METABOLOMICS-BASED BIOLOGICAL MARKERS OF PERINATAL IMPRINTING: EVIDENCES IN THE FIELD OF TOXICOLOGY, FROM STUDIES IN ANIMALS EXPOSED TO ENDOCRINE DISRUPTORS AND HUMAN EXPOSED TO PESTICIDES

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Introduction

Untargeted metabolomics is a global approach aiming at the analysis of the metabolome, e.g. the largest possible set of small metabolites present in biological samples. Metabolomics is increasingly used in the field of toxicology, with the aim of characterizing the global and dynamic response of living systems. Human tissues, cells and accessible samples (blood, urine)from groups exposed to various doses of potentially harmful chemicalscan be used. Metabolomics can be based on a variety of spectrometric techniques, such as Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS). Results are processed using multivariate statistics to seek for variables (key metabolites) that discriminate between groups (“metabolic fingerprints”). Novel developments in MS such as High Resolution MS (HRMS) have further extended spectral possibilities, and bio-informatics pipelines have been developed for the interpretation of these data in the context of “metabolic networks” paving the way for the study of the metabolomemodulation by chemicals. In the present study, we show that metabolomics can be used in the field of toxicology, to unveil a modulation of the metabolic network in animals exposed during critical windows of vulnerability, even for very low doses of contaminants.This conclusion is basedon perinatal studies on endocrine disruptors such as bisphenol A (BPA) and diethylstilbestrol (DES).The persistence of metabolomic changes long after the end of exposure suggests the feasibility of developing markers of past exposure. Metabolomics can also be used to gain a better understanding of cell physiology. Although the human situation *in vivo* is by far more complex (exposure to mixtures of compounds, genetic variability, physiological status)preliminary evidence suggests metabolomics studies on pesticide exposure can contribute successfully to highlight differences connected with the exposure status.

Materials and methods

All samples from animals and human used in this study (rats/mice: serum, tissue extracts; human: urine) were prepared for spectral analysis (NMR or MS) using the protocols detailed in Cabatonet al.[1] and Bonvallot et al.[2]. Samples were submitted to ^{1}H-NMR spectroscopy at 600.13MHz (BrukerAvance DRX-600 spectrometer fitted with a cryoprobe). For MS extracts, we used ultra highperformance liquid chromatography coupled to high resolution MS (UHPLC-HRMS). The UHPLC system was a RSLC3000 (Dionex-Thermo Scientific, Les Ulis, France). Eluted compounds were detected using a LTQ-Orbitrap XL mass spectrometer (Thermo Scientific les Ulis, France) equipped with an electrospray ionization source. For each spectrum, baseline and phase correction, data reduction, and bucketing multivariate analyses were used to evaluate the treatments on the metabolome. We first performed PCA to reveal intrinsic treatment-related clusters and detect eventual outliers. PLS-DA was then used to model the relationship between group and spectral data. We used orthogonal signal correction filtering[3] to remove variation not linked to the treatment. Filtered data were mean centered and scaled seven-fold cross-validation was used to determine the number of latent variables to be included in the PLS-DA models and to estimate the predictive ability (Q^2 score) of the adjusted models. The model is considered to be valid for a Q^2 score above 0.4[4]. Discriminant variables were determined using VIP (variable importance in the projection). We used this global measure of the influence of each variable on the PLS components to derive a subset of the most

important metabolites for the separation of experimental groups. Then, we used the Kruskal–Wallis test to determine which metabolites were significantly different between groups. SIMCA-P software (V12; UmetricsAB, Umea, Sweden) was used to perform the multivariate analyses. Modeling of metabolic networks was carried out as detailed elsewhere[5] and with the help of the INRA Metexplore server we have developed (www.metexplore.fr).

