ASSESSING THE RELATION BETWEEN POPs INTERNAL EXPOSURE LEVELS AND HUMAN HEALTH: WHO AND WHERE ARE THE GOOD MARKERS?

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Introduction

The fact that environmental and food chemical exposure may play a role in the observed increased incidence of some human health problems today appears undisputed. However, epidemiological studies aiming to reveal associations between such exposure levels and health outcomes sometimes present non convergent, if not contradictory, conclusions. Even more importantly, the demonstration of causality between this environmental factor and human health considered *in vivo* and at a populational scale, is facing multiple levels of difficulty. Thus, this issue still represents a major challenge of the 21th century to be addressed both from a methodological (limits of current existing analytical and statistical approaches) and scientific (need for new paradigms in toxicology and more interdisciplinary research) points of view. Within the panel of questions to be considered in this field, a more precise knowledge of the nature, biological localization, and temporal windows of representativeness / accessibility, of relevant biomarkers of exposure appears as a crucial element. Are different type and/or localization of adipose tissues collected in same individual equivalents in terms of persistent organic pollutant (POPs) concentration levels? Are the already known relationships between POPs levels in adipose tissue and age or body mass index (BMI) extendable from historical (dioxins or PCBs) to emerging (BFRs or PFAS) pollutants? In what extend an internal dose measured from biological fluids such as blood or milk is reflecting the long term exposure and global body burden of individuals? Finally, is the measurement of native (parent) compounds in fat or blood in typical control case studies the more relevant approach for investigating the possible link between chemical exposure and a given health outcome? Based on various and non previously published data sets and results generated from several research projects, but using comparable analytical workflow, the present work is aiming to illustrate these different issues from an expology point of view.

Materials and methods

Human biological samples considered in this work were collected in the frame of several research programs. The ADIPOTOX project (coord. INSERM U747, R. Barouki; partnership INSERM U755, K Clément) concerned control *versus* obese French subjects involved in a gastric surgery program recruited between 2006 and 2008 in the Department of Nutrition, Center of Reference for Medical and Surgical Care of Obesity, Pitié-Salpêtrière Hospital (Paris, France)[1]. The ENDOMET project (PhD thesis work of S. Ploteau (MsD, MCU-PH); dir. LABERCA, JP Antignac and B Le Bizec) concerned control *versus* endometriosis affected French women recruited between 2013 and 2014 in the Gynecology-Obsteric department of the CHU Hotel Dieu (Nantes, France). The DEER project (coord. Turku University, J Topari) concerned a Danish mother-child cohort recruited from 1997 to 2001 in the Department of Growth and Reproduction of the Copenhagen University Hospital (Rigshospitalet, K Main, NE Skakkebaeck, Copenhagen, Denmark)[2]. The NUPEM (coord. Regional Center of Human Nutrition, CRNH, M Champ) and LACTACOL (coord. INRA U1280 PHAN, CY Boquien) projects concerned French mothers recruited in 2009-2011 and 2012-2014 respectively in the Lactarium structure of the CHU Hotel Dieu (C Bosher, A Legrand, Nantes, France)[3]. The CONTREPERF project (coord. LABERCA, JP Antignac) concerned French mothers recruited from 2010 to 2012 at the Gynecologic-Obstetric Department of the Paule de Viguier Hospital (A Berrebi, Toulouse, France).

As National Reference Laboratory (NRL) for regular control of various classes of chemicals residues and contaminants in foodstuff of animal origin, including several classes of POPs, LABERCA has developed for years suitable methodologies for measuring environmental chemicals in food matrices. The Unit has also adapted these methodologies to animal including human biological matrices. The determination of dioxins (PCDD/PCDF, n=17 congeners), polychlorobisphenyls ("dioxin-like" and "non dioxin-like" PCB, n=12 and 6 congeners, respectively), and polybromobisphenyls (PBDE, n=7 congeners) in particular is based on fully validated (2002/69/CE, 2002/70/CE, 2004/44/CE directives) and accredited methods (ISO 17025 standard).

