



Added value of plasma metabolomics to describe maternal effects in rat maternal and prenatal toxicity studies

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ARTICLE INFO

Keywords:

Metabolome analysis

Pregnant rats

Maternal toxicity

ABSTRACT

For regulatory purposes prenatal developmental toxicity (OECD No. 414) studies are routinely performed in our laboratories. The suitability of metabolomics as technology to identify maternal toxicity in such studies was investigated. Plasma was sampled from pregnant, non-fasted rats on gestation day 20 before cesarean section. Metabolite profiling was performed by gas- and liquid-chromatography-tandem mass spectrometry techniques. The sensitivity of routinely examined maternal toxicity parameters (OECD No. 414) was compared to those of metabolome analysis. Evaluating 44 studies, the metabolome-derived NOEL was more sensitive in 45% of the cases in detecting maternal toxicity than the maternal NOAEL. Metabolome patterns indicative for liver effects and 4-hydroxyphenylpyruvate dioxygenase (HPPD) enzyme-inhibition were established in pregnant rats based on regulated metabolites using reference compounds. The HPPD inhibition and liver toxicity patterns in pregnant rats were reasonably comparable to the ones established in non-pregnant, fasted rats. Metabolomics is a useful tool for an improved and mechanism-based identification of maternal toxicity in maternal and prenatal toxicity studies. The data suggest that the current classical maternal toxicity parameters may underestimate the extent of effects of compounds on the dams.

1. Introduction

In metabolomics, metabolites are defined as small endogenous molecules such as carbohydrates, amino acids, nucleic acids, fatty acids or derivatives thereof (Lindon et al., 2004, 2006). Metabolomics was used to study metabolite changes in (human) maternal blood, urine, amniotic fluid and umbilical cord blood during pregnancy (Diaz et al., 2011; Luan et al., 2014) and, since more than a decade, to identify toxicological mechanisms in rat studies (Kamp et al., 2012; Mattes et al., 2014, 2013, van Ravenzwaay et al., 2007, 2010a, 2012; Strauss et al., 2009). In 28-day classical toxicity rat studies (Organisation for Economic Co-operation and Development (OECD) No. 407) metabolome analysis showed similar sensitivity with respect to the no observed adverse effect level (NOAEL) compared to classical toxicological investigations (van Ravenzwaay et al., 2014). Within the last years, BASF and metanomics established a database (MetaMap®Tox) for the detection of toxicological modes of action (MOA) based on metabolic profiles in plasma of rats. Approximately 274 different endogenous metabolites are measured and more than 40 modes of action (MoA) can be differentiated. However, the database MetaMap®Tox was built up with

plasma from non-pregnant rats which were fasted overnight prior to blood sampling.

In toxicology, assessment of developmental and maternal toxicity according to official test guidelines (OECD No. 414, Office of Prevention, Pesticides & Toxic Substances (OPPTS) 870.3700) is required by regulatory authorities for chemicals, agrochemicals and in a modified form also for pharmaceutical active ingredients (OECD, 2001). For this reason, prenatal toxicity (OECD No. 414) and range-finding - maternal toxicity - studies are routinely performed in our laboratories.

In prenatal toxicity studies (PTS) according to OECD No. 414, the test substance is administered via gavage to pregnant animals, in most cases rats or rabbits. This type of study gives insight into the prenatal developmental toxicity including fetal malformations or variations. Besides the fetus, focus is also placed on the maternal organism, related toxicity and subsequent effects that maternal toxicity may have on the developing fetus. Maternal-mediated toxicity might lead to secondary (adverse) effects on developing rat fetuses (ECETOC, 2004; Khera, 1985; 2012), such as decreased litter size and birth weight, fetal growth retardation (Chernoff et al., 1990; Euker and Riegler, 1973; Glöckner

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<https://doi.org/10.1016/j.toxlet.2018.10.032>

Received 13 April 2018; Received in revised form 14 September 2018; Accepted 29 October 2018

Available online 09 November 2018

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and Karge, 1991) and variations like ‘supernumerary ribs’ (Beyer and Chernoff, 1986; Chernoff et al., 1990). Furthermore, embryofetal hypoxia caused by maternal anemia or uterine clamping in pregnant rats was able to induce fetal skeletal malformations, such as shortening of extremities or terminal transverse limb reduction defects (Ritchie et al., 2017; Webster et al., 1987; Wilson, 1953). With respect to classification of a compound as teratogen (no classification, category 2 and category 1b), developmental toxicity is assessed considering also the extent of maternal toxicity (Danielsson, 2013).

The OECD No. 414 requires clear (but not lethal) maternal toxicity, described as “clinical signs and decrease in body weight”, in the highest tested dose (OECD, 2001). However, precise criteria for maternal toxicity are still lacking and assessment of maternal toxicity in guideline studies is often limited to a small set of study parameters, e.g. in a standard OECD No. 414, such as clinical observation, food consumption and body weight. The study design is, in some cases, complemented by clinico-chemical (hematology and clinical chemistry) and pathological (organ weights and histopathology) data. As extensive maternal toxicity investigations during pregnancy may affect the development of the fetus, there is a risk that maternal toxicity is not explicitly noticed in these studies (ECETOC, 2004; Giavini and Menegola, 2012; Rogers et al., 2005).

In order to investigate the suitability of metabolomics as technology to better identify and qualify maternal toxicity, metabolome analysis was performed in 44 maternal and prenatal developmental toxicity studies in rats exposed to test substances undergoing toxicity testing at BASF. Within these regulatory required studies, plasma was sampled from pregnant, non-fasted rats on day 20 of gestation shortly before cesarean section. The sensitivity of routinely examined maternal toxicity parameters was compared to those of metabolome analysis using the classical maternal no observed adverse effect level (NOAEL) and the NOEL derived by metabolomics (van Ravenzwaay et al., 2014).

2. Material and methods

2.1. General

The studies were carried out in accordance with the OECD Principles of Good Laboratory Practice or were referring to them (OECD, 1992). They were performed in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care)-approved laboratory in accordance with the German Animal Welfare Act and the effective European Council Directive. Most of the studies were conducted in accordance with OECD test guideline No. 414 (OECD, 2001), Commission Regulation (European Commission, EC) No. 440/2008 (Commission Regulation 2016) and OPPTS 870.3700 (EPA 1998). The range-finding studies were referring to the above-mentioned guidelines but mostly lack examination of the fetuses (maternal toxicity studies) or used a lower number of animals per test group. All studies were performed under the reference number 23 177-07 of the local authority.

2.2. Test substances

A total of 44 test substances were tested within regulatory toxicity studies (new active ingredients, pre- or main studies required for regulatory submission). All substances were produced, provided and

analytically characterized by BASF SE, Ludwigshafen Germany. The stability of the test substance and their preparations at least during the administration period were analytically confirmed.

2.3. Test animals

Wistar Han rats CrI:WI(Han) were supplied by Charles River Laboratories, Research Models and Services, Germany GmbH, Sandhofer Weg 7, 97633 Sulzfeld. The animals which were paired by the breeder (time-mated animals) were supplied at noon on the day of evidence of mating; this day is referred to as GD 0 (GD: gestation day) and the following day as GD 1. From GD 0 (day of supply) to the beginning of administration (GD 6), the animals were accustomed to the environmental conditions and to the diet.

They were allowed free access to mouse/rat laboratory diet (Provimi Kliba SA, Kaiseraugst, Switzerland) and water. The animals were housed individually in polycarbonate cages type III cages, floor area around 800 cm² (TECHNIPLAST, Hohenpeißenberg, Germany) on dust-free wooden bedding. Environmental enrichment consisted of wooden gnawing blocks (Typ NGM E-022, Abedd® Lab. and Vet. Service GmbH, Vienna, Austria). Temperature was maintained at 20 to 24 °C, with a relative humidity of 30 to 70%, a light/dark cycle of 06.00 to 18.00 h light and 18.00 to 06.00 h dark. The random distribution of the test animals to the individual test groups was carried out on the day of supply (= GD 0) by randomly removing the animals from the transport boxes. Animals were around 10–15 weeks old at start of the administration period.

