**Study design**

**IMPLEMENTATION OF ALIGNEMENT STUDY IN CROATIA- ISSUES IN CONDUCTING A RESEARCH PROJECT**

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Although parts of several phases in research conduction were suggested or decided by HBM4EU, Croatian Institute of Public Health (CIPH) team encountered inevitable challenges in all phases (Planning, Preparatory, Concretisation, Starting and Fieldwork Phase) of the study. As well as other participating countries, Croatia represents one of primary sampling units (PSU) of 300 participants which in this case targets one of the seven age groups (20-39y) set by the Protocol. In the Planning Phase three out of nine prioritized substances/ substance groups were identified as substances of interest; Cd, PAHs and Bisphenols. Accordingly, the selected substances determined the matrix to be collected, matrix volume and the sampling time-frames. Moreover, geographical areas were selected within a country, followed by suitable sampling frame identification, indicated by the given target population. The survey is to be conducted in five different region in Croatia (5 counties and its centres) in order to provide an insight into the exposure of individuals. One of the most commonly reported issue referred to fieldwork, especially the first contact with individual participants (invitation, clarification of inclusion and appointment fixing) and corresponding, response rates. In addition to that, a low number of contacted individuals responded the non-responder questionnaire. Under these events, health literacy concerns are raised. Pre-existing knowledge on low health literacy suggests issues related to disease understanding as well as medication and self-care managing. On the other hand, in Public Health context, low health literacy could result in lower participation rates in health promoting behaviours, screening programs, preventive services as well as studies. In line with all mentioned, the survey will be followed up within participants by a questionnaire regarding health literacy, achieving awareness raising, better problem understanding and possible new directions in practices.

**Study design**

**HARMONIZED FRAMEWORK FOR THE IDENTIFICATION OF EMERGING CONTAMINANTS IN HUMAN URINE BY NON-TARGET SCREENING WITH LC-QTOFMS**

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Non-target screening analysis of human urine samples by liquid chromatography coupled to high resolution mass-spectrometry (LC-HRMS) is able to provide an overview of the presence of emerging chemicals (ECs) in the population. However, despite the attractive features this novel strategy is facing a lack of harmonization in order to obtain comparable and high-quality results.

To facilitate the development of comparable non-target/suspect screening workflows for ECs in human urine by LC-HRMS, we developed a generic quality assurance-quality control (QA/QC) framework that involves each step of the analytical method. Crucial QA/QC measures have been implemented in the analytical workflow, such as the addition of a set of labeled (internal) standards for a wide range of chemical properties to the urine samples or duplicate analysis in parallel. Considering the large number of possible technical and parameterization approaches, various lists with QA/QC measures can be established. The most important measures were: 1) daily QTOF calibration 2) definition of a minimal value for the signal to noise ratio, 3) prevention of detector saturation, and 4) coherence between scan speed and number of cycles.

The new framework has been applied to the analysis of 35 urine samples belonging to a Flemish cohort. This study is part of the EU H2020 HBM4EU project that assesses the human exposure to ECs and their metabolites. The urine samples were analysed using QuEChERS as sample treatment combined to injection of the extracts on the LC-QTOFMS. More than 20 ECs were identified at identification levels 2 to 4 according to Schymanski´s scale [1]. The highest detection frequencies (DF) were observed for the benzotriazole metabolite hydroperoxide-tolytriazole (DF 100%), the phthalate metabolite mono-iso-nonyl phthalate (DF 97%), Bisphenol B (DF 95%) and the UV filter metabolite [hydrox[y2-ethylhexyl 4-(dimethylamino)]benzoate (DF 77%).

[1] E. L. Schymanski et al. Environ. Sci. Technol. 48 (2014)2097–2098.

