HORIZON2020 Programme
Contract No. 733032 HBM4EU

Granada Workshop WP14 and WP13: Effect biomarkers & establishing exposure and health relationships

Additional Deliverable AD 14.2

WP14 - Effect Biomarkers

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UNIVERSIDAD DE GRANADA

1 Abstract/Summary

Background: Work Package 14 (WP14) “Effect Biomarkers” general aims can be summarised as follows:

i) conduct literature searches on effect biomarkers relevant for HBM, so an inventory of effect biomarkers in relation to both the most relevant adverse outcomes and specific environmental exposures can be created;

ii) identify gaps in knowledge related to effect biomarkers;

iii) implement and/or research on novel or understudied effect biomarkers that can address those data gaps and provide an added value to HBM;

iv) contribute to the understanding of exposure-health relationships through effect biomarkers.

Objective: UGR, as leader of WP14, has hosted the first ‘’Granada Workshop’’ with the aim to present preliminary results and discuss important issues related to Task 14.2 recorded in D14.2 (List of effect biomarkers for the 1st list of prioritised substances) and D14.4 (Report on results of the selected biomarkers of effect [Proof of concept]). Additionally, UGR agreed to also host a WP13 workshop during the same days with the aim to plan the interaction between WP14 and WP13 ‘’Establishing exposure and health relationships’’. The present deliverable (AD14.2) aims to provide a summary of the workshop, presenting the state of development of all the tasks and interactions conducted in WP14.

Results: Preliminary results regarding the different literature searches on effect biomarkers conducted were discussed during the workshop, setting the basis for deliverable 14.2 (D14.2). Analogously, preliminary results on the “Proof of concept” regarding biomarkers of combined effect to chemical mixtures were also presented and discussed, setting the basis for deliverable 14.4 (D14.4). A training in Adverse Outcomes Pathways (AOPs) was also carried out, fundamental for the interaction between WP14 and WP13, which aim is to support the knowledge from effect biomarkers used in Human Biomonitoring (HBM) with the available experimental knowledge for each chemical family of the first set of prioritised substances. Moreover, a brief update on the recruitment and characteristics of HBM4EU aligned studies was presented, so future proposals for the implementation of effect biomarkers in HBM4EU can be made not only based on available HBM effect biomarkers and experimental knowledge, but also taking into account the specific chemical families that will be measured in each window of development (children, adolescents and/or adults), and the biological matrices available.

Conclusions: The “Granada Workshop” represented an important step forward for the consolidation of both WP14 and WP13 objectives, and all the attending participants realised the crucial relevance of person-to-person interactions in order to pursue specific goals inside such a big project like HBM4EU. Since many complex transversal actions are being conducted within WP14, this document will help to clarify how all WP14 objectives and deliverables are interdependently arranged to accomplish their function inside HBM4EU.

2 Workshop general information

Target group: The workshop is dedicated to everyone interested in

- Discussing preliminary results from D14.2: The literature search.
- Discussing preliminary results of D14.4: The proof of concept.
- Planning of future interactions between WP14 and WP13.

Registration: Mid-January 2018 – end of February 2018

Funding of travels and accommodation: Participants themselves will cover their travel and accommodation costs.

Capacity: Maximum 60 participants. Priority was given to partners of Work Package 14 if the number of participants exceeds 60 persons.

Location, agenda and attending participants

'Centro de Investigación Biomédica (CIBM)'. This research center is located within the '*Parque Tecnológico de la Salud'* in front of the University Hospital and the School of Medicine.

Address: Av. del Conocimiento, s/n
Phone: +34 958 24 10 00
Location: 18100 Granada, Spain

Figure 1: Location of the Centre of Biomedical Research ''Centro de Investigación Biomédica (CIBM)''.

