PROJECT FINAL REPORT - SUMMARY

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Project acronym: OBELIX

Project title: OBesogenic Endocrine disrupting chemicals: LInking prenatal eXposure to the development of obesity later in life

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OBELIX
ENDOCRINE DISRUPTORS AND OBESITY

Executive Summary

OBELIX (**OB**esogenic **E**ndocrine disrupting chemicals (EDCs): **LI**nking prenatal e**X**posure to the development of obesity later in life) is a multidisciplinary European research project that was performed from May 2009 to November 2013. The main goal of OBELIX was to investigate if early life exposure to EDCs plays a role in the development of obesity and related disorders later in life. OBELIX investigated six major classes of EDCs, including dioxins, polychlorinated biphenyls, brominated flame retardants, organochlorine pesticides, phthalates, and perfluorinated alkyl acids (PFAAs). More information on the project team, approach and outcomes can be found at www.theobelixproject.org. The main objectives and main conclusions of OBELIX were:

1. *To **assess early life (perinatal) exposure** in humans to major classes of EDs in food, using mother-child cohorts from various European regions with different food contaminant exposure patterns.*

OBELIX demonstrated that perinatal exposure to major classes of EDCs is widespread across Europe. New methods were developed for the sensitive analysis of phthalate metabolites and PFAAs in cord blood and breast milk. Novel postnatal exposure models to EDCs were developed, indicating divergent effects of pre- and postnatal exposure to EDCs.

2. *To relate early life exposure to EDCs with **effect biomarkers** and **health outcome data in children**.*

In epidemiological studies in children, health outcomes such as birth weight, growth, body mass index (BMI) and levels of serum hormones such as thyroid hormones, leptin, adiponectin and insulin were examined. Postnatal exposure to PCB 153 was related to reduced birth weight, body mass index (BMI) and serum leptin levels at 6 years. Prenatal DDE was associated with rapid growth in children up to 2 year, increased BMI and serum leptin levels at 6 years. Preliminary results indicate a relation between perinatal exposure to dioxin-like chemicals and elevated BMI at 6 years.

3. *To perform in vivo **hazard characterization** of early life exposure to EDCs*

Hazard characterization studies in mice showed that perinatal dietary exposure to the EDCs BPA, PFOA, TCDD, DEHP and PFOA resulted in altered serum lipid and/or adipokine hormone levels in adulthood. These effects on metabolic pathways did not consistently coincide with increased body weight or adiposity in mice. In many cases, a reduced body weight was found, and effects were clearly gender-specific. Perinatal BPA exposure showed increased lipid accumulation in adipose tissue in males. Perinatal TCDD exposure showed increased fat pad weight in females.

4. *To determine **mechanisms of action** of EDCs*

Animal studies showed that perinatal exposure to the tested EDCs caused compound- and sex-specific effects on metabolic pathways that occurred with a latency, suggesting programming. No generalized mechanism of action could be ascertained from the animal studies. In vitro studies with mouse preadipocyte cells indicated that EDCs induced fat cell differentiation which was related to altered adipokine gene expression and changes in global DNA methylation, as well as altered methylation of specific genes such as PPAR. Gene expression profiling in cord blood demonstrated an association between prenatal EDC exposure and glucocorticoid, estrogen and progesterone receptor signalling.

5. *To perform **risk assessment** of early life exposure to EDCs for the risk of obesity and related disorders later in life.*

Risk assessment revealed that for some EDCs tested in OBELIX, such as BPA and PFOA, critical effect concentrations in animal studies in OBELIX were lower than those used to set current tolerable daily intake (TDI) levels, suggesting that current TDIs may not be sufficiently protective. The margin of exposure between critical concentrations in animals and human exposure levels was relatively wide, suggesting limited risk to human health. Health outcomes measured in children, however, were observed at lower exposure levels of EDCs than could be detected in animal experiments. This may be due to species differences or to combined exposures of the human population which is also much more genetically diverse. This discrepancy between animal and human studies warrants the consideration of epidemiological data in risk assessment. Taken together, the results of the OBELIX project indicate that early life exposure to EDCs can alter metabolic pathways that play an essential role in energy metabolism and weight regulation. Perinatal exposure to EDCs such as PCB, DDE and dioxin-like chemicals were associated with altered early childhood growth. The long term consequences of these effects in early childhood warrant further investigation.

Project context and objectives

The incidence of childhood obesity has reached epidemic proportions globally. Despite advancements in knowledge on the endocrine and neuronal regulation of energy balance, there is still much uncertainty related to the etiology and underlying physiological mechanisms of obesity. There is accumulating evidence that factors that influence long-term risk of obesity and related disorders begin very early in life. Early life exposure to environmental contaminants has been implicated in altering developmental metabolic programming, resulting in possible higher susceptibility to obesity. The OBELIX (OBesogenic Endocrine disrupting chemicals: LInking prenatal eXposure to the development of obesity later in life) project examines the hypothesis that prenatal exposure to endocrine disrupting compounds (EDCs) in food plays a role in the development of obesity later in life. This project focused on assessing prenatal exposure to chemicals from six major classes of EDCs found in food including dioxins and dioxin-like polychlorinated biphenyls (PCBs), non-dioxin-like PCBs, brominated flame retardants (BFRs), organochlorine pesticides, phthalates and perfluorinated alkyl acids (PFAAs), e.g. perfluorooctanoic acid (PFOA) and perfluorooctanesulfonate (PFOS) (Legler et al, 2011).

List of OBELIX participants

Beneficiary no.	**Organization and Principal Investigator**	**Country**
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2	Flemish Institute for Technological Research (VITO) Prof. G. Schoeters	Belgium
3	Rijksinstituut voor Volksgezondheid en Milieu (RIVM) Dr. L. van der Ven	Netherlands
4	National Institute of Public Health (NIPH) Dr. M. Eggesbø	Norway
5	Ecobaby Foundation Prof. J. Koppe	Netherlands
6	National Institute for Agricultural Research (INRA) Dr. M. Feinberg	France
7	Slovak Medical University (SMU) Prof. T. Trnovec	Slovakia

More information on OBELIX can be found on the website www.theobelixproject.org

OBELIX has a multidisciplinary approach that combines epidemiology, neonatology, endocrinology, toxicology, analytical chemistry and risk assessment. The objectives of the project, which are divided over 4 workpackages (WP, see also figure below), are:

1) To **assess prenatal exposure** in humans to major classes of endocrine disrupting chemicals in food identified as potential inducers of obesity later in life, using mother-child cohorts from various European regions with different food contaminant exposure patterns. (WP 1)

