COMPLEMENTARY ANALYSIS OF INFANT FORMULAE-CHEMICAL TARGET ANALYSIS VERSUS IN-VITRO BIOASSAYS

Pandelova M[1], Henkelmann B[1], Levy Lopez W[1], Piccinelli R[2], Leclercq C[2], Maggioni S[3], Benfenati E[3], Pinto C[4], Bondesson M[4], Gustafsson J-A[4], Pongratz I[5], Balaguer P[6], Riu A[7], Zalko D[7], Cravedi J-P[7], Schramm K-W[1,8*]

[1]Helmholtz Zentrum München, German Research Center for Environmental Health, Institute of Ecological Chemistry, Ingolstädter Landstrasse 1, 85764 Neuherberg, Germany; [2]National Research Institute on Food and Nutrition, Rome, Italy; [3]Mario Negri Institute for Pharmacological Research, Department of Environmental Health Sciences, via La Masa 19, 20156 Milan, Italy; [4]University of Houston, Center for Nuclear Receptors and Cell Signaling, Houston, TX 77204, USA; [5]Department of Biosciences and Nutrition, Karolinska Institutet, Huddinge, SE-141 83; [6]Institut de Recherche en Cancérologie de Montpellier, 34298 Montpellier Cedex 5, France; [7]INRA, UMR 1331 TOXALIM (Research Centre in Food Toxicology), F-31027 Toulouse, France; [8]TUM, Wissenschaftszentrum Weihenstephan für Ernährung und Landnutzung, Department für Biowissenschaften, Weihenstephaner Steig 23, 85350 Freising, Germany
*Email: schramm@helmholtz-muenchen.de

Introduction

Obviously, infants are particularly vulnerable to at least certain chemicals in food and cannot be considered to be "small adults". Moreover, infants tend to be exposed to relatively high levels of chemicals in food, since they consume more food per kilogram body weight (bw). Some food chemicals can interfere, even at low levels, with the function of the hormone systems in the body. Those food contaminants, which are able to activate most susceptible nuclear receptors named as endocrine disruptors (EDs), are of major concern. Chemicals that are known human EDs include PCDD/F, PCB, DDT, and other pesticides and industrial chemicals. Many of the direct toxicological effects of PCDD/F which have been studied in animals seem to be related to the Ah-receptor (Aryl hydrocarbon receptor, also called 'dioxin receptor')[1]. Dietary exposure to PCDD/F is higher in breast-fed infants[2] but breast feeding has many beneficial aspects, including a measurable positive influence on immunological development. According to the statistics from 2007 European women are increasingly reluctant to breastfeed their infants exclusively at six months of age and only 33.1% of infants in the USA are exclusively breastfed up to 3 months of age[3]. Thus, commercial infant formulae play an important role in the infant's diet. Soy-based formulae (Sf) account for nearly 25% of the formula market in the USA[4]. The predominant phytoestrogen in soy and derived products is the isoflavone genistein, which accounts for approximately two thirds of the soy isoflavone content. The adequacy and safety of soy-based formula for infant use is still controversial[5]. Several methods including chemical target analysis or in vitro estrogenicity bioassay were developed to identify the potency of the EDs potency of commercial infant foods. The objective of the present study was to complementary examine both approaches and investigate the impact of the most consumed infant formulae in Europe on nuclear receptor based modulation.

Materials and methods

Infant formulae included in the study

The 2007 market data made available by "Food for Thought" (www.fft.com) was used to identify the major brands of infant formulae and their shares of the market in 22 EU countries considered to represent the entire Europe[6]. A total of 3 pooled samples of "starting" infant formulae of milk-based (Mf), soy-based (Sf), hypoallergenic–based (HAf) were prepared separately for chemical target analysis and in-vitro bioassay. The proportion of each product in the pooled sample was the same as its share of the European market. Finally 22 different infant formulae products were sampled in 2008 from six different countries including France, Germany, Italy, Portugal, Sweden, and UK. Furthermore, one product of Sf with highest market share within EU was sampled from Germany in 2010 for further bioassay testing.