Briefly[1], the sample preparation includes a first high pressure-high temperature extraction step using an accelerated solvent extraction system (ASE), followed by three successive purification and fractionation stages on activated silica, florisil, and celite columns, respectively. The measurement of each fraction is then performed using gas chromatography coupled to high resolution mass spectrometry (GC-HRMS, electromagnetic sector instruments) and quantification is achieved according to the isotopic dilution method (i.e. using ^{13}C labeled analogous as internal standards). For hexabromocyclododecanne (α-, β- and γ-HBCD, n=3 isomers), a measurement by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS, triple quadrupole instrument) is preferred, still using isotopic dilution for quantification and with two diagnostic signals (MRM transitions) for unambiguous identification. For Perfluorinated Alkylated Subtances (PFAS, n=16 substances), a liquid/liquid extraction (LLE) and a purification on two successive Solid Phase Extraction (SPE) systems, i.e. Oasis® HLB and carbon graphitized (Envicarb®) cartridges is applied, followed by a LC-MS/MS measurement[4].

Results and discussion

A main biological compartment used to get a quantitative measurement of POPs in human is adipose tissue. Indeed, considering their lipophilic and bioaccumulable character, the POPs concentration level in fat is expected to reflect the historical and cumulative exposure of individuals. Now, the equivalence of any type of adipose tissue depots present in the body in terms of POPs content is often questioned, depending on each particular clinical protocol and other practical considerations in multidisciplinary studies. Available data on this topic remains extremely scarce. In the present work, we have measured and compared the internal levels of historical (PCDD/F and PCBs) and emerging (BFRs) POPs in at least two different adipose tissue depots (visceral *versus* subcutaneous, parietal *versus* epiploon) from two French women populations. The obtained results (Fig. 1) show both a very good positive correlation between these two distinct fat samples collected from the same individuals (R^2 better than 0.90), and also an extremely good equivalence of the two adipose tissue depots in terms of POPs levels (slope around 1). Interestingly, this observation was made both for PCDD/F, PCBs, PBDE and HBCD. In that case, the equivalence of each type of investigated adipose tissue depot seems to be demonstrated in the scope of assessing biological markers of chemical exposure to POPs in human.