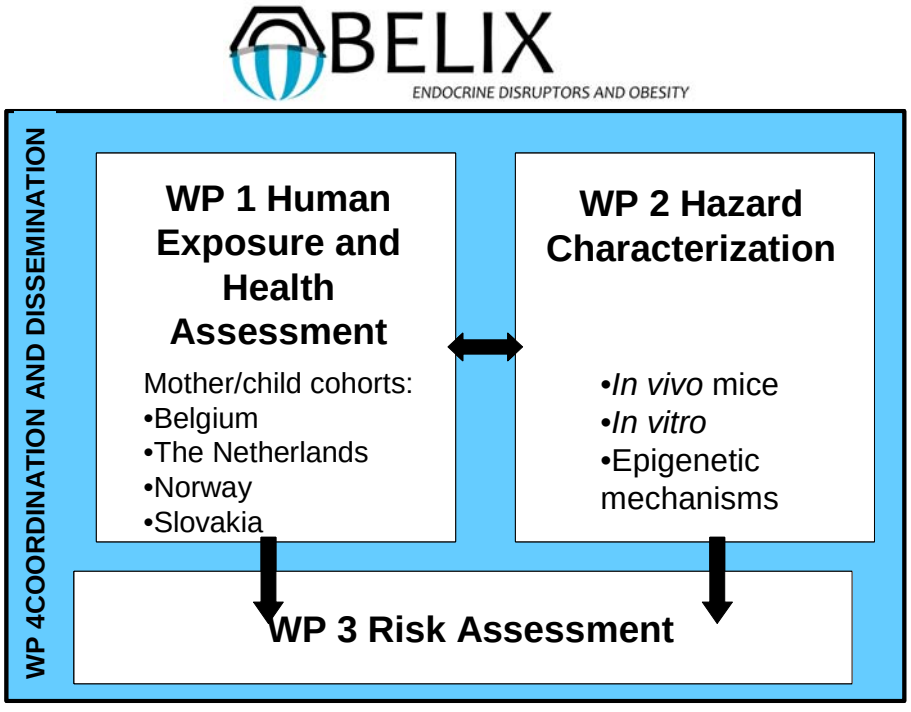
2) To relate early life exposure to EDCs with **effect biomarkers** and **health outcome data** which are related to risk for obesity later in life. (WP 1)

3) To perform **hazard characterization** of early life exposure to representatives of major classes of EDCs in food with respect to the development of obesity later in life, using dose-response analysis in a mouse model. (WP2)

4) To determine **mechanisms of action** of obesogenic EDCs using analysis of effect biomarkers, gene expression and epigenetic analysis. Mouse models, *in vitro* models and analysis in peripheral mononuclear cells of biological samples from the cohorts, will be used as complementary tools. (WP 2)

5) To perform **risk assessment** of prenatal exposure to obesogenic EDCs in food, by integrating maternal exposure through food, prenatal contaminant exposure and health effect data in children, and hazard characterization and mechanistic information in animal and in *in vitro* studies. (WP 3)

6) To **coordinate** the project and **disseminate** the results (WP 4).



In order to achieve these overall objectives, each WP has the following goals:

WORKPACKAGE 1 (Exposure assessment and health effects):

1) To assess prenatal exposure in cord blood or human milk and obtain information on the full range of selected exposure markers for EDCs with different endocrine disrupting properties in at least 200 participants from each of the four participating EU cohorts;
2) To collect effect biomarkers and health outcome data from at least 200 participants of each cohort as well as information on life style factors, ethnicity, and anthropometric parameters of the parents;
3) To relate exposure markers of EDCs with the effect biomarkers and health outcome data via multiple regression and multivariate analysis, while taking into account relevant confounders and covariates, including information from maternal dietary intake (WP3);
4) To analyse in a subset of the participants changes in gene expression and epigenetic changes in peripheral blood mononuclear cells, and to correlate the patterns and profiles with exposure and effect markers.

WORKPACKAGE 2 (Hazard Characterization):

1) To test the developmental origin of obesity induced by EDCs by prenatal exposure assay with mice.
2) To examine the *in vivo* mechanisms of action through altered gene expression or epigenetic regulation
3) To examine the *in vitro* effects of EDCs on cell differentiation and epigenetic regulation

WORKPACKAGE 3 (Risk assessment):

1) To analyze the risk of EDC exposure for the development of obesity and related disorders later in life, taking in account human exposure levels and contaminant intake through food
2) To evaluate the causality between developmental exposure and obesity later in life by integrating effect biomarkers, transcriptome and epigenetic analysis, in animal studies
3) To identify potential mechanisms of action of obesogenic EDCs
4) To evaluate the weight of evidence for the association between prenatal exposure to endocrine disrupting chemicals (EDCs) in food and the development of obesity and related disorders later in life

WORKPACKAGE 4 (Management and Dissemination):

1) To ensure that the project objectives are achieved and to make sure that the deliverables are finished according to the allocated time and budget;
2) To disseminate relevant information to the public, policy makers, end-users and stakeholders on the possible link between early exposure to EDCs and the development of obesity and related disorders later in life

Main Science and Technology

Summary of results based on objectives

Workpackage 1 Exposure assessment and health effects

1) To assess prenatal exposure in cord blood or human milk and obtain information on the full range of selected exposure markers for EDCs with different endocrine disrupting properties in at least 200 participants from each of the four participating EU cohorts.

Five birth cohorts have been included in the OBELIX project: the Flemish birth cohorts FLEHS-I (n=1196) and FLEHS-II (n=250) initiated respectively in 2004 and 2009 with cord blood samples available, the Norwegian HUMIS cohort (n= 2600, initiated in 2003) with milk samples available, the Slovak Michalovce cohort (n= 1134, initiated in 2003) with cord and milk samples available and the Dutch LINC cohort initiated through OBELIX in 2011 (n=143), which collected cord and milk samples. The cohort studies have focused on the following endocrine substances with expected different modes of action: PCB153 as a marker of non-dioxin like PCBs, organochlorine pesticides (DDE and HCB), brominated flame retardants (BDE47, BDE99 and HBCD), dioxins (PCDD/Fs and dioxin-like activity measured with CALUX), perfluorinated compounds (PFOS and PFOA) and the secondary metabolites of the phthalate DEHP. New sensitive methods have been developed to analyse PFOS and PFOA and the DEHP metabolites in human cord and human milk samples (de Cock et al, submitted). It was not possible to quantify Bisphenol A in cord blood or milk samples due to high background levels. In addition, a method for organotin analysis in human milk from the HUMIS cohort was developed and organotins were analysed, but most samples were not above the limit of detection (LOD).

The OBELIX project successfully carried out additional chemical analysis of EDCs in cord blood and milk samples of 4 cohorts to complement the already available information. New data of secondary phthalate metabolite levels were obtained in LINC and FLESH-II, and PFOA and PFOS were measured in LINC, Michalovce and HUMIS milk samples. Additional data of PCB153, HCB and DDE in cord and milk samples were obtained in the LINC cohort. Dioxin like compounds (measured with CALUX reporter gene assays), BDE47, BDE99 and HBCD were additionally analysed in Michalovce milk samples and in LINC samples. These data offer a picture of the distribution of EDCs in birth cohorts from different regions in Europe (Norway, Slovakia, Flanders and the Netherlands) and cover a broad time range between 2002 and 2013. This exposure data has been used in various studies, both published studies (Thomsen et al, 2010; Govarts et al, 2012; Sonnenschein-van der Voort et al, 2013; Gascon et al, 2014; Ten Tusscher et al, 2014) and publications in preparation by OBELIX scientists.