Determination of PCDD/F and dioxin-like PCB amounts in infant formulae

Extraction of 20 g of fresh weight (fw) infant formula was carried out on Accelerated Solvent Extractor (ASE) (Dionex GmbH, Idstein, Germany) using a mixture of n-hexane: acetone (75:25, v/v) at 120 ºC and at a pressure of 12 MPa. PCB and PCDD/F analyses were performed by HRGC/HRMS[7]. The tetra to octa PCDD/F and tetra

to hepta PCB were identified and reported in pg WHO_{98}-TEQ/g fw. The lower and upper bounds (LB and UB) approach was applied for quantification of the results. The analytical laboratory involved is quality assured according to DIN EN ISO/IEC 17025 and accredited for the analysis of PCDD/F and PCB.

Determination of genistein in infant formulae
Aliquots of 0.5 g of fw powdered infant formula were double extracted in ultrasound bath with acetonitrile, the extracts were centrifuged and the supernatants were filtered, concentrated and diluted with MilliQ water. Subsequently, the extracts were purified by solid phase extraction using N-vinylpyrrolidone/divinyl benzene copolymer cartridges and concentrated before injection in the LC-ESI-MS/MS system. A C8 column was employed for the chromatographic separation and 0.05% acetic acid in MilliQ water and acetonitrile were used as mobile phases. The monitored transitions were 269-133, 269-159 for genistein and 175-133, 175-119 for its internal standard 4-methylumbelliferone.

Micro-EROD bioassay
Samples of 20 g of fw infant formula were Soxhlet extracted with 800 ml toluene for 24 h. The extract was concentrated to 4 ml and then a clean-up step was carried out on a sandwich column. Then, the eluate was concentrated and transferred to 200 µl DMSO under N_2 stream at 45°C. EROD bioassay was performed using the rat liver cell line (HII4E) expressing cytochrome P4501A1 upon exposure to AhR agonists, as described elsewere[3]. The results were given as pg 2,3,7,8-TCDD toxicity equivalent values (TE values) per gram sample.

Isolation and identification of estrogenic substances using recombinant ERα-based affinity columns
Extraction of 20 g of fw infant formula was carried out on ASE using a mixture of n-hexane: acetone (75:25, v/v) as describe above. Then, the estrogenic activity of the food extracts was evaluated using luciferase assays based on the use of reporter MELN and HELN cell lines, as described elsewere[8,9]. In order to trap estrogenic compounds from food extracts, 50 µl extracts diluted in washing buffer were applied on 12 nmol of recombinant ERα-ligand binding domain (ERα-LBD) immobilised on 500 µl Ni-NTA phase. Elution was performed with 3 ml of elution buffer. Aliquots from eluted fractions were analysed using HPLC coupled to a UV detection. Affinity columns were also used in order to identify estrogenic biotransformation products of genistein obtained following incubation with rat liver microsome incubations[10]. Activation of estrogenic signaling by the German soy-based infant formula and BPA was investigated using HELN ERα and ERβ cell lines, described above.

Results and discussion:
Levels of PCDD/F and dioxin-like PCB and TE-EROD value in infant formulae
Table 1 presents the LB and UB for the levels of PCB and PCDD/F in infant formulae based on chemical analysis and EROD-bioassay.

Table 1
Lower bound (LB) and upper bound (UB) levels of PCDD/F and PCB (pg (WHO-TEQ)/g fw) and TE- EROD values (pg/g fw) in Mf, Sf and HAf.

Type infant formula		Mf	Sf	HAf
PCDD/F (WHO-TEQ) pg/g fw	LB	0.04	0.05	0.11
	UB	0.09	0.09	0.11
PCB (WHO-TEQ) pg/g fw	LB	0.0010	0.0003	0.0005
	UB	0.0021	0.0013	0.0006
TE EROD pg/g fw		<LOQ*	<LOQ	<LOQ

* for EROD: LOD=0.5 pg/g fw, LOQ=3.5 pg/g fw

PCB were detected at low levels (up to 0.021 pg (WHO-TEQ)/g fw) in infant formulae. LB of 0.04 and 0.05 pg PCDD/F (WHO-TEQ) /g fw that correspond to 0.16 (WHO-TEQ) pg/g lipid (the package labelled lipid content