2.4. Experimental procedure

2.4.1. Studies and test groups

In this work, three different study types were used:

- Maternal toxicity studies (MTR)
- Prenatal toxicity studies (PTS)
- Prenatal screening studies (sPTS)

The details of each study type are presented in Table 1:

In general, the aim of all studies was to evaluate the potential effects of different chemicals or agrochemicals on embryonic and fetal development following repeated gavage administration to female Wistar rats from gestation day (GD) 6 through GD 19. In this work, the influence of the test substance on the maternal organism during gestation was examined. Two (high and mid) or three (high, mid and low) different dose levels were used. The two highest dose levels were used for metabolome analysis and further evaluation in this work. A further group of animals receiving a vehicle preparation only served as a control. Animals numbers varied between 25 (PTS), 10 (sPTS) and 10, 7 or 5 (MTR) animals per group.

Gavage administrations were done daily in the morning between 07:30 and 12:00 h. Each test substance was gavaged for 14 days (GD 6–19).

2.4.2. Clinical signs, body weight and food consumption

All animals were checked for moribund state and death twice daily (once daily on weekends and public holidays), and at least once daily

Table 1
Overview of the different study types.

Study type	Purpose	Number of dose levels ^a	Number of animals per group	Evaluation of the fetuses
MTR	Range-finding study for dose selection before a PTS	2	5, 7 or 10	No
PTS	OECD 414	3	25	Yes
sPTS	Prenatal screening study for new compound development	2 or 3	10	Yes/No

^a For each study, a control group of similar size was used.

for clinical signs. During the administration period (GD 6–19) all animals were checked daily for any abnormal clinical signs before the administration as well as within 2 h and within 5 h after the administration. Abnormalities and changes were documented for each animal.

Food consumption and body weights were recorded regularly.

2.4.3. Blood sampling for hematology and clinical chemistry

Blood was taken on GD 20 from the retrobulbar venous plexus in the morning from non-fasted pregnant animals (dams) anaesthetized with isoflurane. Blood sampling and blood examinations were carried out in a randomized sequence. The list of randomization instructions was compiled with a computer system (Ascentos, PDS Life Sciences). The following parameters were determined in blood with K3-EDTA as anticoagulant using a particle counter (Advia 120 model; Bayer, Fernwald, Germany): leukocyte count (WBC), erythrocyte count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT), differential blood count, reticulocytes (RET). An automatic analyzer (Cobas c501; Roche, Mannheim, Germany) was used to examine the clinicochemical parameters (enzymes and blood chemistry): alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ -Glutamyltransferase (GGT), inorganic phosphate (INP), calcium (CA), urea (UREA), creatinine (CREA), glucose (GLUC), total bilirubin (TBIL), total protein (TPROT), albumin (ALB), globulins (GLOB), triglycerides (TRIG), cholesterol (CHOL).

2.4.4. Blood sampling for metabolomic profiling

Blood was taken on GD 20 from the retrobulbar venous plexus in the morning from non-fasted animals anaesthetized with isoflurane. Blood of the first 10 pregnant animals (out of 25 in PTS) or of all animals (MTR, sPTS) of each group was collected.

One mL per animal was collected into Eppendorf tubes, coated with K3-EDTA as anticoagulant (10 μ L of a 10% solution) and kept cool until centrifuged under cooling. Afterwards, separated plasma samples were covered with gaseous N_2 and deep frozen at 80 °C. Additional blood samples were taken from control animals after decapitation. Metabolome analysis was performed by Metanomics GmbH, Tegeler Weg 33, 10589 Berlin, Germany.

After blood sampling, all surviving dams were sacrificed and examined.

2.4.5. Metabolite profiling

Mass spectrometry based metabolite profiling of plasma samples was performed by GC–MS (gas chromatography-mass spectrometry) and LC–MS (liquid chromatography-tandem mass spectrometry) techniques as described in detail in previous works (Kamlage et al., 2014; van Ravenzwaay et al., 2007, 2010b, 2012) and below.

Proteins were removed from 60 μ L plasma samples by precipitation using 200 μ L acetonitrile. Subsequently, polar and non-polar fractions were separated for both GC–MS and LC–MS/MS analysis by adding water and a mixture of ethanol and dichloromethane (1:2, v:v). For GC–MS analysis (CTC GC PAL, Agilent 6890 GC gas chromatograph, 5973 MSD mass spectrometer), the non-polar fraction was treated with methanol under acidic conditions to yield the fatty acid methyl esters derived from both free fatty acids and hydrolyzed complex lipids. The non-polar and polar fractions were further derivatized with O-methyl-hydroxylamine hydrochloride and pyridine to convert oxo-groups to O-methyl-oximes and subsequently with a silylating agent before analysis (Roessner et al., 2000). For LC–MS analysis, both fractions were reconstituted in appropriate solvent mixtures. HPLC was performed by gradient elution on reversed phase separation columns. Mass spectrometric detection technology was applied which allows target and high sensitivity MRM (Multiple Reaction Monitoring) profiling in parallel to a full screen analysis as described in patent WO2003073464 (Walk and Dostler, 2003). For GC–MS the acquisition in scan mode m/z 15–600 for

polar compounds and m/z 40–600 for lipid compounds was applied. For LC–MS MRM and a Q3 Scan of m/z 100–1000 was used. MRMs off all analytes were determined using solutions of the authentic standard.

GC–MS conditions: CTC GC PAL, Agilent 6890 GC gas chromatograph, 5973 MSD mass spectrometer, gradient: 70 °C–340 °C, carrier gas: helium, acquisition in scan mode m/z 15–600 (polar compounds) / m/z 40–600 (lipid compounds).

LC–MS conditions: Agilent 1100 HPLC System, AB Sciex API 4000 mass spectrometer, gradient elution for polar compounds with water / acetonitrile / ammonium formate, gradient elution for lipid compounds with water / methanol / methyl tert-butyl ether / formic acid, MRM and Q3 Scan m/z 100–1000.

For GC–MS and LC–MS/MS profiling, data were normalized to the median of reference samples which were derived from a pool formed from aliquots of all samples to account for inter- and intra-instrumental variation. In plasma, 274 semi-quantitative metabolites could be analyzed using the single peak signal of the respective metabolite and a normalization strategy according to the patent WO2007012643A1 (Walk et al., 2006) resulting in ratio values which represent the metabolite change of treated versus control animals. Steroids hormones, catecholamines and their metabolites were measured by online SPE-LC–MS/MS (Solid phase extraction-LC–MS/MS) (Yamada et al., 2002; Zhang et al., 2011). The methods applied resulted in 274 plasma analytes for semi-quantitative analysis, of which 248 were chemically identified and 26 were structurally unknown. This list of 274 metabolites contains metabolites which are measured more often than once in the different analytical methods (e.g., LC- or GC–MS, lipid or polar phase). These metabolites can/are also (be) used to confirm the validity of the measurements. The number of unique metabolites is 104. For the analyzed metabolites, changes were calculated relative to untreated control animals resulting in an up- or downregulation of these metabolites. The detailed evaluation of metabolic changes was already published previously (Kamp et al., 2012).

2.4.6. Post-mortem examination and pathology

On GD 20, the dams were sacrificed under isoflurane anesthesia by decapitation, in randomized order. After the dams had been sacrificed, they were necropsied and assessed for gross pathology.

Target organs were weighed, fixed in 4% buffered formaldehyde solution and stored for further potential examinations (e.g. histopathology). In MTR and some sPTS studies no further examinations were done. The uteri were not opened.