The exposure assessment data was essential to study exposure response relations in the cohorts, addressing prenatal exposure and postnatal exposure with cord levels and maternal milk levels as exposure metrics. Conversion factors were derived to calculate exposure of PCB, HCB , DDE, PFOS and PFOA at birth for cohorts which only had data on levels in maternal milk samples. Postnatal exposure was modelled based on cord levels and maternal milk levels, information on age at delivery, pre pregnancy weight, gestational age and duration of breastfeeding. The modelling was based on methods developed by partner 7 (SMU) (Trnovec et al, 2011; Wimmerová et al 2011; Patayová et al, 2013). Measured blood levels at 6, 16 and 45 months of age that were available from the Slovak cohort were used to develop a state of the art toxicokinetic model of exposure to organochlorine compounds from birth to 45 months of age (Verner et al, 2013).

2) To collect effect biomarkers and health outcome data from at least 200 participants of each cohort as well as information on life style factors, ethnicity, and anthropometric parameters of the parents;

The OBELIX cohorts collected data at birth on clinical outcomes such as birth weight and the risk for being born small (SGA) or large for gestational age (LGA). Additional data were collected on thyroid levels measured in cord samples or through heel prick after birth. Data on weight and length during the first years of life were obtained in FLEHS-I until 3 years of age, in Michalovce and in HUMIS up to age 6 years. Metabolic markers insulin, leptin, and adiponectin were measured at birth (LINC and FLEHS-II) and at age 6 years in the Michalovce.

3) To relate exposure markers of EDCs with the effect biomarkers and health outcome data via multiple regression and multivariate analysis, while taking into account relevant confounders and covariates, including information from maternal dietary intake (WP3);

In collaboration with the ENRIECO network of mother/child cohorts (http://www.enrieco.org/), a meta-analysis was performed in 12 European birth cohorts. The results suggested a negative association between PCB exposure and birth weight, while no significant association was found with p,p'-DDE. On average, birth weight declined by 150g per 1 µg/L increase in PCB153 cord serum concentration (Govarts et al., 2012). For the cohorts with information on gestational weight gain (GWG) a sensitivity analysis was performed, which suggested that GWG mediates part of the association between PCB153 and birth weight, but the association was still there when GWG was included in the model (Govarts et al., 2014).

A database of pooled cohort data was constructed in which the data of the OBELIX cohorts have been uploaded after the necessary data transfer agreements have been signed by each partner. This database has been distributed to the partners and following 4 research questions have been answered by analysis of the individual cohort data and of the pooled cohort data if appropriate:

i) Prenatal exposure to EDCs in relation to birth weight and the risk on being born SGA or LGA

The pooled results of our OBELIX cohorts (FLEHS-I and II, Michalovce and Humis) confirmed the negative association between PCB153 and birth weight found in the meta-analys of Govarts et al 2012, but it was not statistically significant, indicating that larger cohorts are needed to find a significant effect. Prenatal exposure to phthalates metabolites (LINC and FLEHS-II cohorts) suggested a negative association with birth weight, but this association has to be examined further in detail in a larger sample (de Cock et al, 2014, submitted). For the binary outcome Small for Gestational Age (SGA) in our pooled OBELIX cohorts, an association was found between PCB153 and the odds of having an SGA-baby. For HCB, an association with both SGA and Large for Gestational Age (LGA) was suggested, but these results should be further confirmed as there were contradictions between the cohorts (Govarts et al, in preparation).

Preliminary results showed that exposure to dioxin-like compounds was associated with a higher risk of having an LGA-baby, but the numbers were small and associated with large uncertainty. Exposure to the perfluorinated compound PFOA showed an increased risk of SGA. For the binary outcomes SGA/LGA that are rare outcomes, this pooling of databases appeared to be very useful, as with the separate cohort data no reliable statements could be made (Govarts et al, in preparation).

ii) Prenatal exposure to EDCs and thyroid hormone levels at birth

We examined thyroid hormone levels at birth due to their important role in metabolism (Leijs et al, 2012). Non-linear dose-response relations were observed between EDCs and TSH/T4 levels after birth (de Cock et al, 2014, submitted). The associations changed if gestational weight gain was no

longer included in the model. For most compounds gender effects were noticed; effects were observed predominantly in either boys or girls, not in both genders. These results warrant confirmation in a larger sample size.

iii) Perinatal exposure to EDCs in relation to insulin, leptin and adiponectin levels at birth and at 7 years

Studies in the Michalovce cohort indicate that changes in metabolic markers measured at 7 years were associated with early life exposure to EDCs (Palkovicova et al, 2014, in preparation). Prenatal exposure levels of DDE and HCB were positively related to serum levels of leptin at 7 years, in addition increased DDE was related to higher insulin levels but only in girls. In contrast, postnatal exposure to PCB153 and HCB, measured at the age of 45 months or at 6 yrs were negatively associated with leptin levels at 7 yrs. Breast milk exposure to dioxin-like compounds and to PFOS/PFOA was negatively related to serum levels of adiponectin at 7 years. Metabolic markers measured at birth showed no changes in relation to exposure at birth, but the size of the cohort with these measures may be too small to detect significant changes. Interestingly, the effects of PCB 153 on leptin levels were corroborated by gene expression studies that indicate a down-regulation of leptin receptor gene expression in 45-month old children from the Michalovce cohort (Ghosh et al, 2013).

iv) Pre- and postnatal exposure to EDCs in relation to early childhood growth and BMI at 6-8 years

Growth during early childhood and BMI at 6 years showed an relation with early life exposure to EDCs. Pooling cohorts gave a large sample size, allowing distinction between investigation of effects due to (modelled) prenatal and postnatal exposure to PCBs, DDE and HCB. Distinct effects were observed for the different chemicals depending on pre- or postnatal exposure. Our studies indicated that an increase in growth and BMI at 6 years was associated with prenatal DDE exposure. In contrast, a significant decrease in growth was associated with postnatal PCB-153 exposure (Iszatt et al, 2014, submitted). Preliminary studies also indicate a relation with perinatal exposure to dioxin-like compounds and BMI at 6 years (Iszatt et al, in preparation).

4. To analyse in a subset of the participants (lowest and highest exposure percentiles in each cohort) changes in gene expression and epigenetic changes in peripheral blood mononuclear cells (PBMCs), and to correlate the patterns and profiles with exposure and effect markers.