**Study design**

**STRATEGIES FOR SUSPECT AND NON-TARGETED SCREENING OF NEW EMERGING CHEMICALS IN HUMAN BIOMONITORING**

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Humans are exposed to a large number of chemicals from consumer products, food and through environmental media. Current human biomonitoring has to challenge on one hand the high variety of chemicals in focus with a high range of physical properties, as well as including different metabolism products as possible biomarkers of exposure in the analysed human material. This is a revealing challenge for classical targeted chemical analysis. The development of high resolution mass spectrometry combined with high performance computation for data acquisition provides an opportunity to simultaneously detect a large number of compounds in various matrices. Our study inside of the work package 16 (New emerging chemicals) of the European project HMB4EU demonstrates opportunities and limitations of state-of-the art methods and how they can be applied in a qualitative screening of suspects on a sample set representing the general European population. It applies open source high performance computational tools for high throughput data acquisition and identification of compounds in human samples analysed by liquid chromatography coupled to high resolution mass spectrometry.

**Occupational**

**SYSTEMATIC REVIEW OF BIOLOGICAL MONITORING DATA FROM OCCUPATIONAL EXPOSURE TO HEXAVALENT CHROMIUM**

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According to IARC, Cr(VI) compounds are carcinogenic to humans (Group I). In addition, ECHA states that occupational exposure to Cr(VI) levels of more than 1 µg/m³ result in cancer risks of > 4 extra lung cancers per 1000 workers. Nowadays, the principal biomarker used for the biomonitoring of Cr(VI) exposure at the workplace is urinary (total) Cr. The most stringent OEL currently set in workplace in EU, namely in France and the Netherlands, is 1 µg/m³. The biological limit value (BLV) that corresponds to this OEL is 2.5 µg/L or 1.8 μg/g creatinine. Since this BLV is close to background urinary Cr levels in occupationally non-exposed populations, new and more specific exposure biomarkers are needed. Furthermore, urinary Cr is not specific for Cr(VI) since it measures exposure both to Cr(III) and Cr(VI). Consequently, it has become challenging to interpret urinary Cr levels. Therefore a systematic literature review will be presented to provide an overview of historical biological monitoring data from occupational exposure to Cr(VI). In addition to this state-of-the-art overview, occupational exposure to chromium among Belgian workers are assessed using a Job Exposure Matrix (JEM) approach based on local historical biomonitoring data. JEM is an epidemiological instrument providing occupational exposure inferences by using job titles. Historical biomonitoring dataset that comprised of urine measurements was obtained from an occupational health service in Belgium. The goal of this review is to provide a scientific base for research into more specific biomarkers within the HBM4EU hexavalent chromium occupational exposure study.

**Priority substances**

**DIETARY INTAKE OF ACRYLAMIDE AND RISK OF BREAST, ENDOMETRIAL AND OVARIAN CANCER: A SYSTEMATIC REVIEW AND DOSE-RESPONSE META-ANALYSIS**

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**Background**: Acrylamide is a probable human carcinogen that occurs naturally in starchy foods during cooking processes at high temperatures. Aside from occupational exposures and smoking, the main source of human exposure is diet, particularly consumption of potatoes, grain products, and coffee. High acrylamide intake has been associated with altered sex-steroid hormone concentrations and increased risk of hormone-dependent gynecologic neoplasms.

**Objective**: We performed a systematic review of the papers investigating the association between acrylamide intake and risk of breast, endometrial and ovarian cancer in humans. We also examined a possible dose-response relation by carrying out a dose-response meta-analysis of these studies.

**Methods**: We searched in PubMed up to September 10, 2019 the non-experimental human studies investigating risk of breast, endometrial, or ovarian cancer in relation to dietary intake of acrylamide. We also carried out a dose-response meta-analysis using a restricted cubic spline model.

**Results**: We retrieved 18 studies: 11 cohort, 5 case-cohort, and 2 case-control studies. Since some studies assessed more than one cancer type, we found a total of ten studies on risk of breast cancer, seven on endometrial cancer, and seven on ovarian cancer. In the dose-response meta-analysis, acrylamide intake was associated with slightly increased risks of endometrial and ovarian cancers, with a stronger and almost linear increased risk among never smokers. Conversely, for breast cancer we found no evidence to support an increased risk following acrylamide exposure, except for a positive association among premenopausal women exposed to at least 20 µg/day of acrylamide.