HBM4EU. Granada Workshop 2018

Hour	Monday 19		Tuesday 20		Wednesday 21	
	WP14	WP13	WP14	WP13	WP14	WP13
9:00-10:00			D14.2 Summary and General Discussion	Task 13.1 AOPs	D14.4 Summary and General discussion	Task 13.2 Cohorts (continued)
10:00-11:00						
11:00-11:30			Coffee Break		Coffee Break	
11:30-12:30	Partners Arrival		D14.4 Proof of Concept	Task 13.2 Cohort	Summary and Future directions (WP13-WP14 together)	
12:30-13:30						
13:30			Lunch		Workshop Closing	
14:15-14:45	Registration-Coffee					
14:45-15:15	**Welcome:** Biomarkers of effect by WP14 Nicolas Olea and Vicente Mustieles		Coffee			
15:15-16:00	**D14.2:** Literature Search: **Bisphenols**	**WP13 Get-together** Introduction, Summary of outcomes so far	**Interaction WP14-WP13** Initial Training on AOPs by **Mirjam Luijten** **Ludek Blaha** **Vicente Mustieles**			
16:00-16:30	**D14.2**: Literature Search: **Pthalathes**					
16:30-17:00	Coffee Break					
17:00-17:45	**D14.2:** Literature Search: **PFAS-PFOAS**	**WP13 Round table discussion** Cooperation and linking of tasks 13.1&13.2				
17:45-18:15	**D14.2**: Literature Search: **FRs**					
18:15-18:45	**D14.2**: Literature Search: **PAHs**		**Round Table and Discussion**			
18:45-19:15	**D14.2**: Literature Search: **Cadmium**					
19:15			**Join-Together**			

DAY 1		
14:00-14:45	**Partners Arrival-Registration-Coffee**	**HALL**
14:45-15:00	**Welcome Speech:** Dr. Nicolas Olea	***Room 1***
15:00-16:00	**Introduction:** Effect Biomarkers by Vicente Mustieles (15') Bisphenol group will inform about their current state and preliminary results. UGR-CNRS-NIPH-INSERM ***Moderator (Vicente Mustieles)-Secretary (Andrea Rodriguez)***	***Room3***
16:00-16:30	Phthalates group will inform about their current state and preliminary results. VITO and UCY ***Moderator (Greet Schoeters)- Secretary (Nathalie Lambrechts)***	***Room 3***
16:30-17:00	**Coffee Break**	**HALL**
17:00-17:45	PFAS-PFOA group will inform about their current state and preliminary results. AU-RIVM-VITO-DTU ***Moderator (Eva Cecilie Bonefeld-Jorgensen)- Secretary (Vicente Mustieles)***	***Room 3***
17:45-18:15	FRs group will inform about their current state and preliminary results MU-EASP ***Moderator (Ludek Blaha/Zuzana Novakova)- Secretary (Beatriz González)***	***Room 3***
18:15-18:45	PAHs group will inform about their current state and preliminary results NRCWE-BfR-UCY ***Moderator (Anne Thoustrup)- Secretary (Christiana Neophytou)***	***Room 3***
18:45-19:15	Cadmium group will inform about their current state and preliminary results BfR-UH-MUW ***Moderator (Alfonso Lampen)- Secretary (Stephan Couderq)***	***Room 3***

DAY 2		
9:00-9:30	**D14.2-** Summary and General Discussion regarding the Literature Search	***Room 3***
9:30-10:00		
10:00-10:30		
10:30-11:00		
11:00-11:30	**Coffee Break**	**HALL**
11:30-12:00	**D14.4.-** UGR; AU: Preliminary results from the Proof of Concept	***Room 3***
12:00-12:30	**D14.4.-** CNRS; CEA: Preliminary results from the Proof of Concept	***Room 3***
12:30-13:00	**D14.4.-** UH; MU: Preliminary results from the Proof of Concept	***Room 3***
13:00-13:30	**D14.4.-** DTU; INSERM: Preliminary results from the Proof of Concept	***Room 3***
13:30-13:45	**Large Group Photo**	
13:45-14:45	**LUNCH: Menu from University Canteen 3.5€ (normal and vegetarian option)**	
14:45-15:15	**Coffee**	**HALL**
15:15	**Interaction WP14-WP13:**	
17:00	Training on AOPs by Mirjam Luijten Ludek Blaha Vicente Mustieles	
18:00		
19:00	**Round Table and Discussion**	***Room 1***
19:00-19:30	**Join-Together**	**HALL**
19:30-20:00		
20:00-20:30		

DAY 3		
9:00-10:00	D14.4 Summary and General discussion regarding the Proof of Concept	*Room 3*
10:00-11:00		
11.00-11.30	**Coffee Break**	**HALL**
11.30-12.30	Summary and Future directions (WP13-WP14 together)	*Room 1*
12.30-13.30		
	Workshop Closing	*Room 1*