This objective is directly linked to our goal to identify biomarkers in human samples that can be predictive of chemical exposure and obesity development and that contribute to the mechanistic understanding and hence to the proof of concept that EDC levels trigger pathways related to growth and metabolism. We performed analysis with whole genome arrays as well as global DNA methylation analysis in cord blood samples (FLEHS-II cohort) (Remy et al, in preparation). In the last stage of the project the observed changes in gene expression patterns and global DNA methylation status have been evaluated in relation to the EDC exposures measured in the FLEHS-II cord samples. Cord DDE levels only were positively associated with DNA methylation status after adjustment for age and BMI of the mother and gender of the baby. The gene expression analysis of 200 cord blood samples showed that gestational age, gender, blood lipid content, and season of birth are major covariates for gene expression among the whole transcriptome. Prenatal EDC exposure to DDE, PCB153, PFOS and PFOA were associated with changes in gene expression patterns at birth related to endocrine signalling pathways such as glucocorticoid, estrogen and progesterone receptor signalling.

WORKPACKAGE 2 (Hazard Characterization):

1. To test the developmental origin of obesity induced by EDCs by prenatal exposure assay with mice.

A total of 5 long term animal studies were carried out in OBELIX. The selected test EDCs were BPA, PFOA, TCDD, DEHP and PCB 153. Maternal exposure to the chemicals at low concentrations (generally below NOAEL for developmental toxicity) was given through the diet during pre-mating, gestation and lactation (total of 9 weeks). C57BL/6 (B6) dams were mated with FVB males, and after weaning, exposure was stopped, and the F1 progeny were maintained for a period of 20 to 55 weeks of age.

Perinatal exposure to BPA at 0-3000 μg/kg body weight/day resulted in sex-specific effects with increased body weight in male offspring, as well asa dose-dependent decrease in circulating glucagon. Physical activity was also decreased in exposed males. In females, decreases in body weight, serum lipids and fat pad weights with concordant changes in the endocrine profile were found (van Esterik et al, 2014). Detailed statistical analysis revealed less variation in metabolic parameters such as serum lipids or glucagon as compared to body weight, suggesting that these may be better parameters to describe perturbations in metabolic and energy homeostasis than body weight per se. Further statistical revisions in the final period showed that for body weight effects in males, confounding by litter size could not be excluded.

The PFOA study showed that perinatal exposure to 0-3000 μg/kg body weight/day resulted in decreased body weight in females only. The high fat diet that was supplied during the last weeks of the study did not trigger a reversal of the underweight phenotype in females, nor an overweight phenotype in males. For females, a reduction in perigonadal and perirenal fat pad weight and low serum triglycerides and cholesterol were also seen, indicating metabolic dysregulation.

Subsequent studies with TCDD, PCB 153 and DEHP were extended from a half year for BPA and PFOA to a final duration of one year. This extension also allowed the determination of non-metabolic parameters which have known associations with obesity, i.e. selected neurobehavioural and immunological parameters. In the study with perinatal exposure to TCDD at 0 to 10000 pg/kg/day, we observed changes in body composition measured as effects on fat pads, with a leaner phenotype in males and a heavier phenotype in females. Additionally, there were effects in splenocyte stimulation response. These results suggest that perinatal exposure to TCDD can have lasting effects, or effects that appear after a latency period.

In the study with DEHP, perinatal exposure to 0 to 100 mg/kg bw/day resulted in no effects on body or fat pad weight of offspring at 55-57 weeks of age. However, males showed effects on serum lipid profiles (increased free fatty acid and cholesterol), as well as altered serum glucose levels. Effects were shown in the immune domain, i.e. effects on spleen weight in females and cytokine responses of cultured spleen cells after stimulation with immunomitogens in both sexes. Another remarkable observation were differences in the object recognition test, hinting at changes in attention performance due to perinatal exposure to DEHP.

The final study with perinatal exposure to 0-1406 μg/kg body weight/day PCB153 produced limited effects at adult age (52 weeks) in C57BL/6JxFVB hybrid mice. Throughout the study, stage-specific decreases in body weight were seen, particularly in males. Males also showed a dose-dependent increase of serum free fatty acids and of glucose at necropsy. Females showed increased serum

glucagon levels, as well as specific effects on organ anthropometrics (increase in femur length and thymus weight, decrease in pancreas weight).

Overall, the mouse in vivo studies in WP2 showed compound- and sex-specific effects on metabolic endpoints that were dose-related. These observations support the validity of the prenatal programming concept. The observed effects were not necessarily towards an obese phenotype; on the contrary, a lean phenotype was often observed. Our studies do therefore not support the obesogen hypothesis in a consistent manner. However, effects on underlying metabolic parameters may be translated in different apical phenotypes depending on the species (different outcome pathways). It is important to note that each of the five chemicals tested in animal studies in OBELIX showed that perinatal exposure resulted in metabolic changes that persisted into adulthood, long after termination of exposure at weaning.

Our studies, supported by other studies in the scientific literature, have also led to hypothesize that the outcome of programming may be conditional, i.e. other internal or environmental factors than the studied contaminant, may confound the effect. Such differences may exist in basal toxicokinetics, feed (e.g. concentration of methyl donors), microbiome, and model sensitivity. These confounders or combined effect inducers should be researched further.

2. To examine the *in vivo* mechanisms of action through altered gene expression or epigenetic regulation

Epigenetic analysis in liver DNA of the BPA study (males only) suggested decreased global methylation, and methylation changes in specific loci, including estrogen receptor-1 (*Esr1*), as detected with methylation sensitive restriction digestion and subsequent sequencing. A DNA preprocessing protocol based on the same principle, but followed-up by methylation microarrays, was not sufficiently discriminative. The suggested specific markers were verified through dedicated PCR. This verification revealed a single marker as informative, namely *Esr1*, which was thereafter validated by bisulphite sequencing. Resulting dose responses of methylation revealed small methylation differences, possibly too small to be conclusive, and prone to experimental bias. No dose-related differences in gene expression of *Esr1* was found. Furthermore, as for in vivo data, litter size was a confounding factor, hampering conclusive interpretation. Global methylation analysis and analysis of *Esr1* in females did not show methylation differences between control and high dose animals. Subsequent analysis in females was based on another methylation sensitive restriction digestion method (DREAM), revealing possible differentially regulated methylation of 21 loci. Validation of a first set of these 21 (5 targets) could not reproduce differences suggested by DREAM (van Esterik et al, submitted).

Overall, we demonstrated that the observed altered metabolic phenotype in female offspring after maternal exposure to BPA is not associated with liver DNA methylation changes. Our studies should be considered as explorative, and follow-up studies are needed to reveal further details and underlying mechanisms of the suggested programming effects, including epigenetic analysis in affected tissues, or in the tissues that regulate their functions.