**Conclusions:** Based on the relatively small number of studies published to date, acrylamide intake was associated with increased risk of endometrial and ovarian cancer in a dose-response fashion, with a slightly stronger association observed among never smokers. Acrylamide intake was associated with an increased risk of breast cancer only among premenopausal women and at intakes greater than 20 µg/day.

**Keywords**: acrylamide; dietary intake; breast cancer; endometrial cancer; ovarian cancer.

**Priority substances**

**CADMIUM EXPOSURE AND RISK OF BREAST CANCER: A DOSE-RESPONSE META-ANALYSIS OF COHORT STUDIES**

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**Background**: Cadmium is a toxic heavy metal that has been implicated in breast cancer etiology, albeit with inconsistent results. The general population is exposed to cadmium through dietary intake, cigarette smoking, emissions of motorized traffic and industrial facilities. We carried out a systematic review and dose-response meta-analysis of the cohort studies investigating the association between cadmium exposure and breast cancer risk.

**Methods**: Following a literature search through September 10, 2019, we carried out a systematic review and a dose-response meta-analysis to investigate the relation between cadmium exposure and disease risk. We used a restricted cubic spline model and the ‘one-stage’ approach, stratifying for exposure assessment method and menopausal status.

**Results**: We identified eleven studies on breast cancer risk, six based on cadmium dietary intake, and five on urinary excretion levels. In dose-response analysis, we observed a positive, statistically imprecise linear relation between dietary cadmium intake and disease risk. The risk ratio at 20 µg/day compared with no intake was 1.12 (95% confidence interval 0.80-1.56). Conversely, we detected a very imprecise negative association between urinary cadmium excretion and risk (risk ratio=0.89, 95% confidence interval 0.37-2.14 at 2 µg/g creatinine of cadmium excretion). Analysis restricted to post-menopausal women showed substantially no association, as was true for all meta-analyses carried out by comparing the highest versus the lowest exposure category.

**Conclusions**: Overall, we found scant evidence of positive association between cadmium and breast cancer. Available data were too limited to carry out stratified analyses according to age, smoking and hormone receptor status. Therefore, possible associations between cadmium exposure and breast cancer risk in selected subgroups cannot be entirely ruled out.

**Keywords:** cadmium, breast cancer, dietary intake, urine excretion, dose-response meta-analysis

**Priority substances**

**BIOMONITORING OF POLYCYCLIC AROMATIC HYDROCARBONS IN LACTATING MOTHERS: URINARY LEVELS AND RISK ASSESSMENT**

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Polycyclic aromatic hydrocarbons (PAHs) are persistent organic pollutants generated during the incomplete combustion of organic materials. Humans may be exposed to PAHs through inhalation of polluted air or cigarette smoke, dietary intake and dermal contact. Since several PAHs are considered by International Agency of Research on Cancer as carcinogenic or potentially carcinogenic in humans, a risk assessment of exposure to PAHs in vulnerable population is necessary.

First-morning urine samples were collected from 110 lactating mothers participating in the BETTERMILK project in Spain (2015). The urine sample (2.5 ml) was spiked with labeled internal standards and subjected to an enzymatic treatment. After that, a liquid-liquid extraction was carried out and the total organic layer was evaporated under N2 stream. The dry residue was redissolved with the mobile phase. In total, 11 urinary metabolites of PAHs (1-, 2-hydroxynaphthalene, 2-, 3-hydroxyfluorene, 1-, 2-, 3-, 4-, 9-hydroxyphenanthrene, 1-hydroxypyrene and 3-hydroxybenzo[a]pyrene) were analyzed in the final extract using HPLC-MS/MS (Thermo Fisher Scientific) with a KINETEX F5 column (2.1 x 150 mm, 2.6 μm) (Phenomenex) and ESI source.