HBM4EU-Granada Workshop 2018 Participants					
	WP13 (MU)	**WP14 (UGR)**	**WP16 (INRA)**	**Total Partners** (without overlapping)	**Confirmation**
Partners: **Attending** **Not Attending**	AUTH EAA FIOH FMUL IEM IMROH INRS INSA **INSERM** ISGlobal **MU** MUW NKUA **NIPH** RegionH RIKILT **RIVM** RSU SDU SZU Umweltbundesamt GmbH UNIMORE **UGR** **UH** UI **VITO**	AU BfR CNRS DTU EASP **INSERM** IRAS MUW **MU** NCRWE **NIPH** **RIVM** UCY **UGR** **UH** **VITO**	INRA	AU (1)	Eva Cecilie Bonefeld-Jorgensen
				AUTH (1)	Spyros Karakitsios
				BfR (1)	Alfonso Lampen
				CEA	
				CIM (1)	Eiva Bernotiene
				CNRS (2)	Jean-Baptiste Fini Stephan Couderq
				DTU (2)	Anne Marie Vinggaard Hanna Johansson
				EASP (3)	Marina Lacasaña Beatriz González-Alzaga Antonio Hernández
				EPIUD (1)	Flabio Barbone
				FIOH (1)	Pasi Huuskonen
				FMUL (1)	Joana Costa
				IEM	
				IMROH	
				INRA	
				INRS (1)	Sophie Ndaw
				INSA (1)	Henriqueta Louro
				INSERM (2)	Arthur David Shereen Cynthia
				ISGlobal	
				ISCIII	
				ISS (2)	Alessandro Alimonti Beatrice Bocca
				LSMU (1)	Loreta Strumylaitė
				MU (2)	Ludek Blaha Zuzana Novakova
				MUW (1)	Claudia Gundacker
				NCRWE (1)	Anne Thoustrup
				NIPH (2)	Inger-Lise Karin Steffensen Tim Hofer

				NKUA	
				RegionH (1)	Anna-Maria Andersson
				RIKILT (1)	Ad Peijnenburg
				RIVM (2)	Aldert Piersma Stella Fragki
				RSU (1)	Žanna Martinsone
				SDU (1)	Tina Kold-Jensen
				SZU (1)	L´ubica Murínová
				UoA (1)	Georgios Baltatzis
				Umweltbundesamt GmbH (1)	Maria Uhl
				UCY (1)	Christiana Neophytou
				UNIMORE (2)	Marco Vinceti; Tommaso Filipini
				UGR (15)	Nicolás Olea Marieta Fernández Vicente Mustieles Andrea Rodríguez Juan Pedro Arrebola Fernando Vela Soria José Manuel Molina Carmen Freire Francisco Artacho Inmaculada Jiménez Luz Maria Iribarne Iris Reina Francisco Peinado Alicia Olivas Raquel Quesada
				UH (1)	Tim Nawrot
				UI (1)	Thorhallur I Halldorsson
				VITO (3)	Greet Schoeters Nathalie Lambrench Sylvie Remy
Total	59				

3 Introduction

The 5-year strategy of WP14 "Effect Biomarkers" can be summarised in the following points: i) to conduct literature searches on effect biomarkers relevant for HBM, so an inventory of effect biomarkers in relation to both the most relevant adverse outcomes and specific environmental exposures can be created; ii) to identify gaps in knowledge related to effect biomarkers; iii) to implement and/or research on novel or understudied effect biomarkers that can address those data gaps and provide an added value to HBM; iv) to contribute to the understanding of exposure-health relationships through effect biomarkers.

The first 2-years plan for WP14 includes: i) literature searches on the first list of prioritised substances (D14.1 and D14.2); ii) discussion of preliminary results and identification of gaps in knowledge (AD14.2 "Granada Workshop", the present deliverable); iii) implementation and/or research on novel or understudied effect biomarkers (D14.4 "Proof of concept", research on biomarkers of combined effect to chemical mixtures); iv) contribution to the understanding of exposure-health relationships (AD14.2 "Granada Workshop", the present deliverable, through the interaction of WP14 with WP13, joining both the experimental knowledge from Adverse Outcome Pathways (AOPs) and from effect biomarkers used in HBM).

Biological markers or biomarkers have been traditionally classified in 3 groups: biomarkers of exposure, biomarkers of effect, and biomarkers of susceptibility. During the Granada Workshop, it was agreed that, for Human Biomonitoring (HBM) purposes, we will follow the World Health Organization definitions:

- **Biomarker of exposure**: The chemical or its metabolite or the product of an interaction between a chemical and some target molecule or cell that is measured in a compartment in an organism.
- **Biomarker of effect**: A measurable biochemical, physiologic, behavioral, or other alteration in an organism that, depending on the magnitude, can be recognised as associated with an established or possible health impairment or disease.
- In addition, and based on the preliminary results from the literature searches, another categorisation within effect biomarkers was needed: i) Classical or traditional effect biomarkers that have been widely studied for a particular chemical family, ii) Classical but understudied effect biomarkers, and iii) Novel effect biomarkers.
- **Biomarker of susceptibility**. An indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific chemical substance.