3. To examine the *in vitro* effects of EDCs on cell differentiation and epigenetic regulation

The in vitro mechanistic research in this project focussed on a murine adipocyte differentiation model (murine 3T3-L1), and human umbilical cord mesenchymal stem cells (uMSC), as well as gene

expression in human peripheral blood lymphocytes. Effort went into optimization of detection of adipocyte phenotype, and fluorescent measurement of adipored/nile red staining has now been adapted. In the 3T3-L1 model, adipogenic differentiation could be induced by reference compounds (TBT, troglitazone (TROG)), as well as BPA and BDE 47 (Bastos Sales et al, 2012). The adipogenic effect of BDE 47 was a novel finding. Dioxin (TCDD) clearly inhibited adipogenesis in the 3T3-L1 cells (Bastos Sales et al, 2013). Studies with uMSC suggesting DEHP as a stimulator of adipogenic differentiation were preliminary and require further optimization. 3T3-L1 cells were further used for analysis of epigenetic regulation in vitro following exposure to BDE 47. Analysis of locus specific changes of methylation was finalized on targets which were selected following gene expression analysis of a dedicated set of 13 genes involved in adipogenesis. A newly developed method for detection of methylation changes, methylation sensitive high resolution melting PCR (MS-HRM), showed changes in methylation of the PPARγ2 promoter after BDE 47 and TROG. Analysis on the leptin promoter did not reveal any significant alterations in DNA methylation (Kamstra et al., 2014). *In vitro* exposure of human peripheral blood lymphocytes with a subset of key compounds of the project and subsequent gene expression analysis showed that the used compounds induced specific genes, which can be linked to chronic diseases, including obesity related conditions (Wens et al, 2013).

WORKPACKAGE 3 - Risk assessment

1. To analyze the risk of EDC exposure for the development of obesity and related disorders later in life, taking in account human exposure levels and contaminant intake through food

In this task, a compilation of food questionnaire data forwarded by WP1 was performed in order to assess the maternal dietary intake of EDCs in the various cohorts. According to available data, it was possible to study 18 different EDCs that cover the major field of OBELIX project. The detailed procedures used to estimate two dietary exposures estimates:

i. Classical dietary point estimate, which is simply obtained by multiplying, for each individual, all food quantities by the corresponding food contamination levels;

ii. Body burden which is assumed to represent, for a given EDC, the total burden of one individual. It was developed for cumulative contaminants, such as EDCs. Calculation integrates several biological parameters, such as age, weight and blood concentration.

A detailed report has been compiled with the results. In this report, the individual dietary exposures for all cohorts were put together or for individual cohorts. Several points of view are presented: average exposures, EDC distribution histograms and most significant percentiles of distributions. The results of this exposure assessment are compared to literature data in Task 3.4 below. Among cohorts, highest exposure may vary depending on the EDC. For instance, for TCDD, HCBDs, PCBs we see that FLEHS > MICHALOVCE > ZWOLLE. For PFOA, DDE, PFOS we see that MICHALOVCE > FLEHS > ZWOLLE.

Food contributions to EDC exposures show detailed repartition of foods as contamination vectors for each EDC and each cohort. Vector foods could be identified but may also vary between cohorts. “Classical” vector foods (fish for PCBs, meat for BDE) are visible. This approach may help in defining risk management rules. Dietary exposures can be compared with corresponding exposure biomarkers in the cohorts. However, it is difficult to relate dietary exposure to covariates because of the high uncertainty of dietary estimates. The major methodological problems that limit the

conclusions of this study are described below, and include non-standardized food frequency questionnaires (FFQs) among cohorts, lack of information on the whole diet, and limitations of using FFQs rather than 24-hour dietary recalls. For some cohorts, food intakes were recorded more than 10 years ago, while dietary contamination data were from food analysis reported in 2011. This important time shift may also contribute to a flawed estimate of exposure. Thus, final conclusions on the influence of dietary intake on biomarker levels and on the obesogenic risk are quite difficult to draw. There are many important sources of uncertainty in the data. However, the major shortcoming is that researched effects, i.e. the influence of dietary exposure on early child obesity indicators, may be smaller than the uncertainty on calculated EDC exposure estimate.

Some other scientific issues emerged from this task and indicate that when dealing with persistent chemicals that accumulate, more attention should be paid to the body burden modelling that better reflects the pharmaco-dynamical process of accumulation. Also, classical risk assessment is concerned with high exposures while OBELIX hypothesis is based on low exposures. If so, which toxicological threshold values could be used? In addition, the experimental organization allowed simultaneous exposure estimates. Some complementary work demonstrated that multi-contaminant study was possible using classification trees as a statistical technique.

2. To evaluate the causality between developmental exposure and obesity later in life by integrating effect biomarkers, transcriptome and epigenetic analysis, in animal studies

Of the five compounds tested in animal studies in WP2, BPA and TCDD showed effects that are consistent with an obese phenotype, i.e. body weight gain and/or increase in fat pad size. However, litter size was a confounding factor in BPA exposed male offspring which does not allow us to reliably infer causality. Methylation studies in mouse liver from males did not reveal clear BPA-mediated effects. Support for the “obesogenic” effects of BPA was provided by the in vitro model used in OBELIX. BPA exposure enhanced differentiation of adipocytes in the murine 3T3-L1 preadipocyte cell line and was associated with epigenetic effects, i.e. global DNA hypermethylation (Bastos Sales et al, 2013).

Perinatal exposure to TCDD showed increased fat pad weights in females only, though no other effects were observed in the measured metabolic/biochemical parameters (serum hormones) or serum lipid profiles. Transcriptome and epigenetic analysis were not performed in the TCDD mouse study. In the mouse in vitro 3T3-LI cell line, TCDD exposure led to reduced adipocyte differentiation, but with no concurrent changes in global DNA methylation (Bastos Sales et al, 2013). In microarray studies with cultured human peripheral blood mononuclear cells, exposure to the dioxin-like PCB126 lead to the significant alteration of expression of six obesity-related genes (ADIPOQ, CEL, DUSP9, HSD11B2, MMP11 and TUB) (Wens et al, 2013). These in vitro studies indicate that dioxin-like compounds can alter metabolic and adipogenic pathways, but more mechanistic information is needed to understand the increases in fat pad weight in female offspring following perinatal TCDD exposure.

3. To identify potential mechanisms of action of obesogenic EDCs

The mouse studies showed that perinatal exposure to the test EDCs caused compound- and sex-specific effects on metabolic pathways that occurred with a latency, suggesting programming. Given the complications with litter size in the BPA study, a clear obese phenotype was not observed in OBELIX, with the exception of the effects of TCDD exposure in female mice. As mentioned above, insufficient biochemical and genetic data was obtained to provide a conclusive mechanism of TCDD

action that may explain the observed effects. Most of the test compounds showed a leaner phenotype, with related effects on serum lipid profiles and metabolic hormones. It is important to keep in mind that these upstream molecular effects may translate differently among species, leading to variable apical phenotypes. Although obesity is the focus of the project, this may not be the most important parameter in the animal model. As in humans, many other perturbations are underlying the obese phenotype, and such underlying parameters may be equally or more important to study in the animal model, as they are indicative of a perturbed energy balance. We observed a number of changes in biochemistry, gene expression and epigenetics and cell differentiation that may point to mechanisms of disturbed metabolic action. Compound-associated differentially regulated gene expression in human cord blood cells was observed, as well as differences of global methylation in human cord blood. There were also effects of specific compound exposure on gene expression in cultured human peripheral blood lymphocytes, and compound directed adipogenic differentiation in murine preadipocytes and indications in human mesenchymal stem cells. In the mouse preadipocytes in vitro, compound induced adipogenesis was related to adipokine gene expression regulation and to changes of global and locus specific methylation.