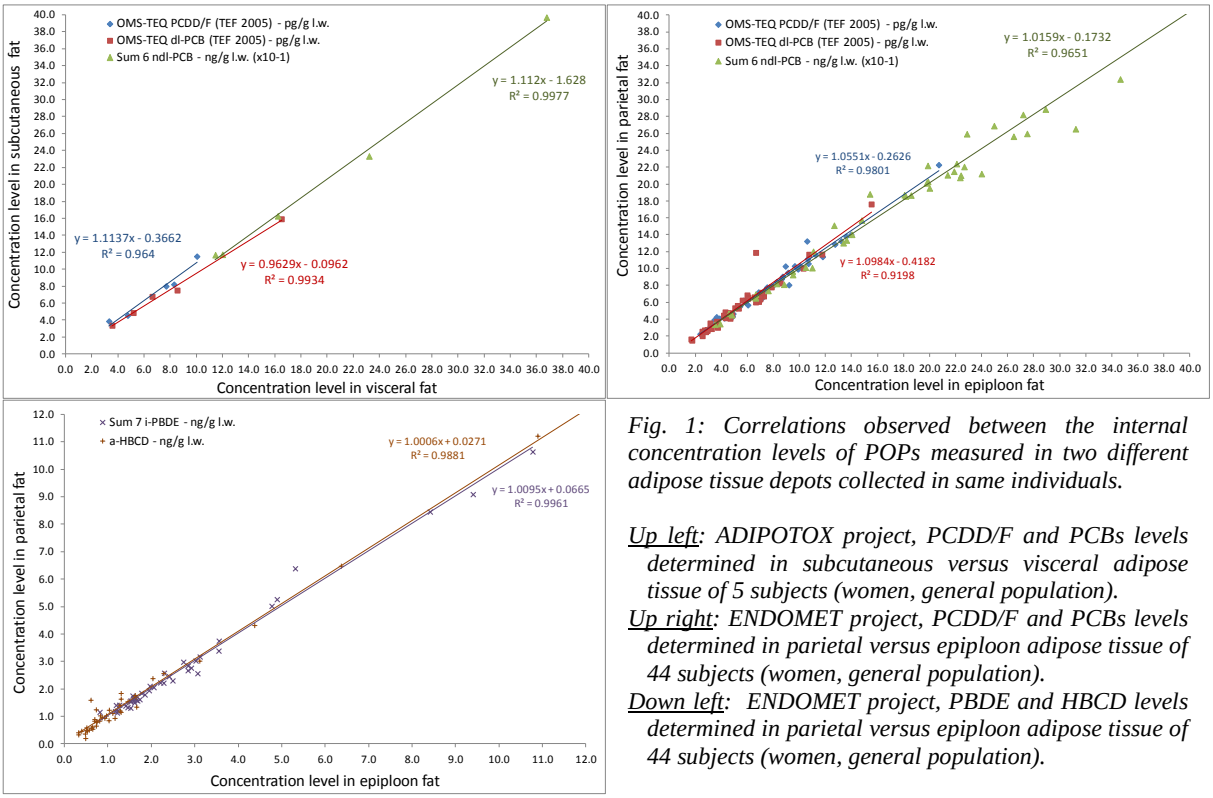

Fig. 1: Correlations observed between the internal concentration levels of POPs measured in two different adipose tissue depots collected in same individuals.

Up left: ADIPOTOX project, PCDD/F and PCBs levels determined in subcutaneous versus visceral adipose tissue of 5 subjects (women, general population).
Up right: ENDOMET project, PCDD/F and PCBs levels determined in parietal versus epiploon adipose tissue of 44 subjects (women, general population).
Down left: ENDOMET project, PBDE and HBCD levels determined in parietal versus epiploon adipose tissue of 44 subjects (women, general population).

A second major issue in studies aiming to link an observed internal exposure with a health outcome is related to the statistical adjustment of the exposure data on several confounding factors. Age and body mass index are two

main examples of such adjustment factors, as a relation is now identified for a while between the internal levels of POPs and these two variables. As the global environmental exposure tends to decrease for decades, at least in Europe and for historical POPs, the date of sampling also appears as a factor to consider when results from different studies are simultaneously considered or compared (in meta-analyses for instance). In the present work, we have stratified two data sets constituted by concentration levels of POPs measured in adipose tissue from French women, according to the considered sampling date (2006-2008 versus 2013-2014), age class (<35 versus >35 years old) and BMI (<25 versus >25). The obtained results (Fig. 2) first confirmed some already known tendencies, i.e. more elevated POPs levels, classically expressed per unit of lipid, in old *versus* young individuals and in lean *versus* obese subjects (explained by a dilution factor in the higher body fat content). They also indicates a near 2 fold decrease between the 2006-2008 and 2013-2014 period of sampling, that can be due also to the different geographical location of the recruited populations (Paris *versus* Nantes). This tendency is however not observed for the "young obese" sub-class of individuals. Interestingly, the rate of bioaccumulation (slope of the regression curve) appeared very similar for the two considered populations (recruited in 2006 *versus* 2013). The same tendencies observed for PCDD/F were also noticed for PCBs. Last but not least, such relation between the levels of historical POPs was not retrieved for emerging POPs. Indeed as illustrated in Fig. 2, the correlation between PBDE or HBCD levels in adipose tissue and age of the considered individuals is not significant, the same absence of correlation being also noticed for PFAS levels measured in breast milk. Finally, statistical adjustment of exposure data undoubtedly merits to be deeply considered without being applied as a systematic procedure with equal relevancy for any type of POPs. The challenge is here to avoid the risk of overadjustment or unnecessary adjustment that can directly influence the conclusion of epidemiological studies.