In PTS, the following examinations or the following weight determinations and counts were carried out: weight of the unopened uterus, number of corpora lutea, number of implantations (differentiated according to live and dead fetuses and early or late resorptions), site of implantations in the uterus. The fetuses were removed from the uterus by caesarian section and further examinations or weight determinations were carried out according to the test guideline OECD 414 (OECD, 2001).

2.4.7. MetaMap®Tox evaluation of metabolic profiles

Patterns of characteristically changed metabolites for a certain toxicological mode of action were developed from the metabolite profiles in the MetaMap®Tox database for over 500 compounds (Kamp et al., 2012). Briefly, characteristic metabolite changes correlating with specific toxicological modes of action are identified based on at least three different chemicals from the MetaMap®Tox data base, which commonly share this particular toxicological mode of action (reference compounds). After identification of the significantly changed metabolites and a consistency check through an expert panel, the pattern is validated against the data base. A pattern should correctly identify at least one further reference compound sharing the same mode of action, which has not been used to establish the pattern. Furthermore, reference compounds in MetaMap®Tox which do not share this particular toxicity should not be assigned. Using MetaMap®Tox, reference

Table 2
Overview of the 44 compounds, study design, dose levels, effects and classical maternal NOAEL as well as metabolome NOEL.

Study No.	Compound class / use	Study type	HD [mg/kg]	LD [mg/kg]	Effects	NOAEL [mg/ kg] maternal	% changed metabolites HD	% changed metabolites LD	NOEL [mg/kg] metabolome
1	(Petro-)Chemical / auxiliary and additive	MTR	1000	300	1000: BIL-, PROT-, GLOB-, liver a/rw + 300: liver a/rw +	300	23.0	21.2	< 300
2	Chemical / intermediate	MTR	1000	300	1000: liver a/rw +	1000	16.8	2.4	300
3	Chemical / battery material	MTR	300	100	300: RET +, WBC +, NEUT +	< 100	13.0	13.0	< 100
4	(Petro-)Chemical / processing aids	MTR	300/60	100	100: RET +, WBC + 300/60: FC-, BW-, BWC-, cBWG-, CREA-, liver hypertrophy/necrosis 100: WC+, FC-, BWC-, CREA-, spleen a/rw-, liver rw +, liver hypertrophy/necrosis	< 100	9.3	12.1	< 100
5	Chemical / aromatic substance	MTR	1000	300	1000: salivation, liver w +, kidney w +, adrenal w + 300: salivation	300	13.6	5.4	< 300
6	Chemical / intermediate	MTR	250	80	250: WC+, FC-, BW-, BWC-, CW-, UREA +, PROT-, BIL +, adrenal rw +, kidney rw +, liver rw +, spleen rw + 80: salivation, BWC-, WC+, BIL +, liver rw +	80	42.0	11.4	< 80
7	Chemical / processing aids	MTR	1000	300	1000: FC-, liver a/rw + 300: liver a/rw +	300	12.3	3.4	300
8	Chemical / aromatic substance	MTR	1000	300	1000: mortality, clin obs 300: clin obs, WC+, FC+, TRIG +, ALT +	< 300	ND	26.7	< 300
9	Chemical / aromatic substance	MTR	1000	300	1000: WC+, RBC, HGB, HCT-, RET +, PROT-, ALB-, GLOB-, BIL +, ALT +, liver a/rw +, adrenals a/rw +, kidney rw + 300: HGB-, HCT-, liver a/rw +	< 300	34.1	12.5	< 300
10	Chemical / antiwear agent	MTR	500	150	500: FC-, NEUT-, LYMPH +, PLT-, BIL-, liver a/rw +, adrenals a/rw + 150: FC-, BIL-, liver rw +	< 150	22.0	18.8	< 150
11	Chemical / processing aids	MTR	1000	300	NAD	1000	3.4	2.3	1000
12	Chemical / processing aids	MTR	1000	300	1000: clin obs, WC-, BWC-	300	2.1	5.1	< 300
13	Chemical / UV absorber	MTR	1000	300	1000: WBC-	1000	4.6	4.2	1000
14	Chemical / intermediate	MTR	25	8	25: PROT-, ALB-, GLOB-, liver a/rw +, liver hypertrophy, nuclear inclusions, single cell necrosis, multifocal necrosis 8: FC-, liver rw +	8	28.2	7.5	< 8
15	Chemical / battery material	PTS	300	100	300: WBC+, NEUT +, LYMPH-, 100: WBC+, NEUT +	< 100	14.4	8.8	< 100
16	(Petro-)Chemical / processing aids	PTS	80	25	80: FC-, BW-, BWC-, cBWG-, CW-, liver single cell necrosis; 25: liver single cell necrosis	< 25	26.1	11.9	< 25
17	Chemical / starting material in the polymerization of compounds	PTS	180	60	180: FC-, BWC-, CHOL +	60	28.3	22.4	< 60
18	Chemical / intermediate	PTS	500	150	60: CHOL + 500: RBC-, HGB-, HCT-, ALT +, CHOL +, TRIG +, liver a/rw +, spleen a/rw +, adrenal a/rw +, ovaries a/rw + 150: liver a/rw +, spleen a/rw +	150	27.3	19.8	< 150
19	Chemical / aromatic substance	PTS	750	250	750: salivation, liver a/rw + 250: salivation, liver rw +	750	44.5	13.3	< 250
20	Chemical / processing aids	PTS	800	200	800: CHOL +, PROT-, GLOB-, liver a/rw +, liver centrilobular hypertrophy	200	24.0	18.0	< 200
21	Chemical / aromatic substance	PTS	450	150	200: liver hypertrophy 450: TRIG +, CHOL +, liver a/rw +; 150: liver a/rw +	150	16.9	8.1	< 150
22	Chemical / intermediate	PTS	60	20	60: BWC-, RBC-, HGB-, HCT-, MCHC-, RET +, BIL +, duodenum thickening, liver w + 20: duodenum thickening, liver w +	< 20	19.2	9.8	< 20
23	Chemical / antiwear agent	PTS	500	150	500: FC-, BWC-, liver a/rw +; 150: liver a/rw +	150	35.5	36.3	< 150

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Table 2 (continued)