World Health Organization & International Program on Chemical Safety (2001). Biomarkers in risk assessment: validity and validation. Geneva: World Health Organization.

The importance of using effect biomarkers in conjunction with exposure biomarkers was highlighted based on previous Human Biomonitoring Initiatives and Committees.

"The ultimate objective of the biomonitoring research is to link biomarkers of exposure to biomarkers of effect and susceptibility to understand the public-health implications of exposure to environmental chemicals.'' (*Human Biomonitoring for Environmental Chemicals. Committee on Human Biomonitoring for Environmental Toxicants. National Research Council, Washington DC, 2006*)

4 Literature search (Deliverable D14.2)

One of the main objectives of WP14 is to create an inventory of effect biomarkers relevant for HBM and for each chemical family under study in HBM4EU. To pursue this, a series of wide and comprehensive literature searches were conducted for each chemical family of the first set of prioritised substances. Different groups of WP14 partners were in charge of the literature search for each chemical family, based on their previous expertise and knowledge. A common methodology was shared among WP14 partners, specifying exposure search terms and search terms for the health endpoints of highest interest, as previously planned in Deliverable 14.1 "Criteria for prioritisation of biomarkers of effect". Thousands of references were gathered, selected and analysed. The results of this task will be extensively reported in Deliverable 14.2 "Literature search on effect biomarkers for the first list of prioritised substances".

Although a brief summary can be found below for each chemical family, the most important slides presented during the workshop by each team can be checked at the end of this deliverable in the Annex section.

4.1 Bisphenols (coordinated by UGR)

- **UGR**-Biochemical-Physiological effect biomarkers (Presented by Vicente Mustieles): A brief description of the methodology followed by the implementation of the literature search fully described in the D14.2 "List of effect biomarkers for the 1st list of prioritised substances".
- Effect biomarkers discussed: Kisspeptin, blood pressure (children), cortisol and adrenal androgens, renal function: Albumin to creatinine ratio in children and low- grade albuminuria in adults. Inflammatory markers.
- Identification of an important gap in knowledge for biochemical/physiological effect biomarkers for Neurodevelopment.
- **CNRS**: Thyroid effect biomarkers (presented by Stephan Couderq): serum TPO, TSH, FT4, FT3, FT4 as well as Thyroid volume. Potential effect biomarkers related to metabolic disease, pro-inflammatory state: leptin, TNF-alpha, master genes of adipogenesis, macrophage related genes.

  The neurotrophins **BDNF** and **GDNF** were discussed as important potential effect biomarkers for neurodevelopment health.

  Glial cell-line derived neurotrophic factor GDNF is implicated in the maintenance of neurons through glial cells. Zinc finger transcription factor Sp4 is important for energy generation and consumption in neurons, and could be a target for BPA based on a rodent study. Placenta potential biomarkers of effect: CRH; Homemobox gene Hoxa10 was proposed as a biomarker of effect for uterine organogenesis.
- **INSERM**: OMICS-Epigenetics effect biomarkers (presented by Shereen Cynthia): Brief introduction about DNA methylation. LINE1 hypomethylation in placenta, in the saliva of prepuberscent girls (HOXA 10, BRCA1 and BEX2, hypomethylation among women with IVF, promoter CpG). **BNDF IV DNA methylation** at two CpG levels (GpG1A, CpG1B). Sperm DNA Hydroxymethylation. Increased DNA Methylation ESR1, IL-6st, STAT3. Micro-RNA expression. Gene expression studies: Kisspeptin **(KISS1)** gene expression in placental samples. BPA was also associated with gene expression of some nuclear estrogen receptors in blood. Hormones assay: male reproductive hormones E2, T, INHB, SHBG.

- **NIPH**: Oxidative Stress (presented by Tim Hofer): Brief introduction about Oxidative Stress. Urinary 8-OHdG and 8-isoprostane discussed as potential biomarkers of effect.

4.2 Phthalates (coordinated by VITO)

- **VITO**: (Presented by Nathalie Lambrechts). Among others, they presented: Genetic polymorphisms; Metabolic effect biomarkers and Metabolomics; Associations between phthalates and Kisspeptin. Era, ERb, PPAg, PXR…etc. No novel effect biomarkers were found for cardiovascular disease. Gene expression of several nuclear receptors were proposed as candidates for biomarkers of effect (ERα, ERβ, PPARγ, PXR, etc.).
- **UCY**: Effect biomarkers related to cancer (presented by Christiana Neophytou): Detection of polymorphisms of ESR CYP17A1. Glutation-N transferase M1 enzyme.