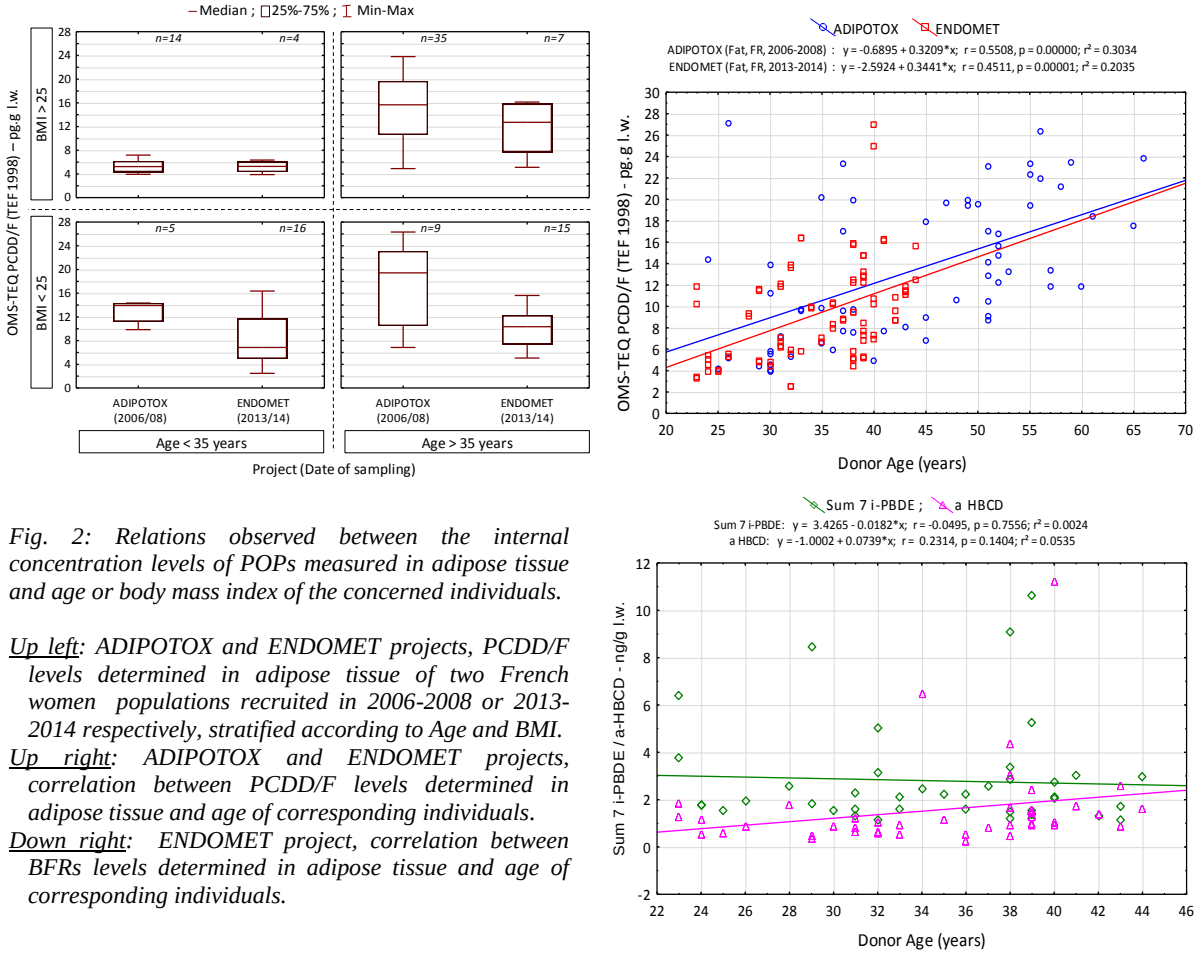

Fig. 2: Relations observed between the internal concentration levels of POPs measured in adipose tissue and age or body mass index of the concerned individuals.

Up left: ADIPOTOX and ENDOMET projects, PCDD/F levels determined in adipose tissue of two French women populations recruited in 2006-2008 or 2013-2014 respectively, stratified according to Age and BMI.

Up right: ADIPOTOX and ENDOMET projects, correlation between PCDD/F levels determined in adipose tissue and age of corresponding individuals.

Down right: ENDOMET project, correlation between BFRs levels determined in adipose tissue and age of corresponding individuals.