Study No.	Compound class / use	Study type	HD [mg/kg]	LD [mg/kg]	Effects	NOAEL [mg/ kg] maternal	% changed metabolites HD	% changed metabolites LD	NOEL [mg/kg] metabolome
24	Chemical / curing agent	PTS	100	25	100: MONO +	100	11.9	5.1	< 25
25	Chemical / aromatic substance	PTS	1000	300	1000: BWC, cBWG, liver aw +, rw +, kidney rw +, PROT-, GLOB-300: BWC, cBWG-	< 300	37.7	13.8	< 300
26	Agrochemical / herbicide	MTR	1000	300	NAD	1000	6.4	17.1	< 300
27	Agrochemical / herbicide	MTR	100	25	100: FC, BWC, RBC +, HGB +, HCT +, CREA +, adrenal glands w +, hyperplasia/ hypertrophy, kidney vacuolar degeneration; 25: adrenal glands w +, hyperplasia/ hypertrophy; kidney vacuolar degeneration	< 25	25.4	13.7	< 25
28	Agrochemical / fungicide	MTR	750	500	750: liver a/rw	750	7.8	9.2	< 500
29	Agrochemical / fungicide	MTR	60	20	500: liver a/rw + 60: FC, BW, BWC, GLOB-, TRIG-, liver rw-, kidney a/rw +, adrenal rw +	< 20	47.0	30.0	< 20
30	Agrochemical / fungicide	MTR	750	500	20: FC, BW, BWC-, liver rw +, kidney rw + 750: liver a/rw +	750	15.0	8.0	< 500
31	Agrochemical / insecticide	MTR	50	15	500: liver aw + 50: FC, BWC, CW-, cBWG-, PROT-, ALB-, GLOB-	< 15	18.8	5.0	< 15
32	Agrochemical / insecticide	MTR	1000	300	15: cBWG- 1000: WC +, UREA +, CREA +, kidney a/rw +, liver rw +, glandular stomach focus	300	10.7	5.0	< 300
33	Agrochemical / insecticide	MTR	200	60	300: WC +	60	16.2	9.3	< 60
34	Agrochemical / fungicide	Screening PTS	1000	300	200: salivation, piloerection, FC, spleen rw- 1000: WC +, BWC-, CREA +, GLOB-, BIL-, liver rw +	< 300	43.0	13.7	< 300
35	Agrochemical / fungicide	Screening PTS	250	80	300: WC +, BWC, CREA +, BIL-, liver rw + 250: clin obs, FC, BW/BWC-, RBC, HGB, HCT-, RET +, MCV +, WBC +, PROT-, ALB-liver rw +, spleen rw +	< 80	48.5	25.8	< 80
36	Agrochemical / fungicide	Screening PTS	600/400	200	80: clin obs, FC, BW/ BWC, RBC, HGB, HCT-, RET +, WBC +, PROT-, ALB, liver rw +, spleen rw + 600/400* (*from GD 16 onwards): clin obs, WC +, FC, BW/BWC, UW-, CW-, WBC +, LYMPH +, NEUT +, ALT +, GLUC, CHOL +, adrenal a/r w +, liver rw +, spleen a/rw +	< 200	39.9	33.1	< 200
37	Agrochemical / fungicide	Screening PTS	200/150	70	200: WC +, FC, BW/BWC, CHOL +, liver rw +, adrenal rw +, spleen rw + 200/150* (*from GD9 onwards): clin obs, FC, BWC, liver a/r w +, kidney rw +	70	29.2	16.7	< 70
38	Agrochemical / fungicide	Screening PTS	600	200	70: liver rw + 600: sacrificed unscheduled (GD13), clin obs, FC, BW-, BWC-	< 200	ND	28.1	< 200
39	Agrochemical / fungicide	Screening PTS	400	100	200: FC-, BW-, BWC-, cBWG, liver a/rw +, adrenals a/rw + 400: FC-, BW-, BWC-, UW-, cBWG-, RBC, HGB, HCT-, RET +, PLT-, MONO +, NEUT-, LYMPH +, UREA +, GLOB-, INP +, adrenal rw +, liver rw +; 100: MONO +, GLOB-, liver rw +	100	41.9	28.5	< 100
40	Agrochemical / fungicide	Screening PTS	250	80	250: clin obs, FC, BW-, BWC-, UW-, cBWG-, RBC, HGB, MCH +, MCHC-, RET +, NEUT-, MONO +, AST +, UREA +, GLOB-, INP +, liver a/rw +, adrenals rw +, kidneys rw +	< 80	56.1	36.8	< 80
41	Agrochemical / fungicide	Screening PTS	300	100	80: clin obs, FC, BWC-, cBWG-, MCV +, RET +; NEUT-, GLOB- 300: WC +, FC, BWC-, cBWG-, RBC, HGB, HCT-, RET +, UREA +, CREA +, PROT-, ALB-, GLOB-, liver a/rw +	< 100	46.8	24.9	< 100
42	Agrochemical / fungicide	PTS	1000	200	100: PROT-, ALB-, GLOB-, liver a/rw + 1000: BIL-, ALB +, PROT +, liver and thyroid a/rw +, hypertrophy/ hyperplasia of follicular cells of thyroid	< 200	11.8	8.0	< 200
					200: BIL-, ALB +				

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Table 2 (continued)

Study No.	Compound class / use	Study type	HD [mg/kg]	LD [mg/kg]	Effects	NOAEL [mg/ kg] maternal	% changed metabolites HD	% changed metabolites LD	NOEL [mg/kg] metabolome
43	Agrochemical / fungicide	PTS	15	5	15: FC, BW, BWC, UREA, CREA, TRIG, liver a/rw +, kidney rw + 5: FC, BW, BWC, liver rw +, kidney rw +	< 5	39.4	29.6	< 5
44	Agrochemical / insecticide	PTS	120	40	120: salivation, FC, BW, UREA, CREA, CHOL +, liver a/rw + 40: salivation, CHOL +	40	28.2	3.5	40

Abbreviations:

No. number, HD high dose, LD low dose, NOAEL no observed adverse effect level, NOEL no observed effect level, NAD nothing abnormal detected, + increased-decreased GD gestation day, MTR maternal toxicity study, PTS prenatal toxicity study, sPTS screening prenatal toxicity study, ND not determined.

In-life parameter: clin obs: findings in clinical observation, FC: food consumption, BW: body weight, BWC: body weight change, UW: uterus weight, cBWG: corrected body weight gain, CW: carcass weight.

Clinical pathology: RBC: red blood count, HGB: hemoglobin, MCH: mean corpuscular hemoglobin, MCV: mean corpuscular volume, MCHC: mean corpuscular hemoglobin concentration, HCT: hematocrit, RET: reticulocytes, CREA: creatinine, PROT: protein, ALB: albumin, GLOB: globulin, WBC: white blood cells, RET: reticulocytes, MONO: monocytes, LYMPH: lymphocytes, NEUT: neutrophils, PLT: platelets, ALT: alanine aminotransferase, AST: aspartate aminotransferase, INP: inorganic phosphate, BIL: bilirubin, TRIG: triglycerides, CHOL: cholesterol.
Pathology: a/rw: absolute and relative organ weights.

substances and their respective changed metabolites were used to create a specific metabolite pattern indicative for liver toxicity and HPPD inhibition in PTS studies.

2.4.8. Statistics

For blood parameters with bidirectional changes and for organ weights a non-parametric one-way analysis using KRUSKAL-WALLIS test was used. If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the hypothesis of equal medians.

For clinical and necropsy examinations of the dams, simultaneous comparison of all dose groups with the control group using the DUNNETT-test (two-sided) for the hypothesis of equal means was performed. For the parameters female mortality and females pregnant at terminal sacrifice a pairwise comparison of each dose group with the control group using FISHER'S EXACT test (one-sided) for the hypothesis of equal proportions was performed.

The sex- and day-stratified heteroscedastic *t*-test ('Welch test') was applied to compare metabolite levels of dose groups with respective controls. A significance level of $p \leq 0.05$ was applied. All statistics were calculated using the statistical software R.

The development of metabolome patterns associated with specific modes of action, their statistical analysis and the used of the data bases MetaMap®Tox has been described by [van Ravenzwaay et al \(2015\)](#).

3. Results**3.1. Comparison of classical maternal NOAEL and metabolomic NOEL**

The compounds were tested within 44 regulatory toxicity studies (new active ingredients, pre- or main studies required for regulatory submission). An overview of all compounds, dose levels, maternal effects, metabolite changes and respective NO(A)ELs can be found in [Table 2](#). Routinely examined maternal toxicity parameters consisted of clinical signs, record of food consumption, body weight, uterus weight, carcass weight determination and necropsy findings. In some cases, clinical pathology (clinical chemistry and hematology) and/or organ weight determination and histopathology of target organs complemented the standard study design of an OECD 414.

After evaluation of the data, a NOAEL for maternal toxicity based on the above mentioned classical study parameters (as defined in the OECD 414 test guideline) was determined using mid- and high-dose levels, only. The NOAEL for maternal toxicity is the highest dose level at which no adverse treatment-related findings are observed.

For metabolomics, the proportion of up- or downregulated metabolites was calculated. A metabolomic NOEL was derived when less than 14 metabolites (5%) were changed at a significance level of $p \leq 0.05$. A detailed definition of metabolome-based NO(A)ELs was already published in a previous work ([van Ravenzwaay et al., 2014](#)).