Results and discussion

In vivo use of metabolomics and the case of endocrine disruptors: unveiling the effects of perinatal exposure for low doses of contaminants. We recently demonstrated that NMR-based metabolic fingerprints can successfully be used to discriminate among mouse pups whose mothers had been exposed to very low doses of BPA[0, 25, 250, or 2500 ng/kg body weight (BW)] during pregnancy and early lactation[1](Figure 1). BPA is a model xeno-estrogen, to which a major part of the human population is chronically exposed. Human exposure occurs in the ng/kg range[6]. An extensive literature has been published about the low dose effects of BPA. In rodents, perinatal exposure studies have demonstrated BPA's effects on fertility, fecundity, and reproductive tissues (mammary gland, prostate…)[6].

Figure 1. NMR-based metabolomics discrimination of mice tissue extracts from animals exposed to low doses of BPA from gestational day 8 to postnatal day 15. Right plot: two-dimensional PLS-DA scores plot for the brain at postnatal day 21, in males from mothers exposed to 0 (black), 0.025 (blue), 0.25 (red) or 25µg/kg (green) BPA (1st and 2nd latent variable out of 3 components: R^2Y=78.9%, Q^2=0.564). Similar intergroup separation can be obtained based on liver and serum extracts[1].

Fetal exposure to xeno-estrogens and other endocrine disruptors result in pathologies and adverse effects, the incidence ofwhich is increasing in human populations over the last decades. As for other chemicals, a direct link between exposure and adverse effects in humans cannot be demonstrated based on a direct experimentation for obvious ethical reasons. However, one record of such an "experiment" exists for a xeno-estrogen; a large number of pregnant women were prescribed diethylstilboestrol (DES), in the nineteen sixties and seventies. Striking similarities in the effects of DES and other xeno-estrogens have been shown in animal models, and mimic the symptoms observed in boys and girls whose pregnant mothers were exposed to DES.In experiments carried out on mice using a protocol similar to the one developed for BPA[1], during which mothers were exposed to 10 ng/kg BW DES, liver extracts from F1 male animals were examined using the NMR metabolic fingerprints approach. Exposed animals and controls were easily discriminated, with extremely high scores, and a very robust model in which more than 99% of the variability was explained by the treatment (Figure 2).

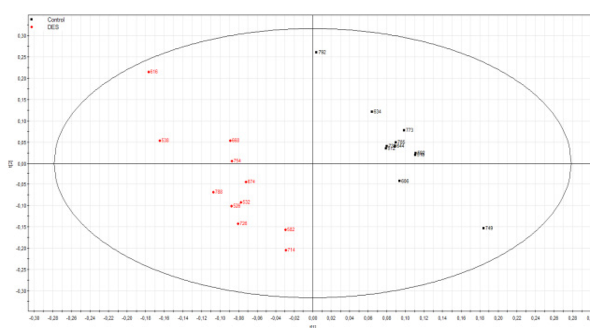


Figure 2. NMR-based metabolomics discrimination of mice liver extracts based on pregnant animals exposed to DES (sub-cutaneous explants in mothers delivering 10 ng DES/kg/day from gestational day 8 to postnatal day 16). Two dimensional PLS-DA scores plot at postnatal day 21. Red : DES exposed mice; Black : controls. 1st and 2nd latent variable out of 3 components are displayed, with R^2Y=99.1% (82.3, 14.5 & 2.3% on the 1st, 2nd and 3rd axis, respectively) and an extremely high Q^2 score of 0.952 (0.617, 0.695 & 0.587, respectively).

In the case of BPA, the variables discriminating among groups, for serum and liver samples (Figure 3) suggest a disruption of the energy metabolism; this is consistent with previous results obtained in conventional studies[7,8], indicating that early exposure to BPA later disrupts energy balance and glucose metabolism. Similar effects of xeno-estrogens have also been reported for DES in rodents[9]. When considered at the level of a single tissue (for instance serum, Figure 3, red box), these variables,can be used as phenotypic biomarkers of early exposure.