Besides the usefulness and relevance of adipose tissue for assessing the chemical internal exposure of human to POPs, the determination of this exposure levels in adult and when the studied health trouble is already observed today appears disputed when this health outcome may be associated to an early and chronic exposure (endocrine disruption for instance). To address this complex issue, a growing interest is noticed for assessing the human

chemical exposure during the perinatal period, in line with the Developmental Origins of Health and Disease (DOHaD) concept. In that context, the determination of POPs in human breast milk is a matter of a high current concern, as indicator of the mother internal exposure and then of the *in-utero* / fetal exposure under the hypothesis that the measured chemicals may cross the placental barrier. One characteristic of this particular biological matrix is that its complex composition (simultaneous presence of lipidic, proteic, and aqueous phases) favorise the presence of a wide range of chemicals with various physico-chemical properties (i.e. persistent and non persistent), making it suitable for assessing the presence of either lipophilic, hydrophilic, or amphiphilic pollutants and/or related markers of exposure. In the present work we have determined the concentration level of PCDD/F, PCBs, PBDE, HBCD and PFAS in several hundreds of breast milk samples originated from French *versus* Danish individuals. The first result observed (Fig. 3) was the non equivalence of the two sample types (country of origin) in terms of quantitative but also qualitative contamination profiles. For instance, samples from French women were globally found around 2-fold less contaminated with in addition lower PBDE and PFAS contributions to the total chemical exposure compared to the samples from Danish women. This observation is independent of the median milk fat content that was found equivalent in the two populations (2.5 versus 2.6 % in French *versus* Danish samples, respectively). At the present times, no clear association was found between the observed POPs levels in Danish breast milk and reproductive health outcomes determined in the breastfed child's. Similar data analysis is still on-going for the French population.

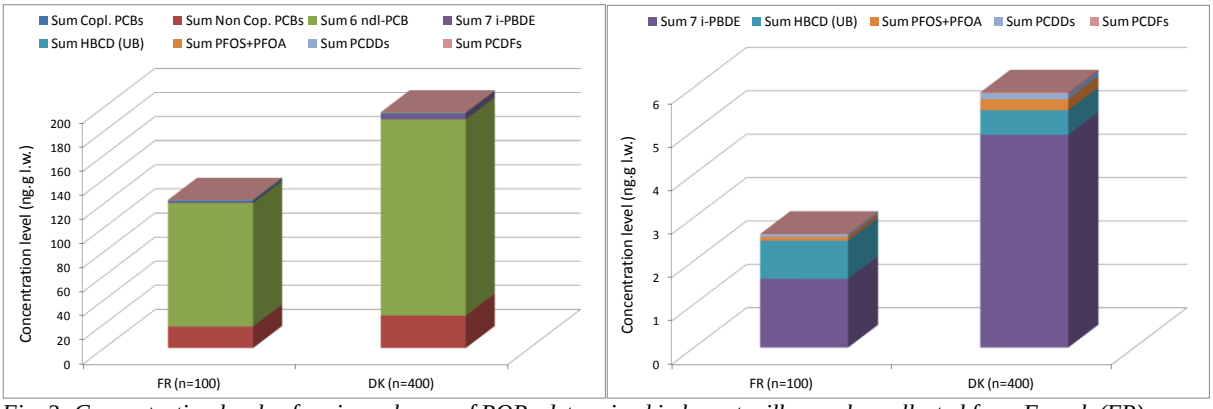

Fig. 3: Concentration levels of various classes of POPs determined in breast milk samples collected from French (FR) versus Danish (DK) mothers. Left: All monitored POPs illustrating the predominant contribution of PCBs to the global exposure profile. Right: all targeted POPs excluding PCBs illustrating the contribution of emerging POPs (BFRs and PFAS).

Conclusion

The quantitative measurement of various classes of POPs in human biological matrices still represents a major challenge in the environmental-health area. The extremely wide panel of substances to consider, the important methodological limitations associated to these *in vivo* studies from study designs, analytical capabilities, and related economical aspects points of view, contribute to the difficulty of establishing a causal link between this chemical exposure and particular health outcomes. Nevertheless, a more integrative view of the human internal chemical exposure still appears as a necessity. Then, the assessment not only of the parent substances but also of their active metabolites (for instance hydroxylated PCBs) also appears an urgent need as they may represent a major element in the researched causality chain.

Acknowledgements

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