The NOAEL for maternal toxicity based on the above mentioned classical study parameters (classical maternal NOAEL) was compared with the NOEL for maternal toxicity described by metabolite changes (metabolomic NOEL). Adverse effects were observed at the high and mid doses in 20 studies and only at the high dose in 16 studies. Eight Studies showed no adverse effect at both dose levels. For metabolome analysis, metabolite changes above 5% were observed at both dose levels in 39 studies and only at the high dose in 3 studies. Only 2 studies showed no changes in metabolomics.

In 24 out of 44 studies, the metabolomic NOEL was equal to the classical maternal NOAEL (see [Table 3](#)). However, in 20 out of 44 studies, the metabolomic NOEL was lower than the classical maternal NOAEL. In no case was metabolomics less sensitive than the classical maternal NOAEL.

Table 3

Comparison of different NO(A)ELs (for interpretation of the references to colour in this Table caption, the reader is referred to the web version of this article).

Study No.	Colour code: red: lower NOA(E)L, green: same NOA(E)L	
	NOAEL maternal	NOEL metabolome
1	300	<300
2	1000	300
3	<100	<100
4	<100	<100
5	300	<300
6	80	<80
7	300	300
8	<300	<300
9	<300	<300
10	<150	<150
11	1000	1000
12	300	<300
13	1000	1000
14	8	<8
15	<100	<100
16	<25	<25
17	60	<60
18	150	<150
19	750	<250
20	200	<200
21	150	<150
22	<20	<20
23	150	<150
24	100	<25
25	<300	<300
26	1000	<300
27	<25	<25
28	750	<500
29	<20	<20
30	750	<500
31	<15	<15
32	300	<300
33	60	<60
34	<300	<300
35	<80	<80
36	<200	<200
37	70	<70
38	<200	<200
39	100	<100
40	<80	<80
41	<100	<100
42	<200	<200
43	<5	<5
44	40	40
Sensitivity	0/44	20/44
	0%	45%

NOAEL: no observed adverse effect level,
 NOEL: no observed effect level,
 MTR: maternal toxicity study,
 PTS: prenatal toxicity study,
 SPTS: screening prenatal toxicity study

3.2. Metabolite pattern in pregnant rats

3.2.1. Liver

As there was a sufficient high number of substances which induced liver effects in pregnant rats, we used the metabolome data of these compounds to establish patterns for liver toxicity. Those liver effects consisted of changes in clinical pathology parameters, increases in absolute/relative liver weights and, if available, morphological changes in liver such as hypertrophy (Table 2).

The generated metabolite pattern is referred to as “liver toxicity pattern” (Table 3). The compounds used for this purpose were compounds with the study Nos. 6, 9, 17 and 20. Seventeen metabolites were

selected to generate the specific pattern. The majority of the metabolites are associated with the class of complex lipids, fatty acids and related lipids with subclasses like fatty acids, sphingolipids and phosphatidylcholins. Few metabolites, such as threonic acid or glucuronic and erythronic acid, belong to different classes, such as vitamins or carbohydrates, respectively. Overall, a total of 16 out of 17 metabolites are upregulated whereas one single metabolite, namely phosphatidylcholine (C16:0, C18:2), is downregulated (Table 4).

This newly generated liver toxicity pattern in pregnant, non-fasted rats was compared with a pre-existing liver toxicity-related pattern which was generated with metabolite analysis in non-pregnant, fasted rats (see Table 5), published previously (van Ravenzwaay et al., 2012). Comparing the two liver toxicity patterns in pregnant and non-pregnant animals, 10 metabolites were upregulated in the same manner. Phosphatidylcholine (C16:0, C18:2) is the only one metabolite which is downregulated and occurred in pregnant rats.

3.2.2. HPPD-inhibition

HPPD inhibitors are herbicides and their mode of action is determined by the inhibition of the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD) which is also present in mammalian species. In pregnant rats, two HPPD inhibitors were tested at a low and a high dose level: one substance in a maternal toxicity study (Table 2; study No. 26) and the second in a prenatal toxicity study. The metabolome data of these two reference substances formed the HPPD inhibition pattern in pregnant rats. Two further HPPD inhibitors were tested and matched the pattern (Table 6).

A metabolite pattern for HPPD inhibition in non-pregnant females was already published previously (van Ravenzwaay et al., 2007). Comparing the non-pregnant, fasted and pregnant, non-fasted HPPD-inhibition pattern, 8 out of 14 metabolites were regulated in the same direction, thus showing a good overlap between both patterns (Table 7). Three metabolites were regulated in the pregnant pattern which could not be confirmed by the non-pregnant pattern. A profound upregulation in tyrosine, which is specific for this mode of action, was also confirmed in pregnant rats.

4. Discussion

4.1. Comparison of classical maternal NOAEL and metabolomic NOEL

In 44 maternal and (screening) prenatal toxicity studies, a NOAEL for maternal toxicity was determined based on classical study parameters referring to OECD test guideline No. 414. Routinely examined maternal toxicity parameters consisted of clinical signs, record of food consumption, body weight, uterus weight, carcass weight determination and necropsy findings. In some cases, clinical pathology (clinical chemistry and hematology) and/or organ weight determination and histopathology of target organs complemented the standard study design of an OECD 414. In parallel, metabolome analysis in rat plasma was performed on gestation day 20 and a metabolomic NOEL was derived based on the proportion of total up- or downregulated metabolites. As adversity cannot be defined only based on the regulation of metabolites, we did not attempt to establish a metabolomic NOAEL. In 45% of the cases, namely in 20 out of 44 studies, the metabolomic NOEL was lower than the classical maternal NOAEL. In no case was the metabolomic NOEL higher than the maternal NOAEL. This indicates a higher sensitivity of metabolomics in detecting maternal toxicity than classical maternal parameters. This was unexpected, as a previous comparison of classical toxicity and metabolomics NO(A)ELs using more than 100 compounds from 28-day studies indicated a similar sensitivity of both methods (van Ravenzwaay et al., 2014).

Given the rather limited nature of parameters used to establish maternal toxicity in prenatal developmental toxicity studies these findings may also indicate that metabolomics is not necessarily more sensitive, but rather that the determination of maternal toxicity is less

Table 4

Metabolite pattern for liver toxicity in pregnant rats (for interpretation of the references to colour in this Table caption, the reader is referred to the web version of this article).

Metabolite	Direction	Chemical 1 HD	Chemical 2 HD	Chemical 3 HD	Chemical 4 HD	Metabolite class
Glucuronic acid	↑	2.05	23.48	3.12	1.20	Carbohydrates and related
Erythronic acid	↑	1.50	1.35	1.26	1.09	
Stearic acid (C18:0)	↑	1.45	1.22	1.74	1.39	Complex lipids, fatty acids and related
Arachidonic acid (C20:cis[5,8,11,14]4)	↑	1.15	1.33	1.30	1.39	
Glycerol phosphate, lipid fraction	↑	1.16	1.67	1.62	1.39	
Lignoceric acid (C24:0)	↑	2.00	1.19	2.04	1.49	
Phosphate, lipid fraction	↑	1.14	1.34	1.20	1.28	
Behenic acid (C22:0)	↑	1.54	1.14	1.98	1.09	
4-Hydroxyphosphatidylcholine (t18:0, Phosphatidylcholine)	↑	2.23	1.34	1.67	1.49	
gamma-Linolenic acid (C18:cis[6,9,12]3)	↑	2.94	1.70	2.19	1.73	
Phosphatidylcholine (C16:0, C18:2)	↓	0.98	0.97	0.94	0.98	
Phosphatidylcholine (C18:0, C18:1)	↑	1.23	1.17	1.40	1.19	
Cholesterol	↑	1.32	1.49	1.38	1.44	Miscellaneous
Sphingomyelin (d18:1, C24:0)	↑	1.22	1.12	1.52	1.14	
Phosphatidylcholine (C18:0, C22:6)	↑	1.02	1.00	1.03	1.03	
Campesterol	↑	1.21	1.10	1.70	1.21	
Threonic acid	↑	1.78	2.52	1.48	1.10	Vitamins, cofactors and related

The four compounds are listed in Table 2 under study Nos. 6,9,17 and 20; HD: high dose, values are presented as x-fold of control; fold change: 1, significant fraction of groups: 0.5, fraction of metabolites: 1. Statistically significant changes (Welch-*t*-test; $p \leq 0.1$) are shown in bold, color indicates direction of regulation: red boxes mean upregulation and yellow boxes downregulation. The intensity of the color corresponds to the magnitude of the fold change.

robust than classical toxicity parameters as determined in OECD 407 (i.e. 28-day) studies.