Detection frequencies ranged from 2% (3-hydroxybenzo[a]pyrene) to 100% (1-hydroxypyrene), and geometric means were between 0.04 ng/ml (4-hydroxyphenanthrene) and 7.15 ng/ml (2-hydroxynaphthalene). An Estimated Daily Intake (EDI) was calculated for interpretation of urinary levels of PAHs metabolites in a risk assessment context. The highest derived EDI was obtained for naphthalene (152 ng/kg-day).

Similar urinary levels of the 11 PAHs metabolites were found in other populations such as USA (0.02 – 4.29 ng/ml) and Czech Republic (0.10 – 5.9 ng/ml). Spanish population seemed to be more exposed to naphthalene. Calculated EDIs were far below their oral exposure Reference Doses established by EPA, so PAHs does not raise a health concern for lactating women living in Spain.

**Priority substances**

# BIOMONITORING OF NON-PERSISTENT PESTICIDES IN URINE FROM LACTATING MOTHERS: EXPOSURE AND RISK ASSESSMENT

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The aim of the present study was to assess the exposure to pesticides in urine from Spanish lactating mothers (n = 116). Six nonspecific (dialkyl phosphates) and 20 specific metabolites of organophosphate pesticides (OPs), herbicides and pyrethroids were analyzed. The most frequently detected biomarkers were diethyl phosphate, p-nitrophenol, 3,5,6-trichloro-2-pyridinol and 3-phenoxybenzoic acid, whose geometric means were 1.9 ng·mL−1, 0.8 ng·mL−1, 1.5 ng·mL−1 and 1.4 ng·mL−1, respectively. Herbicide metabolites were the least frequently detected biomarkers with detection frequencies between 0% (2,4,5-Trichlorophenoxyacetic acid) and 22% (2,4-Dichlorophenoxyacetic acid). Multiple regression analyses showed that the closeness to a farming activity, the place of residence and the presence of garden/plants at home were some of the most important contributors to urinary levels of pesticide metabolites. Estimated daily intake (EDI), hazard quotient (HQ) and hazard index (HI) were obtained in order to interpret urinary levels of the most frequently detected pesticide metabolites in a risk assessment context. The highest EDIs were obtained for chlorpyrifos (0.40–1.14 μg·kg bw−1·day−1) and deltamethrin (0.34–4.73 μg·kg bw−1·day−1). The calculated HQ for chlorpyrifos, dimethoate, parathion and deltamethrin ranged from 0.01 to 0.47, and HI for OPs ranged from 0.09 to 0.33 showing that apparently there were low health risks due to the exposure to these pesticides in this group of Spanish breastfeeding women.

1BETTERMILK: Gormaz M.; Kuligowski J.; Sánchez A.; Torres E.; Correcher P.; Núñez A.; Parra A. and Ramón A.

**Priority substances**

A REVIEW OF PHTHALATE PHARMACOKINETICS IN HUMAN AND RAT: WHAT FACTORS DRIVE PHTHALATE DISTRIBUTION AND PARTITIONING?

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Phthalates are a class of compounds that have been extensively used as plasticizers in different applications. Several phthalates have been recognized as substances of very high concern (SVHCs) in the EU, because of their toxicity for reproduction. However, high amounts of other phthalates are still produced and imported in the European Economic Area. In China and the US, recent studies show increasing concentrations of several phthalates in the air and in human urine, respectively. The understanding of phthalate absorption, distribution, metabolism and elimination (‘pharmacokinetics’) in the organism is still limited. Specifically, phthalate partitioning among tissues is insufficiently understood. Here we estimated partition coefficient (PC) values for different phthalates by using five algorithms and compare them to experimental (*in-vivo* and *in-vitro*) PC values. In addition, we reviewed all pharmacokinetic steps for phthalates in human and rat, based on data from 133 peer-reviewed publications. We analyzed the factors that determine phthalate partitioning and pharmacokinetics. Four processes are particularly relevant to phthalate distribution: protein binding, ionization, passive partitioning and metabolism in different tissues. The interplay of these processes needs to be better represented in methods for determining the PC values of phthalates. More globally, the hydrophobicity of phthalates affects all pharmacokinetic steps, including notably the elimination of phthalates in urine and feces. More studies on protein-bound fraction of phthalates in plasma and pharmacokinetic studies following inhalation and dermal exposure are desirable.

