for ABCG2 but it seems that in human placenta ABCG2 does not limit its transfer [7]. PCB180 has a higher log Kow than PCB52 but is transferred significantly faster across the placenta ($AUC_{120NORM}$). The most likely explanations are higher protein binding of PCB180 (22%) compared to PCB52 (1%), and/or interaction with active transporters. A study in primary placental cells demonstrated that less chlorinated PCBs accumulate in the cells to a greater extent than the more chlorinated PCBs [32].

DON, BP, PCB52, and TCDD are compounds with a slow transplacental transfer rate measured as a low $AUC_{120NORM}$ value. The limited transfer of DON suggests that DON is a substrate for an efflux transporter or that its relatively high ability to form hydrogen bonds to water limits the diffusion rate. Videmann et al. [33] have shown that blocking the ABC transporters (ABCB1 and ABCC2) increases DON levels at the basolateral side in a tight Caco-2 cell monolayer. Furthermore, DON can cross the epithelial barriers via paracellular diffusion through the tight junctions in the Caco-2 cell monolayer [34]. The results from placental perfusions and studies using BeWo cells forming a monolayer implicate a similar mechanism in human trophoblasts [3]. On the other hand, structure-based modeling (QSAR) of placental data has identified hydrogen

bonding as a key descriptor of placental transfer. An increased ability to form hydrogen bonding possibly reduces membrane penetration which could apply for DON [35].

Putatively harmful fetal exposure depends not only on the concentration of the parent compound in the fetal compartment but also concentration of toxic metabolites. Metabolites in the fetal compartment may be transferred from maternal circulation or formed either in placenta or fetal tissues. Original studies were done using only parent compound, except for AA. Therefore we were able to include metabolite data only for glycidamide, the genotoxic metabolite of acrylamide in this meta-analysis. During the human placental perfusions AFB [13] and BP [6] were metabolized while no IQ or acrylamide metabolism was detected [7,12].

In conclusion, using commonly developed protocols, thirteen genotoxic or immunotoxic compounds were studied in three laboratories. The perfusion data were normalized using antipyrine transfer. The presented normalization method generates convincing results by at least partially correcting the bias caused by methodological differences. Based on our results we conclude that NDMA, EtOH, AA, GA, IQ and BPA have a high transplacental transfer rate much similar to passive diffusion. PhIP, PCB180, and AFB1 have a medium transplacental transfer rate whereas DON, BP, and TCDD are slowly transferred based on $AUC_{120NORM}$ values. Thus, although there are differences in transplacental transfer rate the placental barrier is not protective against exposures to the studied compounds.

Competitive financial interest declaration

None of the authors have any financial interest to declare.

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