Our studies have provided some indications of novel mechanisms of action of EDCs related to perturbed energy balance, such as BDE 47-mediated activation of PPARγ, the master regulator of fat cell differentiation. This mechanism was shown for the first time using the 3T3-L1 in vitro model (Kamstra et al, 2014) as well as in cultured human peripheral blood lymphocytes (Wens et al, 2013). Importantly, specific effects on PPARγ methylation and differential expression of genes involved in adipocyte differentiation were shown in 3T3-L1 preadipocytes exposed to BDE 47. For PCB 153, microarrays in human cord blood from the FLEHS-II cohort showed gene expression pathways related to estrogen receptor signalling (inhibition of ESR2) which is in line with findings reported in cultured human peripheral blood lymphocytes in vitro. Microarrays in human cord blood from the FLEHSII cohort showed gene expression pathways relating PFOA concentrations to the progesterone receptor and JAK-Stat signalling pathways, and gene expression pathways relating DDE exposure and glucocorticoid receptor signalling. Importantly, DNA methylation analysis of the cord blood samples also showed a clear association with DDE and global DNA hypomethylation.

4) To evaluate the weight of evidence for the association between prenatal exposure to endocrine disrupting chemicals (EDCs) in food and the development of obesity and related disorders later in life.

Weight of evidence from in vitro, in vivo and human health studies

In human studies in OBELIX, p'pDDE provided the most consistent evidence of effects on energy balance in children that may lead to an obese phenotype. Prenatal exposure was associated with accelerated growth up to age 2, as well as increased BMI at age 6 years. Increased leptin (both sexes) and insulin (girls) levels at age 7 were also associated with prenatal exposure to DDE. At birth, effects were found on thyroid hormone levels in cord blood that were associated with prenatal exposure to DDE, though the sample size of this study was limited. We were not able to perform animal studies with DDE, due to prioritization of other EDCs earlier in the project based on information at the time. To our knowledge, only one study has been published indicating that prenatal exposure to DDE causes weight gain in rodents[1]. Numerous epidemiological studies exist showing similar associations with prenatal DDE and BMI in children (reviewed in De Cock, 2014). Our results in humans would certainly warrant further investigation in animals to examine causality and elucidate the mode of action of the observed obesogenic effects of DDE. Gene expression and methylation studies in cord

[1] Tomatis L, Turusov V, Day N, Charles RT. The effect of long-term exposure to DDT on CF-1 MICE. Int J Cancer. 1972 10(3):489-506.

cells from the FLEHS-II cohort provided an indication of novel mechanisms of DDE action through glucocorticoid receptor signalling and global DNA hypermethylation.

For BPA, we observed enhanced adipocyte differentiation in vitro. In mice, perinatal exposure showed clear effects on female offspring, with decreased body weight, liver and fat pad weights (van Esterik et al, 2014). In males, increased body weight was observed, but this effect was confounded by litter size. A direct comparison with the cohorts in OBELIX was not possible, due to analytical challenges of measuring BPA above background levels in stored cord blood and breast milk samples.

For BDE-47, enhanced in vitro adipocyte differentiation was observed, which was accompanied by differential gene expression of adipogenic genes, and demethylation of PPARγ (Kamstra et al, 2014). Though the in vitro studies showed novel effects of this flame retardant, unfortunately we did not carry out animal studies with this compound because we prioritized other EDCs early in the project. BDE-47 was present at levels often below detection limits in human cord blood from the FLEHS-II and Linc cohorts, and mother's milk from Michalovce cohorts. The HUMIS cohort is the only cohort in OBELIX that had considerable data on milk levels of BDEs. A cohort-specific study indicated no association between BDEs in milk and thyroid-stimulating hormone in neonates (Eggesbo et al, 2011).

For PCB 153, free fatty acids and glucose were increased in male offispring, indicating programming effects relating to metabolic disruption. Interestingly, life-stage specific effects on growth reduction were found in animal studies, as was found in the human studies, in which prenatal exposure was related to lower birth weight and an increased propensity towards small for gestational age babies (SGA). Importantly, postnatal PCB 153 exposure was related to a decreased growth in children up to age 2, as well as decreased levels of leptin in serum from 7 year old children. The divergent pre- and postnatal effect of PCB153 on growth are important and warrant further investigation. Gene expression profiling in cultured human PBMCs indicated the estrogen receptor mediated pathways as a potential mechanistic underpinning of prenatal effects.

For dioxin-like compounds, TCDD inhibited adipocyte differentiation in the murine 3T3-L1 model. Male mice perinatally exposed to TCDD showed decreased growth and fat pad weights. In females, an increase of fat pad weight was found. In humans (N=1009), perinatal exposure to dioxin-like chemicals measured with a bioassay showing TCDD equivalents (TEQ) values indicated a heavier phenotype in children at age 6 years that are in line with the in vivo results in female mice (Iszatt et al, in preparation). In addition, a higher risk for large for gestational age (LGA) weight babies was indicated. Decreased adiponectin levels at age 7 years were related to prenatal TEQ exposure. These are the first reports of this association, and require confirmation in further studies.

For PFOA, gene expression analysis in cord blood cells revealed a novel pathway of progesterone receptor signalling related to exposure to this compound. Perinatal PFOA exposure showed a consistent effect on female offspring in the animal studies, with a leaner phenotype with corresponding serum lipid profiles. In humans, (N=660), a higher risk for SGA babies was associated with PFOA concentrations in cord blood and breast milk. Preliminary analyses indicate an accelerated growth up to 2 years in children. Decreased adiponectin levels were related to postnatal exposure in children at age 7 years. As of yet, no conclusive information is available on possible modes of metabolic disruption of PFOA, though the effects found on PR signalling warrant further investigation.

Risk assessment

One of the main goals of OBELIX is to ascertain if there are risks for obesity or related metabolic disorders in children at current background levels of EDCs. To this end, we performed risk assessment using the 5 EDCs that were tested in animal studies in WP5. Two of the OBELIX EDCs,

namely DDE and BDE-47, were not included in this risk assessment exercise, as we did not perform animal studies on these compounds in OBELIX. It should be mentioned for DDE, however, that a consistent set of observations was found in WP1 indicating that prenatal DDE is associated with accelerated growth in early childhood, and increased BMI and serum leptin levels at age 6 years. This would suggest that current background levels of DDE are not without risk and further examination of health risks of DDE exposure in children is warranted. To our knowledge there are very limited animal experiments in the literature that examine perinatal exposure to DDE and latent effects on metabolism and energy balance. This is an important knowledge gap that should be studied in the future.