of approximately 25 g per 100 g fw) were estimated for Mf and Sf, respectively. A higher PCDD/F level of 0.11 PCDD/F (WHO-TEQ) pg/g fw (0.44 PCDD/F (WHO-TEQ) pg/g lipid) was observed in the hypoallergenic infant formula. Similar PCDD/F UB levels were observed in the three types infant formulae. All these concentration levels are in good agreement with the recent EFSA report on monitoring of dioxin level in "Infant and baby food" group that suggested mean total dioxins equal to 0.42 pg TEQ WHO_{98}/g lipid[11].
With regard to the induction of the EROD activity, no TE levels higher than the LOD (0.5 pg/g fw) and LOQ (3.5 pg/g fw) were observed, which is reassuring since it means that unknown dioxin-like compounds are unlikely to be abundant. The European Commission set an action level of 2 pg PCDD/F (WHO-TEQ)/g lipid and 2 pg dioxin-like PCB (WHO-TEQ)/g lipid in milk and milk products[12]. The current EROD bioassay is sensitive enough to identify this level of concentration. The use of a bioanalytical tool is important since it allows to investigate possible interactions between different even unknown chemicals present in the sample. These possible effects are not considered when single analytical determinations are performed.

Identification of estrogenic contaminants present in infant formula food extracts by immobilised recombinant ERα

Estrogenic activities of infant formula extracts were evaluated using MELN cell luciferase assay (Fig. 1A). No or weak transactivation signal was observed for both milk and hypoallergenic infant formula extracts. However, when soy-based infant formula (corresponding to 6.4 µg of Sf mixture) was applied to MELN cell culture medium, a transactivation signal equivalent to that obtained with 16 pM 17β-estradiol (E_2) (according to E_2EC_{50}) was observed. Sf extract was further applied to HELN ERα and HELN ERβ cells with the aim to characterize their activity with each ER subtype (Fig. 1B). The measurement of the transactivation signal showed one order of magnitude more potency at the level of ERβ than at the level of ERα, for the tested Sf extract.

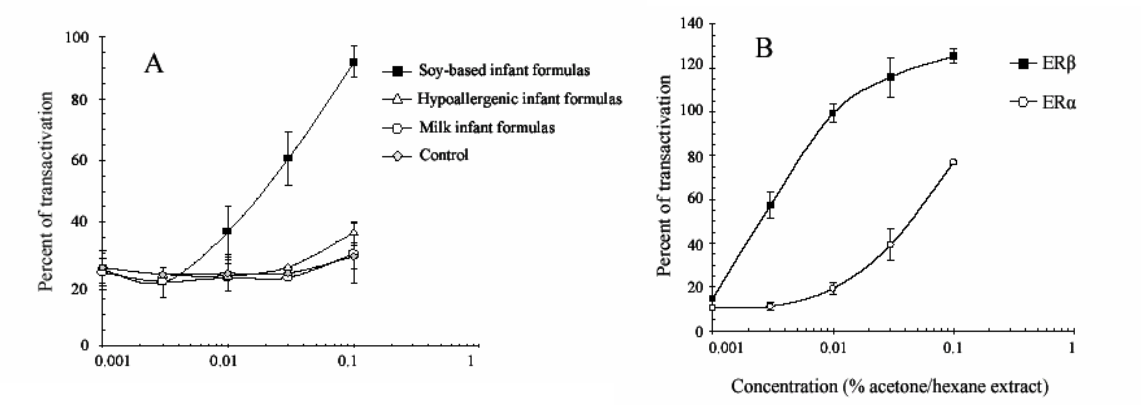

Figure 1
MELN (A) and HELN (ERα and ERβ) (B) cell luciferase assays of soy-based infant formula extract. Results are expressed as a percentage of luciferase activity measured per well (mean ± SEM, n = 4) as a function of acetone/hexane extract percentage. The value obtained in the presence of 10 nM E_2 was taken as 100.

A similar finding of estrogenic signaling by Sf was confirmed in soy-based infant formula recently purchased from the German market. This Sf induced transcriptional activation by both ERα and β, which is in accordance with previous results on the European Sf extract. It was observed that final concentrations of 0.01% and 0.001% Sf extract induced transcriptional activation comparable to approximately 50% of the response induced by 10nM E_2 in HELN ERα and ERβ cells, respectively. Based on published values for genistein's EC_{50} (5.8nM)[13], it was estimated concentration levels of 50mg/kg of genistein equivalent in Sf. Moreover, treatment of HELN ERα cells with 100nM BPA in combination with Sf extract resulted in increased transcriptional activation relative to Sf treatment alone. In the ERβ cells, 10nM of BPA combined with Sf extract activated the luciferase reporter more efficiently than the extract alone.