Overall, plasma metabolomics in pregnant rats is a technique which could easily be added to the study protocol before cesarean section having no influences on the maternal organism and matching the 3R's animal welfare concept. Furthermore, it is a useful tool to additionally describe maternal toxicity in range-finding or prenatal guideline toxicity studies. In pregnant rats, adversity in metabolomics is, however, currently not assessable. Therefore, in a second step, specific metabolite patterns which allow assessment of substance's mode of action and organ toxicity have to be developed.

4.2. Metabolite patterns in pregnant rats

4.2.1. Liver

Since liver effects are the most common findings in toxicity studies, a metabolite pattern linked to liver toxicity in pregnant rats was created based on 4 reference compounds derived from maternal or prenatal toxicity studies.

The liver toxicity pattern in pregnant, non-fasted rats was compared with a liver-related pattern of non-pregnant, fasted female rats. Parts of this pattern in non-pregnant, fasted rats have been published previously but were also refined during the years (van Ravenzwaay et al., 2012). The aim was to reveal similarities or differences in the metabolite regulation related to liver toxicity in pregnant and non-pregnant rats.

Comparing the two liver toxicity patterns in pregnant and non-pregnant animals, 10 metabolites are upregulated in the same manner showing high overlap (Table 5). In general, this indicates that liver-related toxicity can be detected by metabolomics in pregnant, non-fasted rats similarly to non-pregnant, fasted rats.

Metabolites which are only regulated in pregnant, non-fasted animals involve an upregulation of campesterol, erythronic acid, glucuronic acid and a number of complex lipids (such as phosphatidylcholine, sphingomyelin) or fatty acids. Campesterol is a diet-related metabolite, erythronic and glucuronic acids are sugar acids belonging to the class of Carbohydrates. The observed upregulation is consistent with the observations made in a previous work: levels of triacylglycerols, phospholipids and degradation products were increased in blood metabolome levels of non-fasted, non-pregnant females compared to a fasted control group (Mellert et al., 2011). Phosphatidylcholine (C16:0,

C18:2) is the only one metabolite which is downregulated. The difference in metabolite composition of both patterns may be explained by the factors non-fasting and pregnancy and their strong influence on the respective metabolite changes. There is ongoing work which evaluates metabolite differences between non-pregnant and pregnant rats taking fasting and non-fasting conditions into account.

4.2.2. HPPD-inhibition

HPPD inhibitors are herbicides and their mode of action is determined by the inhibition of the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD) which is also present in mammalian species. Upregulation of tyrosine was pointed out as key event and its pronounced increase is a well-known change (Lock et al., 1998). The metabolite profile for HPPD inhibition in non-pregnant, fasted female rats was established previously and an increase of Tyrosine levels in plasma was identified as classical biomarker (van Ravenzwaay et al., 2007). Within this work, HPPD inhibitors (herbicides, coumarons) were tested in pregnant, non-fasted rats and their metabolite profiles were evaluated. Consistent to non-pregnant rats, the mode of action of HPPD inhibitors could be detected in pregnant rats forming a specific pattern. This pattern was created based on two reference substances which were known to be HPPD-inhibitors. In the course of performing routine maternal or prenatal toxicity studies, two further substances were tested and could be identified as HPPD-inhibitors by metabolomics matching the HPPD-inhibition pattern in pregnant rats (Table 6). In total, four substances were tested at two dose levels. An upregulation in tyrosine levels compared to control values were observed in all four cases by metabolomics in pregnant rats. Tyrosine plasma concentrations ranged from around 28-fold up to 42-fold compared to control.

Comparing the non-pregnant, fasted and pregnant, non-fasted HPPD-inhibition pattern, 8 out of 14 metabolites were regulated in the same direction showing an overlap between both patterns (see Table 7). Three metabolites were regulated in the pregnant pattern which could not be confirmed by the non-pregnant pattern. The metabolites are phosphatidylcholine (C16:0, C16:0), homovanillic acid (HVA) and 3,4-dihydroxyphenylglycol (DOPEG); and the latter two belong to the class of hormones. The amino acids related metabolites citrulline and lysine were statistically significantly down- and upregulated, respectively, in non-pregnant rats and a tendency for correctly directed up- or down-regulation of these metabolites without statistical significance was also

Table 5

Comparison of metabolite pattern for liver toxicity in pregnant rats and non-pregnant rats (for interpretation of the references to colour in this Table caption, the reader is referred to the web version of this article).

Metabolite	Pattern "Liver toxicity pregnant"	Pattern "Liver toxicity non-pregnant"	Metabolite class
Erythronic acid			Carbohydrates and related
Glucuronic acid			
Arachidonic acid (C20:cis[5,8,11,14]4)			Complex lipids, fatty acids and related
Behenic acid (C22:0)			
Cholesterol			
Ceramide (d18:1,C24:1)			
Dihomo-gamma-Linolenic acid (C20:cis[8,11,14]3)			
Docosahexaenoic acid (C22:cis[4,7,10,13,16,19]6)			
Docosapentaenoic acid (C22:cis[7,10,13,16,19]5)			
Erythro-Sphingosine (d18:1)			
Gamma-Linolenic acid (C18:cis[6,9,12]3)			
Glycerol, lipid fraction			
Glycerol phosphate, lipid fraction			
Heptadecanoic acid (C17:0)			
4-Hydroxy sphinganine (t18:0, Phytosphingosine)			
Lignoceric acid (C24:0)			
Linoleic acid (C18:cis[9,12]2)			
Nervonic acid (C24:cis[15]1)			
3-O-Methylsphingosine (d18:1)			
5-O-Methylsphingosine (d18:1)			
Palmitic acid (C16:0)			
Phosphate, lipid fraction			
Phosphatidylcholine (C18:0,C18:1)			
Phosphatidylcholine (C16:0,C18:2)			
Phosphatidylcholine (C18:0,C22:6)			
Sphingomyelin (d18:1,C24:0)			
Stearic acid (C18:0)			
TAG (C16:0,C18:2)			
TAG (C18:1,C18:2)			
TAG (C18:1,C18:2,C18:3)			
TAG (C18:2,C18:2)			
TAG (C18:2,C18:3)			
Threo-Sphingosine (d18:1)			
Tricosanoic acid (C23:0)			
Campesterol			Miscellaneous
Threonic acid			Vitamins, cofactors and related

Statistically significant changes (Welch-t-test; $p \leq 0.1$) are indicated by color: red boxes mean upregulation and yellow boxes downregulation, metabolites in bold represent common metabolites of both patterns.

apparent in pregnant rats. Furthermore, a downregulation of some amino acids in plasma in healthy pregnant Wistar rats was previously confirmed by NMR based metabolomics (Shen et al., 2016). Pyruvate plays a role in the glycolysis/gluconeogenesis within the energy metabolism and is downregulated in the HPPD inhibition pattern in non-pregnant and fasted rats. It was shown previously that non-fasting conditions of non-pregnant untreated female rats resulted in higher pyruvate plasma levels compared to fasting conditions (Mellert et al., 2011). Consistently, it is to be expected that pyruvate is not

downregulated in pregnant rats under non-fasting conditions in our case.