4.3 PFAS-PFOA (coordinated by AU)

- **RIVM**: Molecular and genetic testing effect biomarkers (presented by Stella Fragki). Associations between PFAS and gen expression of cholesterol metabolism in peripheral blood samples, and altered gene expression of genes related to reproduction. Some effect biomarkers related to liver function could be relevant for PFAS-PFOA. Possible effects on the immune system were also discussed.
- **AU:** Ex vivo cell-based and biochemical/physiological epidemiological effect biomarkers (presented by Eva Cecilie Bonefeld-Jorgensen): combined serum xenoestrogenic activity related to reproductive outcome and cancer. **Gap** in epidemiological studies: knowledge on E1 and E3, related to metabolism of endogenous estrogens, for thyroid hormones no studies on 3,3-T2; 3,5-T2 and rT3. Association between serum PFASs and urinary levels of Clara Cell Protein.16 (CC16). Few studies on vitamins but a single for weak association between PFASs and 25-OH vitamin D. GAP; no studies on IgD.
- **VITO:** Oxidative stress (Presented by Nathalie Lambrechts): Leucocyte telomere length; DNA methylation in leukocytes; LINE-1 DNA methylation in umbilical cord blood.
- **DTU** (Anne Marie Vinggaard and Hanna Johansson): The animal experimental literature was searched in order to find novel molecular and biochemical targets that could be investigated as possible effect biomarkers.

4.4 Flame Retardants (coordinated by MU and EASP)

- **MU**: Brominated flame retardants (presented by Zuzana Novakova). The methodology and exploratory search was presented. Main issue found: the huge amount of references related to the exposure. After elimination of non-relevant papers, they found 170 papers related to human studies and 210 to animals studies. No effect biomarkers were proposed since the search was not finished.
- **EASP**: Organophosphate flame retardants (presented by Antonio Hernández). Less articles than expected at first: 642 references. After screening, they kept 23 human studies (in vivo: 11; in vitro: 12) and 113 animals studies.

  Experimental studies: most of them related to neurotoxicity (in vivo/in vitro). In addition, reproductive and genotoxicity had a good number of references.

  Human epidemiologic studies: Again, neurotoxicity and genotoxicity got the highest number of references, secondly: endocrine reproductive.

Effect biomarkers found: Sexual and thyroid hormones: (FT4, FT3, TSH). Oocyte, embryo quality, implantation and pregnancy outcomes. Oxidative stress (8-OHdG). Cardiovascular function: SM, Cer, Sph and S1Pm molecular lipids measured in blood, even when they are non-specific, could provide some mechanistic information. Neurodevelopmental tests. Some gaps in knowledge were found for neurodevelopment. Novel information was obtained among in vitro studies.

4.5 PAHs (coordinated by NCRWE)

- **NCRWE:** The methodology and exploratory search was shown (presented by Anne Thoustrup). 16 compounds from EPA and 8 compounds from ECHA were selected to perform the literature search. Main issue found: the amount of references related to the exposure was impossible to approach (>45.000 references after using all the filters). They chose to perform a secondary strategy: to focus only on reviews (1305 references) among 3 partners.
- Effect biomarkers proposed: Chromosomal aberrations, DNA damage 8-OHdG. Specific mutations in TP53-dG. The IARC monography on PAHs was proposed as a good source of information for both, effect and exposure biomarkers for CANCER outcomes.
- **UCY**: Animal studies as means to determine some effect biomarkers (presented by Christiana Neophytou). Some interesting molecular pathways were highlighted and discussed.
- **BfR**: DNA damage effect biomarkers (presented by Alfonso Lampen). 10 potentially relevant papers. DNA damage, nuclear receptors and Plasma lipid damage as potential effect biomarker.

4.6 Cadmium (coordinated by BfR)

- **BfR**: Oxidative stress effect biomarkers (presented by Alfonso Lampen). Associations between Cadmium and oxidative stress (8-OHdG) have been described. Moreover, one hypothesis is that Cadmium could cause early tubular damage through the induction of oxidative stress at the kidneys. The beta-2- microglobulin and N-acetyl-beta-D glucosaminidase (NAG) were proposed as early effect biomarkers of tubular damage useful for HBM purposes.
- **MUW**: Effect biomarkers of renal function (presented by Claudia Gundacker): Kidney damage as health outcome for effect biomarkers proposal. β_2-MG (Beta-2- microglobulin) and NAG (N-acetyl-beta-D glucosaminidase) proposed as early markers of tubular dysfunction. Sex-differences. Need to learn more about the relationship with LDH release, Zinc, Transferrin…

4.7 Summary of the literature search

Effect biomarkers presented during WP14 workshop can be divided in three broad categories:

- **Classical and studied effect biomarkers:** Reproductive hormones, Thyroid hormones; Glucose metabolism, Serum lipids (total cholesterol, LDL, HDL, TG), Blood pressure, anthropometric measures, etc.