Keywords: phthalate; partition coefficient; distribution; pharmacokinetic; algorithm; experiment; binding; hydrophobicity; ionization; metabolism

**Priority substances**

**BIOMONITORING OF URINARY PHTHALATE BIOMARKERS IN LACTATING WOMEN**

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The phthalates are high volume produced chemicals used as plasticisers in a wide range of applications. Phthalates are frequently released into the environment by leaching and migration from the products to the air, food, water and dust. Therefore, general population is continuously exposed to phthalates through ingestion, inhalation or dermal**.** There is a high concern in exposure to phthalates since they are suspected endocrine disruptors for humans**.**

After entering in the human body, phthalates undergo metabolism to monoesters. A high percentage of the absorbed dose is excreted in urine during the first 24 h as free or conjugated metabolites.

The objectives of the present study are i) Determine the urinary levels of phthalate metabolites in a population of Valencian mothers ii) Estimate the risk assessment of the studied population to phthalates.

Women during breastfeeding period (n=104) provided urine samples from the 2nd to the 8th week after delivery as part of the project (BETTERMILK) implemented in Valencia (Spain) during 2015.

Sample treatment included an enzymatic hydrolysis followed by ultracentrifugation and injection of the supernatant in a LC-MS/MS(QqQ) system for the analysis of 14 urinary phthalate metabolites.

In order to evaluate the risk assessment, urinary levels were compared with the biomonitoring equivalent (BE) guidance values present in the literature

Nine phthalate monoesters presented detection frequencies higher than 80%. Levels of phthalate metabolites ranged from <LoQ to 1291 ng/mL, being MEP the metabolite which showed the highest levels (geometric mean (GM) = 34.9 ng/mL). None of the phthalates at the 95th percentile level presented concentrations higher than their BE.

The study shows that the participating women exposure to phthalates is below the BE guidance values.

**New methods**

**DEVELOPMENT AND APPLICATION OF NON-TARGETED APPROACHES FOR CHARACTERIZING HUMAN INTERNAL EXPOSURE TO HALOGENATED CHEMICALS OF CONCERN.**

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Since centuries, humans are exposed to chemicals present everywhere in our environment (food, water, air, dust, etc.). This wide range of contaminants still increases and interferes with the environmental-food-human continuum. In order to keep an eye on this constant evolution and to act as early warning support to policy, the HBM4EU-WP16 “emerging chemicals” aims to establish a workflow able to produce a general overview of chemicals exposure. This approach based on large screening method, suspect/non-targeted screening (NTS), is still at an early stage of development in the human biomonitoring field.

The non-targeted approach, in combination with latest and future generations of instruments, opens the door to holistic characterisation of biological samples. In order to make this work of large screening feasible, we refocused our research on halogenated contaminants which are already known as persistent, toxic and bio-accumulative. The lipophilic character of those compounds supposes to expect them in lipophilic tissue and/or in storage or excretion compartments, such as adipose tissue, breast milk, placenta, meconium.

In this context and in order to partly characterise the early stage of life exposure, we developed an appropriate suspect/NTS workflow able to detect a large range of compounds, including chemicals of emerging concern (CEC), in human breast milk. Non-targeted sample preparation and instrumental method were optimised. The data processing was focused on halogenated compounds thanks to isotopic pattern and mass defect of chlorinated and brominated molecules by developing HaloSeeker application (Léon, 2019). Based on this suspect/non-targeted methodology and as a first proof of concept a pesticide’s metabolite, the 4-hydroxy-chlorothalonil, was identified in breast milk with LC-HESI-HRMS (Q-Orbitrap) analysis. The present work describes challenges, recent promising results and future expectations of this method.

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