Purification and identification of estrogenic compounds present in infant food extracts using HPLC-UV and LC-MS. Since only the soy-based infant formula extract displayed an ER activity when tested using MELN and HELN bioluminescent cells, only the Sf mixture extract was investigated by HPLC-UV. Among all the phytoestrogens detected, in particularly two (the major ones, with respective retention times of 24.5 and 28 min) were trapped by ERα-ligand binding domain (LBD) affinity columns. The use of these columns, combined with high resolution electrospray ionisation (ESI)/MS, allowed to conclude that the estrogenic activity of the soy-based infant formula extract could be attributed to the presence of the isoflavones daidzein and genistein. Both natural phytoestrogens display an agonistic activity for ERs, daidzein being less potent than genistein[14]. Subsequently, the quantity of bioactive compounds present in the corresponding sample was estimated. This was calculated by considering that most of the biological activity measured in this extract was associated with the presence of genistein, and that genistein is 10-fold more potent than daidzein for ERβ. Hence, concentration level of approximately 0.23 mM (31mg/kg) genistein, according to genistein's EC_{50} (5.8 nM)[13] was estimated in soy-based infant formula extract.

Levels of genistein in infant formulae detected by LC-MS/MS and GC-MS analysis
A method based on ultrasonic extraction and purification by solid phase extraction followed by LC-MS/MS analysis was developed for the determination of genistein in investigated infant formulae: the achieved limits of detection were 0.6, 2.1, 10.8 ng/g for Mf, HAf, Sf respectively. As a result, traces of genistein below the detection limits were determined in milk and hypoallergenic formulae, while genistein has been quantified at level of 9.7 µg/g soy formula sample corresponding to 15 nM of E_2 equivalent.

Acknowledgements:
The study was financial supported by the European Union network CASCADE (FOOD-CT-2003-506319) within the frame of WP19 projects (bread project and babyfood project).

References:
1. Lundqvist C, Zuurbier M, Leijs M, Johansson C, Ceccatelli S, Saunders M, Schoeters G, Tusscher GT, Koppe JG (2006); *Acta Paediatr*. 95 (453): 55-64
2. Gies A, Neumeier G, Rappolder M, Konietzka R (2007); *Chemosphere* 67(9): 344-349
3. Pandelova M, Piccinelli R, Levy Lopez W, Henkelmann B, Molina-Molina JM, Arrebola JP, Olea N, Leclercq C, Schramm K-W (2011); *Food Addit Contam*. Part A., in press
4. Cimafranca MA, Davila J, Ekman GC, Andrews RN, Neese SL, Peretz J, Woodling KA, Helferich WG, Sarkar J, Flaws JA, Schantz SL, Doerge DR, Cooke PS (2010); *Biol Reprod*. 83(1): 114-121
5. Bhatia J, Greer F, Committee on Nutrition (2008); *Pediatrics* 121: 1062-1068
6. Piccinelli R, Pandelova M, Le Donne, Ferrari M, Schramm K-W, Leclercq C (2010); *Food Addit Contam*, Part A. 27 (10): 1337-1351
7. Cok I, Donmez MK, Uner M, Demirkaya E, Henkelmann B, Shen H, Kotalik J, Schramm K-W (2009); *Chemosphere*. 76 (11): 1563-1571
8. Balaguer P, Francois F, Comunale F, Fenet H, Boussioux AM, Pons M, Nicolas JC, Casellas C (1999); *Sci Total Environ*. 233 (1-3): 47–56
9. Balaguer P, Boussioux AM, Demirpence E, Nicolas JC (2001); *Luminescence*. 16 (2): 153–158
10. Riu A, Balaguer P, Perdu E, Pandelova M, Piccinelli R, Gustafsson JA, Leclercq C, Schramm K-W, Dagnino S, Debrauwer L, Cravedi JP, Zalko D (2008); *Food Chem Toxicol*. 46 (10): 3268-3278
11. European Food Safety Authority (2010). *EFSA Journal*. 8 (3); 1385 Available from: http://www.efsa.europa.eu/en/scdocs/doc/1385.pdf
12. Commission Recommendation, 2006/88/EC Recommendation of 6 February 2006. *Official J Eur Union* (14.02.06)
13. Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, Van Der Saag PT, Van Der Burg B, Gustafsson JA (1998); *Endocrinology* 139 (10): 4252–4263
14. Escande A, Pillon A, Servant N, Cravedi JP, Larrea F, Muhn P, Nicolas JC, Cavailles V, Balaguer P (2006); *Biochem Pharmacol*. 71 (10): 1459–1469