  These effect biomarkers are known to be predictive of different hormonal and metabolic diseases, and for that reason are considered “classical” or “traditional” effect biomarkers. Additionally, since we found that for most of the chemical families, these biomarkers were previously studied, we considered them to be “Studied”.

- **Classical but less studied effect biomarkers:** HPAdrenal-Axis; Adipokines; Inflammatory markers; Liver enzymes; Renal function; Others (IgE, vitamin D…). Urinary 8-OHdG and 8-isoprostane.

  Although these markers are considered “classical” or “traditional”, their use was not

so common for most of the chemical families under study. In general, perhaps one or two studies evaluated associations between exposure to one of the chemical families included in the first set of prioritised substances and one of these markers, but more studies are needed to reach conclusions. For that reason these markers, although classical, were classified as “less studied”.

- **Novel effect biomarkers:** Kisspeptin, Gene expression of nuclear receptors: ERα, ERβ, AR, PPARγ, AhR, TR, GR, Genes of cholesterol pathways. BDNF and GDNF. DNA methylation and other Omics/epigenetics markers.

  These effect biomarkers are promising effect biomarkers that need to be further studied and that can provide an added value to HBM and help to fill data gap in relation to effect biomarkers of relevance for HBM.

Table 1: Summary list of preliminary effect biomarkers that could be implemented in HBM studies (Please, note that this list contains preliminary information that will be completed in Deliverable D14.2)

Classical (and studied) effect biomarkers	Classical (less studied) effect biomarkers	Novel
Reproductive hormones: LH, FSH, TT, E2, SHBG	HPAdrenal-Axis: CRH-ACTH-Cortisol + Adrenal Androgens (DEAH-S)	Kisspeptin
Thyroid Hormones: TSH, T3, T4	Adipokines: Leptin and Adiponectin	Gene expression of nuclear receptors: ERα, ERβ, AR, PPAR-γ, AhR, TR, GR, Genes of colesterol pathways
Glucose metabolism: [FBG+Insulin= HOMA-IR] + HbA1c	Inflammatory markers: hsCRP, IL-6…	BDNF, GDNF, Sp4
Serum lipids: Total cholesterol, LDL, HDL, TG	Liver enzymes; AST, ALT, GGT, AF	OMICs-Epigenetic markers: DNA methylation and micro RNAs, among others
Blood pressure	Renal function: Urinary albumin, β2-microgobulin, NAG	Biomarkers of combined effect for chemicals mixtures: E-screen
Anthropometric measurements: Anogenital distance (AGD); Waist circumference: Height/Weight; Pertentage of Body Fat; Skinfold-thickness; Birth weight, Birth length	Urinary 8-OHdG and 8-isoprostane	Genetic polymorphisms: CYP17A1, ESR CYP17A1
Chromosomal aberrations and micronuclei (MN) assay	Others: IgE, vitamin D (25-OH-D)	

4.7.1 Traditional/classical and studied effect biomarkers

Regarding classic and studied effect biomarkers, mainly hormonal and metabolic pathways, it is very important to take into account the complete hormonal or metabolic cascade, that is, to follow the whole pathway, in order to provide the most complete picture and interpretation possible.

The entire literature search has highlighted the **role of reproductive, thyroid and adrenal hormones, as well as metabolic parameters** such as serum lipids, glucose and insulin as key effect biomarkers for most chemical families studied. These markers are commonly and routinely analysed in different laboratories and their cost is very affordable. Moreover, there are no big concerns about the validity and reliability of these measurements. **Therefore, these classical and studied effect biomarkers in relation to both adverse outcomes and specific environmental exposures should be regarded as minimum basis**, **as has been done in other HBM initiatives, including the National Health and Nutrition Examination Survey (NHANES).**