The differences in metabolite composition between the non-pregnant and pregnant HPPD inhibition pattern are rather explained by the increase in sensitivity of the current methods and ability to measure further metabolites in the last 10 years than by the factors pregnancy or fasting. However, the influence of pregnancy and (non-)fasting on characteristic metabolites has to be evaluated as mentioned above.

Table 6

Metabolite pattern for HPPD inhibition in pregnant rats (for interpretation of the references to colour in this Table caption, the reader is referred to the web version of this article).

Metabolite	Direction	Substance 1 LD	Substance 1 HD	Substance 2 LD	Substance 2 HD	Substance 3 LD	Substance 3 HD	Substance 4 LD	Substance 4 HD	Metabolite class
Threonine	↑	1.47	1.26	1.40	1.38	1.69	1.71	1.30	1.44	Amino acids and related
4-Hydroxyphenylpyruvate	↑	183.41	162.76	180.72	209.61	224.46	310.28	124.99	198.80	
Glycine	↑	1.38	1.91	2.08	2.22	2.05	3.51	1.57	1.81	
5-Oxoproline	↓	0.64	0.60	0.64	0.63	0.57	0.61	0.72	0.70	
Glutamine	↓	0.60	0.56	0.57	0.59	0.61	0.57	0.69	0.65	
Serine	↑	1.37	1.15	1.38	1.52	1.41	2.05	1.25	1.32	Complex lipids, fatty acids and related Hormones, signal substances and related
Tyrosine	↑	31.59	34.87	29.09	42.23	31.73	47.06	16.94	27.78	
Phosphatidylcholine (C16:0,C16:0)	↓	0.88	0.89	0.90	0.75	0.85	0.82	0.88	0.82	
Homovanillic acid (HVA)	↑	2.20	2.28	2.50	3.17	1.90	4.91	1.51	1.47	
3,4-Dihydroxyphenylglycol (DOPEG)	↑	1.33	1.39	1.41	1.50	1.23	1.53	1.53	1.38	
3,4-Dihydroxyphenylalanine (DOPA)	↑	3.95	4.57	4.35	4.35	2.93	4.62	2.58	2.60	

LD: low dose, HD: high dose; values are presented as x-fold of control, fold change: 1, significant fraction of groups: 0.5, fraction of metabolites: 0.9, substances in bold are reference substances. Statistically significant changes (Welch-*t*-test; $p \leq 0.1$) are shown in bold, color indicates direction of regulation: red boxes mean upregulation and yellow boxes downregulation. The intensity of the color corresponds to the magnitude of the fold change.

Table 7

Comparison of metabolite pattern for HPPD inhibition in non-pregnant and pregnant rats (for interpretation of the references to colour in this Table caption, the reader is referred to the web version of this article).

Metabolite	Pattern pregnant	Pattern non-pregnant	Metabolite class
Citrulline			Amino acids and related
Glutamine			
Glycine			
4-Hydroxyphenylpyruvate			
Lysine			
5-Oxoproline			Complex lipids, fatty acids and related
Serine			
Threonine			
Tyrosine			
Phosphatidylcholine (C16:0,C16:0)			
Pyruvate			Energy metabolism and related
3,4-Dihydroxyphenylalanine (DOPA)			Hormones, signal substances and related
3,4-Dihydroxyphenylglycol (DOPEG)			
Homovanillic acid (HVA)			

Statistically significant changes (Welch-*t*-test; $p \leq 0.1$) are indicated by color: red boxes mean upregulation and yellow boxes downregulation, metabolites in bold represent common metabolites of both patterns.

5. Conclusion

In conclusion, metabolomics is a useful tool to better identify and describe maternal toxicity in range-finding as well as in prenatal guideline toxicity studies and contributes to the compelling need of supporting data in this field. Evaluating 44 studies, the metabolome-derived NOEL was more sensitive in 45% of the cases in detecting maternal toxicity than the maternal NOAEL. Specific metabolite patterns which allow assessment of substance's mode of action and organ toxicity have to be developed. Comparing the patterns for liver toxicity and HPPD inhibition, it is noted that there is a reasonable overlap in similarly regulated metabolites. Given the fact that there is a significant metabolome difference between fasted and non-fasted rats (Mellert et al., 2011) and, in addition, the potential metabolome differences induced by pregnancy and non-pregnancy, this overlap indicates that compound specific metabolome changes are a dominating factor. The potential importance of metabolome-based assessment of maternal toxicity is not only in avoiding to underestimate maternal toxicity but more importantly to provide insight in the nature of the toxic effects on the dams. With developmental toxicity occurring at maternally toxic

dose levels, a mechanistic-based approach for maternal toxicity will open up the “black-box” and should allow a better evaluation of maternal toxicity being causatively related to developmental toxicity.

Moreover, the metabolomics technology also offers the possibility to examine selectively target important constituents of the maternal blood for fetal development, e.g. folic acid, to identify a potential maternal effect responsible for developmental toxicity. Finally, as over time we will learn more about adverse outcome pathways, metabolomics, jointly with other new technologies, will contribute to an overall deeper understanding of toxicity.

Although still somewhat hypothetical, metabolome-based determination of maternal toxicity offers the possibility to assess maternal toxicity on an individual basis with treatment-group comparison by a PCA (principal component analysis). This is of some importance as in prenatal developmental toxicity studies the most important parameters for evaluating adverse effects in the progeny are normally litter based. Thus, outliers, i.e. dams with either particularly high or low metabolome changes, can readily be identified and assessed with respect to the observed effects in their litters.

Conflict of interest

All authors are employees of the BASF Group and declare that there is no conflict of interest.

Transparency document

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

Acknowledgements

The authors are grateful to the lab team of the reproductive toxicology for their (technical) assistance.