For the risk assessment, we selected the most sensitive critical effect observed in OBELIX animal studies and compared this to benchmark dose levels (BMDL) established by EFSA. We then calculated a corresponding tolerable daily intake (TDI) level and compared this to exposure and/or body burden (BB) levels reported by EFSA, taking important exposure routes into account. Where possible we also used calculated food exposure data from WP3, in which food frequency questionnaires (FFQ) from FLEHS, Michalovce and LINC were used to calculate exposure to EDCs through the diet.

In summary, our risk assessment of BPA indicated that the OBELIX BMDL05 for decrease in fat pad weight in female offspring (233 µg/kg bw/d) is >10 times smaller than the most sensitive BMDL10 recently proposed by EFSA in 2014[2]. Using the uncertainty factors (UF) recently proposed by EFSA, this OBELIX BMDL would lead to a TDI of 0.28 µg/kg bw/day, which is considerably lower than the current proposed TDI of 5 µg/kg bw/day. This level is above the estimated human lifetime exposure level of up to 0.132 µg/kg bw/day[3], however, when taking into account combined exposure through diet and other sources such as thermal paper, BPA exposure may exceed this OBELIX TDI for sensitive groups, indicating potential risk of metabolic disruption in humans at background exposure to BPA.

For PFOA, the OBELIX BMDL05 for decrease in fat pad weight in female offspring was also lower than the most sensitive BMDL10 that was proposed by EFSA[3] to derive a TDI. Applying the same UF as applied by EFSA, the BMDL for decreased fat pad weights in female F1 mice observed in OBELIX would lead to a corresponding TDI value of 0.23-0.28 µg/kg bw/day. Regarding PFOA exposure, the EFSA indicative estimate from food and water is 2 ng/kg bw/day, which correspond very well with the intakes estimated for the OBELIX cohorts FLESH, Michalovce and LINC calculated in WP3. Although OBELIX data indicate a lower TDI than derived by EFSA, it should be mentioned that human exposure may be considerably lower than the TDI based on the OBELIX animal study. EFSA estimated a human exposure of 0.002-0.006 µg/kg bw/day[4], which would indicate a indicate a sufficient margin of safety.

For TCDD, the most sensitive effect observed in the OBELIX animal study was a decrease in fat pad weight in male F1 mice, corresponding with a BMDL05 which is well above the dose used to establish the current TDI[4]. This would indicate limited risk of TCDD for metabolic disrupting effects. However, we think human exposure and the results of the epidemiological studies in WP1 should be considered as well in the risk assessment of TCDD. In the OBELIX cohorts, median TEQ levels in blood (FLEHS) and breast milk (Michalovce) ranged from 4.3 to 23.5 pg CALUX-TEQ/g lipid. Assuming a BB equilibrium and a fat percentage of 20%, these values could be calculated into median BB estimates of 0.86 to 4.7 ng CALUX-TEQ/kg bw. Applying the same UF as used by SCF (2001)[4] to calculate the

[2] EFSA 2014. Draft Scientific Opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs. EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF).

[3] EFSA 2008. Perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA) and their salts. Scientific Opinion of the Panel on Contaminants in the Food chain. The EFSA Journal(2008) 653, 1-131.

[4] SCF, 2001. Opinion of the Scientific Committee of Food on the risk assessment of dioxins and dioxin-like PCBs in food. Scientific Committee of Food CS/CNTM/DIOXIN/20 final.

LOAEL-EHDI into a TDI value, the LOAEL-BB for TCDD of 40 ng/kg bw in rat derived for the study of Faqi et al. (1998)[5] can be recalculated into a tolerable human BB of 4 ng/kg bw. Comparing this tolerable human BB to the BB estimates for the OBELIX cohorts demonstrates that human exposure is in the same range as critical exposure levels, or even exceeding these critical levels.

For PCB 153, the most sensitive effect observed in the OBELIX animal study was an increase in glucagon levels in female F1 mice, corresponding with a BMDL05 that is larger than the overall NOAEL range for non-dioxin-like (NDL) PCBs proposed by EFSA (2005a). For NDL-PCBs, EFSA (2005a)[6] estimated an average dietary intake of 10-45 ng/kg bw/day, which is well below the critical effect concentration in OBELIX. However, a "margin of body burden" (MoBB) approach has been used by EFSA for PCB 153 risk assessment in which the NOAEL estimated animal BB is divided by the estimated median human body BB. For the OBELIX mouse study, lipid-based PCB-153 concentrations were used to calculate the serum concentration corresponding to the BMDL05. This serum concentration could be translated into a BB of 1042 µg/kg bw, which is actually lower than the critical BB of 1200 µg/kg bw for PCB-153 in rats. In the OBELIX cohorts, median PCB-153 levels in cord plasma (FLEHS, Michalovce, LINC), maternal serum (Michalovce), or breast milk (HUMIS, LINC) ranged from 0.028 to 0.137 µg/g lipid. These serum levels were calculated into BB values and indicated limited risk with a MoBB of 39-189. Interestingly, although the MoBB estimates for PCB 153 seem large enough to be protective, significant associations were found in OBELIX for background PCB 153 exposure and health effects in children. For example, decreased leptin blood levels have been found in children from the Michalovce cohort at age 7 years that correlate with PCB-153 blood levels at age 6 years (Palkovicova et al, in prep). By applying a benchmark approach to these data, a BMCL of around 0.2 µg/g lipids could be estimated as a critical internal PCB-153 dose. Assuming a fat percentage of 20%, this value can be calculated into a critical BB of 40 µg/kg bw. This value is much lower than the critical BB established in the animal studies performed in OBELIX or reviewed by EFSA (2005a)[7]. It is clear that epidemiological data generated in OBELIX should be considered in risk assessment of EDCs, as the use of animal data only to set critical effect levels may underestimate risk in humans.

For DEHP, the most sensitive effect observed in the OBELIX animal study was an increased level of free fatty acids in male F1 mice resulting in a BMDL slightly smaller than the most sensitive NOAEL of 5000 µg/kg bw/day that is proposed by EFSA (2005b)[7] to derive a TDI. Applying the same UF as applied by EFSA (2005b), the BMDL in OBELIX would lead to a corresponding TDI value of 44 µg/kg bw/day, which is slightly lower than the TDI of 50 µg/kg bw/day derived by EFSA. Examination of exposure data indicated that external exposure values are lower but in the same range as the TDI derived by EFSA (2005b) or derived from the OBELIX animal study. However, improved exposure assessment of DEHP is recommended, in particular in situations of high neonatal exposures.

[5] Faqi, A.S., Dalsenter, P.R., Merker, H.-J., and Chahoud, I. (1998). Reproductive toxicity and tissue concentrations of low doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin in male offspring rats exposed throughout pregnancy and lactation. Toxicology and Applied Pharmacology, 150, 383-392.

[6] EFSA 2005a. Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the commission related to the presence of non dioxin-like polychlorinated biphenyls (PCB) in feed and food. The EFSA Journal (2005) 284, 1 – 137

[7] EFSA 2005b. Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) on a request from the Commission related to Bis(2-ethylhexyl)phthalate (DEHP) for use in food contact materials. The EFSA Journal (2005) 243, 1-20.