4.7.2 Novel or less studied effect biomarkers of interest

- **BNDF**. Proposed as a novel effect biomarker to cover the gap in knowledge for neurodevelopment.
- **Gene expression of nuclear receptors in peripheral blood**. Gene expression of nuclear receptors measured in cell populations in serum could be used to provide novel information about the mechanistic understanding of environmental chemicals: ERα, ERβ, AR, PPARγ, AhR, TR, GR, and genes of cholesterol pathways among others.
- **Kisspeptin**. Proposed a novel effect biomarker for studying reproductive health both during pregnancy and puberty.
- **Urinary 8-OHdG and 8-isoprostane.** Proposed as biomarkers of DNA damage and lipid peroxidation. Although not specific, it seems these markers are related to all the chemical families studied. Further work is warranted to better understand how so many different chemical families could converge to generate oxidative stress, and if this could be related to detoxification pathways, mainly in the liver and kidneys.
- **Omics/epigenetic markers.** Several chemical families were associated with Omics or epigenetic markers including DNA methylation and micro-RNAs among others. This field warrants further investigation. Although some specific markers could be of interest, one concern could be the technical and economic feasibility of these biomarkers .
- **Biomarkers of combined effect to chemical mixtures.** There are several ex vivo cell-based effect biomarkers implemented in the epidemiologic literature, including the estrogenic screen (E-Screen) and the Anti-androgenic screen (A-Screen). These and other biomarkers of combined effect to chemical mixtures will be extensively studied in the Proof of concept (D14.4).
- **Vitamin D.** Although less studied, several associations between chemical families and vitamin D have been observed. The study of vitamin D and other vitamins and markers of nutritional status will be useful to address the area of nutritional-chemical interactions.

4.7.3 Knowledge gaps

- Biochemical/physiological effect biomarkers for **Neurodevelopment** and neurological development.
- Biomarkers of combined effect to **chemical mixtures**.
- Interaction between markers of **nutritional status** and **exposure** to environmental contaminants.
- There is a need to understand how different chemical families could converge to generate **oxidative stress**, and whether this is mainly related to detoxification pathways

in the liver and kidneys.

- Most of the effect biomarkers studied are specific to physiological functions/diseases, but not to chemical families. However, some omics or epigenetic effect biomarkers such as DNA methylation and expression of nuclear receptor genes, could be more specific to chemical families, which warrants further study. Priority will be given to those effect biomarkers that can better predict future diseases, and for which a link between a given exposure and that specific biomarker or disease is substantiated based on the experimental literature.
- Gap in knowledge for biomarkers of effect related to PFAS exposure and **immune system** was pointed out.
- In general, few effect biomarkers for cancer endpoints were found.
- In general, few effect biomarkers for cancer endpoints were found.

5 Proof of Concept

A category for “mixtures” were included among the first set of prioritised chemical substances. In relation to effect biomarkers, there is a clear data gap when it comes to the assessment of the effect of complex and real-life chemical mixtures. Among the different approaches for studying the effect of mixtures, the use cell-based bioassays, especially estrogenic and anti-androgenic bioassays, have produced very promising results, even in epidemiologic studies. Therefore, the aim of Deliverable 14.4 is to develop a pilot study in order to analyse the combined effect of chemical mixtures extracted from human biological matrices. For this purpose, chemical extracts from human samples, representing real-life chemical mixtures, are being tested in different bioassays to evaluate the estrogenic, anti-androgenic, anti-thyroid, aryl-hydrocarbon receptor (AhR), and steroid activities of these extracts, among others screens and biological markers. For the present pilot, we chose placental samples as the biological matrix of election due to the high availability of sample quantity, which allowed us to share the same samples with other WP14 partners. However, in the future other matrices should be tested such as serum. Therefore, chemical extracts obtained from 25 placental samples were extracted by UGR and sent to all the partners involved in D14.4. While preliminary results were discussed during the Granada Workshop, results will be extensively reported in D14.4.

UGR (presented by Vicente Mustieles):

Included in Task 14.3 (identification of needs for the development of new biomarkers of effects and decision criteria for their validation), WP14 partners discussed the proof of concept (D14.4 ‘’Report on results of the selected biomarkers of effect’’) started in year one. Special attention has been paid to both ex-vivo cell based and in vivo functional assays that have the potential to elucidate the combined effect of chemicals. As agreed, WP14 partner explored the use of new biomarkers of effect in human placenta, in order to implement the methodology in ongoing European cohort studies. In fact, agreement with coordinators of relevant European mother-child cohorts was obtained, and technical and ethical issues from the Spanish INMA-cohort were solved.