Metabolites	1H NMR chemical shift Δ (ppm)	Whole body PND2	Serum PND21	Liver PND21	Brain PND21
Cholines	3.20(s); 3.22(s); 3.23(s)	↑	↑	↓	↓
Lactate	1.33(d); 4.11(q)	↑	↓	↓	
Glucose	3.54(m); 3.66(m); 3.70(m); 3.74(m); 3.78(m); 3.82(m); 3.86(m); 3.90(m)	↑	↑	↓	
Creatine	3.03(s); 3.93(s)	↑			
Glutamate	2.08(m); 2.34(m)			↑	↓
Taurine	3.26(t); 3.42(t)		↑	↑	
Glutamine	2.14(m); 2.46(m)				↑
Lipids (LDL/VLDL)	0.86(m); 0.90(m); 1.26(m); 1.30(m)		↓		
Valine/Leucine/Isoleucine	0.94(d); 0.96(d); 1.0(d); 1.01(d);1.05(d)	↓			
Lysine	1.72(m); 2.98(m)	↓			
Glutathione	2.17(m); 2.56(m); 2.94(m)			↑	
Glycogen	5.42(m)			↓	
Aspartic acid	2.65(dd); 2.80(dd)				↑
GABA	1.90(q); 2.27(t); 3.01(t)				↓

Figure 3.Summary of the discriminant metabolites for postnatal days 2 and 21 mice exposed to BPA in the perinatal period (arrows: variation compared to controls; based on the results obtained by Cabaton et al, 2013)[1].

Persistent effects of perinatal imprinting.On-going studies on BPA suggest that gestational exposure not only triggers a significant change in the metabolome at birth, but also that these changes persist long after exposure has ceased. Female mice exposed gestationally and during lactation show a pattern that allows for discriminationamong groups even at 3 month of age, demonstrating persisting effects of perinatal imprinting (Figure 4). At this stage, F1 females exposed both gestationally and through lactation had not been further exposed to BPA for at least 2½ month.

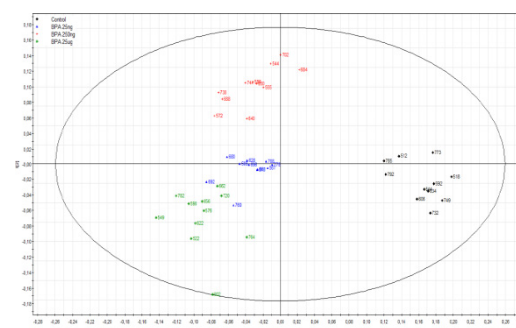


Figure 4. NMR-based metabolomics discrimination of F1 female mouse liver extracts, from animals exposed to BPA solely during the perinatal period (same protocol as detailed for males in Fig. 1). Two-dimensional PLS-DA scores plot at postnatal day 90. R^2Y=69.8%, Q^2=0.595.

Understanding the underlying metabolic shifts through metabolic networksmodeling. Metabolic fingerprints can be successfully used to discriminate between exposed/unexposed groups. They can be applied to *in vivo* or *in vitro* samples. The latter approach, already applied to BPA and halogenated contaminants, can be used to further explore which metabolic pathways are shifted within genome scale metabolic networks (integration of all possible metabolic reactions that can be performed by the organism). This bioinformatics approach also aims at identifying which reactions and related enzymes are involved in the metabolic response. Hence, combining physiological relevance of metabolomics data and recent advances in genomic technologies will result in improving systems biology research in the field of toxicology.

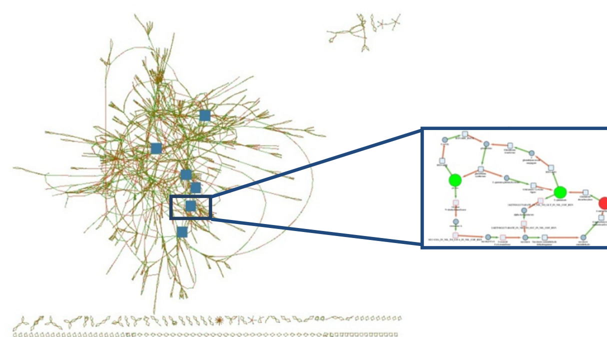


Figure 5 illustrates this approach with the extraction (through modeling) of the brain sub-network corresponding to the pathways involved in metabolic shifts for male mice perinatally exposed to low doses of BPA[1].