Workpackage 4 Management and Dissemination

1) To ensure that the project objectives are achieved and to make sure that the deliverables are finished according to the allocated time and budget;

The OBELIX project achieved its objectives to a great extent, and deliverables have been submitted on time. Legal, administrative and financial aspects of the project ran smoothly. Regular meetings ensured good progress. The constructive atmosphere in the group was notable and the motivation of each partner was remarkable.

Our project has benefitted immensely from our independent scientific advisory board (SAB). The following scientists formed the SAB: Dr. M. Longnecker (epidemiologist from NIEHS, U.S.A.), Dr. R. Simmons (neonatologist from Children's Hospital of Philadelphia and University of Pennsylvania, U.S.A.), and Dr. R. Stöger (University of Nottingham, U.K.), a molecular biologist with epigenetics expertise. In addition to the SAB, OBELIX has benefitted tremendously from the modelling expertise of Dr. Mark Andre Verner. In their final report to the coordinator on their impressions of the project, the SAB stated the following:

"The overall vision for the project was impressive: create an interdisciplinary team of scientists who would generate new data on the toxicology, molecular mechanisms, epidemiology, and risk implications of a panel of obesogens. The subprojects were developed and revised in conjunction with the team. The goals were ambitious but nonetheless relentlessly and cheerfully pursued to much success. The project was a model of how scientific questions should be pursued—holistically. Several strengths of the project were evident both at the final workshop and over its entire course. For the epidemiologists, by having group discussions about the center-specific and pooled projects it seemed clear that the study designs, data analyses, and reports benefitted tremendously. For the toxicologists and molecular biologists, because their contributions to the larger project were so fundamentally important, they responded to the pressure with tremendous productivity considering the time and financial constraints.

Both the [final] workshop and the overall project were well organized. Some of the key points ("lessons learned") were: 1) postnatal exposure to persistent organic pollutants can now be estimated much better than previously and the new methods may reveal previously unidentifiable relationships; 2) candidate obesogens sometimes showed effects in the direction opposite that hypothesized; 3) response to obesogens was sometimes transient or delayed; 4) molecular models of adipocyte differentiation can guide epidemiologic investigations."

2) To disseminate relevant information to the public, policy makers, end-users and stakeholders on the possible link between early exposure to EDCs and the development of obesity and related disorders later in life

Considerable effort has gone into effectively disseminating OBELIX to a very widespread audience, including scientists in and out of our fields, the public, policy makers, and stakeholders. A total of 23 manuscripts have been published and many are in preparation. Published manuscripts up to the date of June, 2014 are shown below under "OBELIX list of publications". Approximately 80 other dissemination activities have taken place, including conference presentations, newspaper articles, lectures to social organizations and medical practitioners, and television appearances. These dissemination activities are described below in the "potential impact of OBELIX" section.

OBELIX list of publications

1. Bastos Sales L, Kamstra JH, Cenijn PH, van Rijt LS, Hamers T, Legler J. Effects of endocrine disrupting chemicals on in vitro global DNA methylation and adipocyte differentiation. Toxicol In Vitro. 2013 Sep;27(6):1634-43. doi: 10.1016/j.tiv.2013.04.005. Epub 2013 Apr 18. PubMed PMID: 23603478.

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Potential impact of OBELIX

Childhood obesity is of great concern because obese children generally become obese adults and childhood obesity is associated with a shorter life expectancy. Furthermore, obesity is associated with a range of metabolic disorders including insulin resistance, type 2 diabetes, and dyslipidaemia, as well as increased risk for cardiovascular disease and cancer. The urgent need for studying the impact of food contaminants on obesity development is based on two observations: (1) Prevalence of overweight and obesity among children has increased in most societies around the world, particular over the last 20 years, and (2) The current obesity epidemic cannot be fully explained by genetic factors or by changes in physical, socio-cultural, economic and political factors. OBELIX has generated new knowledge about perinatal exposure to major classes of EDCs and their potential to form a risk factor for obesity later in life. This knowledge has consider potential impact, for example, to underpin appropriate regulation of EDCs. OBELIX has identified cases in which current food safety standards may not sufficiently take metabolic dysregulation caused by EDCs into account. It also became apparent in OBELIX that effects on growth and body weight related to perinatal EDC exposure occur at lower levels in children than are detected in animal studies. Prof. Legler, OBELIX coordinator, is currently involved in a number of activities to raise awareness of perinatal effects of EDCs within government and general society, including a discussion of OBELIX results with members of the Dutch parliament, and participation in a Dutch television documentary "Zembla" in December 2013 (http://zembla.incontxt.nl/seizoenen/2013/afleveringen/19-12-2013). A major international impact of OBELIX was the coverage of the project in the scientific documentary for the Canadian Broadcasting Company called "Programmed to be Fat?" which was aired in Canada on Jan 12, 2012 (http://www.cbc.ca/natureofthings/episode/programmed-to-be-fat.html) and in many countries since then.

OBELIX has demonstrated that perinatal exposure to major classes of EDCs is widespread in Europe, and exposure to some EDCs is associated with changes in birth weight, early growth and body weight at 6 years of age. This information has raised the interest of the medical community, as demonstrated by a workshop we held for medical practitioners in December 2012, and from society, as reflected in media attention (various TV, newspaper and "non-scientific" articles). A main dissemination highlight was the final OBELIX workshop, held in Brussels in October 2013, which was attended by 60 stakeholders from academia, policy and industry.

Though not directly studied in OBELIX, our results support the premise that prevention of exposure to EDCs during pregnancy and postnatal development could have important socio-economic implications, as strategies aiming at prevention of weight gain and obesity are likely to be more cost effective and to have a greater impact on long-term control of body weight than treating obesity once it has developed. New studies are starting to emerge showing the high medical costs of exposure to EDCs during childhood in terms of health care to treat obesity and related disorders like diabetes (e.g. Trasande L. Health Aff. 2014 Feb;33(2):316-23.)

OBELIX has also made considerable scientific impact, with over 20 publications in high-quality scientific journals (see list of OBELIX publications) and many more under review, submitted or in preparation, as well as numerous presentations at international conferences. Prof. Legler and other members of the OBELIX consortium have been invited to present the OBELIX project at many occasions, in countries around the world, including Canada, U.S.A., Germany, Italy, and Japan. Importantly, OBELIX research has generated new scientific hypotheses, such as candidate mechanisms of action of metabolic disruption that warrant further research, as well as confirming important concepts, such as the gender-specificity of the effects of EDCs, and the importance of timing of exposure. Importantly, our studies provided a "proof-of-principle" for metabolic disrupting effects of selected individual EDCs. This leads to the question of how complex mixtures of chemicals interact, an important up and coming field of toxicological and epidemiologic research. OBELIX, with

its interdisciplinary and integrated approach, has generated insights that will impact scientific research in the future, by providing a solid scientific basis for continued research in the role of chemical exposure in obesity.