References

- Beyer, P., Chernoff, N., 1986. The induction of supernumerary ribs in rodents: role of the maternal stress. *Teratog., Carcinog. Mutagen.* 6, 419–429.
- Chernoff, N., Setzer, R.W., Miller, D.B., Rosen, M.B., Rogers, J.M., 1990. Effects of chemically induced maternal toxicity on prenatal development in the rat. *Teratology* 42, 651–658.
- Danielsson, B.R., 2013. Maternal Toxicity. In *Teratogenicity Testing*. Humana Press, Totowa, NJ, pp. 311–325.
- Diaz, S.O., Pinto, J., Graca, G., Duarte, I.F., Barros, A.S., Galhano, E., Pita, C., Almeida, Mdo C., Goodfellow, B.J., Carreira, I.M., et al., 2011. Metabolic biomarkers of prenatal disorders: an exploratory NMR metabolomics study of second trimester maternal urine and blood plasma. *J. Proteome Res.* 10, 3732–3742.
- ECETOC, 2004. Influence of Maternal Toxicity in Studies on Developmental Toxicity. ECETOC WORKSHOP REPORT No. 4, Brussels.
- Euker, J., Riegler, G., 1973. Effects of stress on pregnancy in the rat. *J. Reprod. Fertil.* 34, 343–346.
- Giavini, E., Menegola, E., 2012. The problem of maternal toxicity in developmental toxicity studies. *Regul. Toxicol. Pharmacol. RTP* 62, 568–570.
- Glöckner, R., Karge, E., 1991. Influence of chronic stress before and/or during gestation on pregnancy outcome of young and old Uje: WIST rats. *J. Exp. Anim. Sci.* 34, 93–98.
- Kamlage, B., Maldonado, S.G., Bethan, B., Peter, E., Schmitz, O., Liebenberg, V., Schatz, P., 2014. Quality markers addressing preanalytical variations of blood and plasma processing identified by broad and targeted metabolite profiling. *Clin. Chem.* 60, 399–412.
- Kamp, H., Strauss, V., Wiemer, J., Leibold, E., Walk, T., Mellert, W., Looser, R., Prokoudine, A., Fabian, E., Krennrich, G., et al., 2012. Reproducibility and robustness of metabolome analysis in rat plasma of 28-day repeated dose toxicity studies. *Toxicol. Lett.* 215, 143–149.
- Khera, K.S., 1985. Maternal toxicity: a possible etiological factor in embryo-fetal deaths and fetal malformations of rodent-rabbit species. *Teratology* 31, 129–153.
- Lindon, J.C., Holmes, E., Nicholson, J.K., 2004. Toxicological applications of magnetic resonance. *Prog. Nucl. Magn. Reson. Spectrosc.* 1–2, 109–143.
- Lindon, J.C., Holmes, E., Nicholson, J.K., 2006. Metabonomics techniques and applications to pharmaceutical research & development. *Pharm. Res.* 23, 1075–1088.
- Lock, E.A., Gaskin, P., Ellis, M.K., Robinson, M., Provan, W.M., Smith, L.L., 1998. The effect of a low-protein diet and dietary supplementation of threonine on tyrosine and 2-(2-nitro-4-trifluoromethylbenzoyl) cyclohexane-1,3-dione-induced corneal lesions, the extent of tyrosinemia, and the activity of enzymes involved in tyrosine catabolism in the rat. *Toxicol. Appl. Pharmacol.* 150, 125–132.
- Luan, H., Meng, N., Liu, P., Feng, Q., Lin, S., Fu, J., Davidson, R., Chen, X., Rao, W., Chen, F., et al., 2014. Pregnancy-induced metabolic phenotype variations in maternal plasma. *J. Proteome Res.* 13, 1527–1536.
- Mattes, W.B., Kamp, H.G., Fabian, E., Herold, M., Krennrich, G., Looser, R., Mellert, W., Prokoudine, A., Strauss, V., van Ravenzwaay, B., et al., 2013. Prediction of Clinically Relevant Safety Signals of Nephrotoxicity Through Plasma Metabolite Profiling.
- Mattes, W., Davis, K., Fabian, E., Greenhaw, J., Herold, M., Looser, R., Mellert, W., Groeters, S., Marxfeld, H., Moeller, N., et al., 2014. Detection of hepatotoxicity potential with metabolite profiling (metabolomics) of rat plasma. *Toxicol. Lett.* 230, 467–478.
- Mellert, W., Kapp, M., Strauss, V., Wiemer, J., Kamp, H., Walk, T., Looser, R., Prokoudine, A., Fabian, E., Krennrich, G., et al., 2011. Nutritional impact on the plasma metabolome of rats. *Toxicol. Lett.* 207, 173–181.
- OECD, 1992. Decision of the Council Concerning the Mutual Acceptance of Data in the Assessment of Chemicals (C(81)30(Final)) (Adopted by the Council at Its 535th Meeting on May 12, 1981).
- OECD, 2001. Test No. 414: Prenatal Development Toxicity Study. OECD Publishing, Paris.
- Ritchie, H.E., Oakes, D.J., Kennedy, D., Polson, J.W., 2017. Early gestational hypoxia and adverse developmental outcomes. *Birth Defects Res.* 109, 1358–1376.
- Roessner, U., Wagner, C., Kopka, J., Trethewey, R.N., Willmitzer, L., 2000. Technical advance: simultaneous analysis of metabolites in potato tuber by gas chromatography-mass spectrometry. *Plant J.* 23, 131–142. <https://doi.org/10.1046/j.1365-3113x.2000.00774.x>.
- Rogers, J.M., Chernoff, N., Keen, C.L., Daston, G.P., 2005. Evaluation and interpretation of maternal toxicity in Segment II studies: issues, some answers, and data needs. *Toxicol. Appl. Pharmacol.* 207, 367–374.
- Shen, G., Li, Z., Zhang, Y., Wu, H., Feng, J., 2016. 1H NMR-based metabolomics study on the physiological variations during the rat pregnancy process. *Mol. Cell. Endocrinol.* 423, 40–50.
- Strauss, V., Wiemer, J., Leibold, E., Kamp, H., Walk, T., Mellert, W., Looser, R., Prokoudine, A., Fabian, E., Krennrich, G., et al., 2009. Influence of strain and sex on the metabolic profile of rats in repeated dose toxicological studies. *Toxicol. Lett.* 191, 88–95.
- van Ravenzwaay, B., Cunha, G.C.-P., Leibold, E., Looser, R., Mellert, W., Prokoudine, A., Walk, T., Wiemer, J., 2007. The use of metabolomics for the discovery of new biomarkers of effect. *Toxicol. Lett.* 172, 21–28.
- van Ravenzwaay, B., Cunha, G.C., Fabian, E., Herold, M., Kamp, H., Krennrich, G., Krotzky, A., Leibold, E., Looser, R., Mellert, W., et al., 2010a. The use of metabolomics in cancer research. An Omics Perspective on Cancer Research. Springer, Dordrecht, pp. 141–166.
- van Ravenzwaay, B., Coelho-Palermo Cunha, G., Strauss, V., Wiemer, J., Leibold, E., Kamp, H., Walk, T., Mellert, W., Looser, R., Prokoudine, A., et al., 2010b. The individual and combined metabolite profiles (metabolomics) of dibutylphthalate and di (2-ethylhexyl)phthalate following a 28-day dietary exposure in rats. *Toxicol. Lett.* 198, 159–170.
- van Ravenzwaay, B., Herold, M., Kamp, H., Kapp, M.D., Fabian, E., Looser, R., Krennrich, G., Mellert, W., Prokoudine, A., Strauss, V., et al., 2012. Metabolomics: a tool for early detection of toxicological effects and an opportunity for biology based grouping of chemicals from QSAR to QBAR. *Mutat. Res.* 746, 144–150.
- van Ravenzwaay, B., Montoya, G.A., Fabian, E., Herold, M., Krennrich, G., Looser, R., Mellert, W., Peter, E., Strauss, V., Walk, T., et al., 2014. The sensitivity of metabolomics versus classical regulatory toxicology from a NOAEL perspective. *Toxicol. Lett.* 227, 20–28.
- van Ravenzwaay, B., Kamp, H., Montoya, G.A., Strauss, V., Fabian, E., Mellert, W., Krennrich, G., Walk, T., Peter, E., Looser, R., Herold, M., 2015. The development of a database for metabolomics – looking back on ten years of experience. *Int. J. Biotechnol.* 14 (1).
- Walk, T.B., Dostler, M., 2003. Mass spectrometry method for analysing mixtures of substances, Patent application PCT/EP2003/001274. WO2003073464, Metanomics, Berlin.
- Walk, T.B., Looser, R., Bethan, B., Herold, M.M., Kamlage, B., Schmitz, O., van Ravenzwaay, B., Mellert, W., Coelho, P.C.G., Ehrhardt, T., Wiemer, J., Prokoudine, A., Krennrich, G., 2006. Means and methods for analyzing a sample by means of chromatography-mass spectrometry, Patent application PCT/EP2006/064628. WO2007012643A1, Metanomics, Berlin.
- Webster, W.S., Lipson, A.H., Brown-Woodman, P.D.C., 1987. Uterine trauma and limb defects. *Teratology* 35, 253–260.
- Wilson, J.G., 1953. Influence of severe hemorrhagic Anemia During pregnancy on development of the offspring in the rat. *Proc. Soc. Exp. Biol. Med.* 84, 66–69.
- Yamada, H., Yamahara, A., Yasuda, S., Abe, M., Oguri, K., Fukushima, S., Ikeda-Wada, S., 2002. Dansyl chloride derivatization of methamphetamine: a method with advantages for screening and analysis of methamphetamine in urine. *J. Anal. Toxicol.* 26, 17–22.
- Zhang, F., Rick, D.L., Kan, L.H., Perala, A.W., Geter, D.R., Lebaron, M.J., Bartels, M.J., 2011. Simultaneous quantitation of testosterone and estradiol in human cell line (H295R) by liquid chromatography/positive atmospheric pressure photoionization tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* 25, 3123–3130.