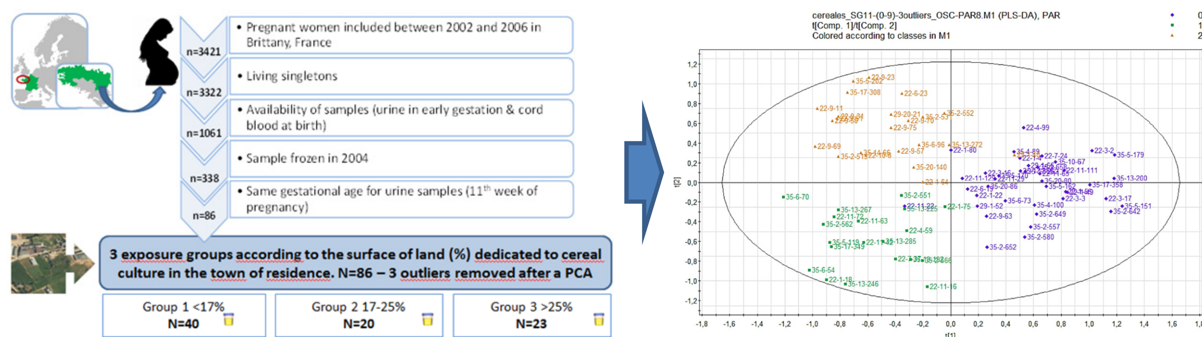
Relevance to the human situation. These results suggest that metabolomics can be used in the field of toxicology, and isespecially suitable to examine the effects of low dose exposure to chemicals. Metabolomic profiling promises to be particularly useful when the compound(s) responsible for these effects cannot be monitored, and when exposure occurred during critical windows of vulnerability.

Still, the situation in humans is rather complex. First, human exposure is not limited to a single compound. Second, human genetic variability is greater than that of laboratory animal models, even if outbred animal strains are used as we did for the BPA studies. Additional factors such as the physiological status can also complicate

metabolomic analyses by enhancing variability. In this context, it is desirable to select specific human sub-populations and specific exposure situations to investigate the use of metabolomics as relevant biomarkers of exposure and effect.

We recently examined the metabolomic profile in humans, in the context of global exposure to agro-chemicals[2] (Figure 6).Our work examined a cohort of pregnant women in Brittany (France), for which we studied the links between exposure to multiple pesticides during early pregnancy, and urinary metabolomic biomarkers. We selected 3 groups of women potentially exposed to pesticides, according to the surface of land dedicated to cereal crops culture in their area of residence. Besides chemometric results based on NMR and PLS-DA, polytomous regression were adjusted for potential confounders (BMI, age, parity, smoking habits).

Figure 6.Flowchart of the selection of the exposure groups and two-dimensional PLS-DA score plot for urinary samples from 83 pregnant women living in towns were 0-17% (purple), 17-25% (green) or more than 25% (orange) of the surface of land is dedicated to cereal crops[2]. The PLS-DA model includes 4 latent variables with a R^2Y=90.7%, and a Q^2=0.564.



Again, it was possible to discriminate among the 3 groups, with a large part of the variability explained by the exposure scenario, and a fully valid model, although characterized by a lower Q² score than for less complex models (e.g. laboratory animals exposed to a single compound). Thus, despite the many factors which enhance variability in human models, exposure to key contaminants can be characterized through the metabolic fingerprints approach. Further investigation of the metabolic pathways involved in this discrimination through metabolic networks quantitative modeling is expected to help gain a better understanding of the mechanisms involved in contaminants toxicity.

Acknowledgements

INRA teams thank the support of the AgenceNationale de la Recherche (ANR projects "Contreperf" and "Nistec"); INRA and the TUFTS team acknowledge the support of NIEHS grants (ES08314 and RC2ES018822). INRA and IRSET acknowledge the support of the EHESP-School of Public Health (Programme "jeuneschercheurs") and the French Ministry of Environment (Grenelle de l'Environnement, programme LOLF 189 EXPiOmark).

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