The scheme below summarises the distribution of tasks (samples and bioassays):

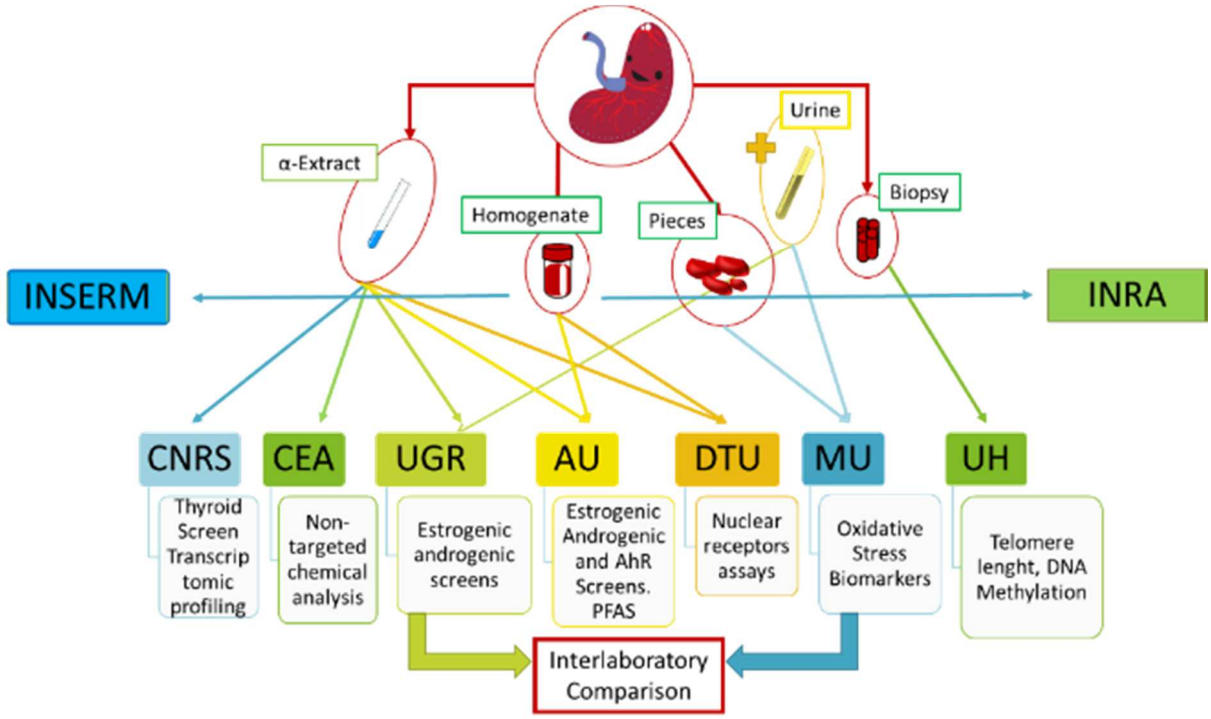


Figure 6: Placental samples obtained from 25 placentas from INMA birth cohort: From the same placentas, 5 alpha extracts, 5 homogenates, 5 pieces and 4 biopsies were obtained. In addition, urine aliquots were obtained from the same individuals that donated the placentas. Finally, a brief description of the bioassays and biomarkers developed by each partner is shown

UGR (presented by Fernando Vela Soria): Explanation of the chemical extraction process of alpha fractions conducted by UGR. In addition, it was contemplated the future possibility of testing the hormonal activity within beta fractions.

AU (presented by Eva Cecilie Bonefeld-Jorgensen): AU will assess **estrogenic and anti-androgenic** effects of alpha-fractions derived from the placentas. AU has a lot of experience implementing these biomarkers of combined effect in epidemiologic studies. Methods and results will be included in D14.4.

INSERM (presented by Arthur David): INSERM will **test gene expression, epigenetic and omics biomarkers** in the 25 placenta samples. Presentation of several potential experimental techniques that could be employed for the **development of effect biomarkers** and exposure: i) Untargeted metabolomics using high resolution mass spectrometry, ii) Gene expression analyses, iii) OMICS approach, iv) Gene sequencing, v) Western blot to assess proteins expressed in the placenta, vi) DNA damage, vii) Micro-RNAs expression in placentas, viii) Analyses of xenoestrogenic potential of placental extracts using transgenic zebrafish with Aromatase enzymatic activity in placenta; ix) Analysis of some metals (As, Cd, Cu, Mn, Pb, Ms, Se, Zn), to asses preliminary trends, x) Preliminary test specifically for metabolomics testing the sensitivity of their bioassay, xi) Annotation of the placenta metabolomics and Identification of xenobiotics that will lead to the identification of prenatal exposure.

CNRS (presented by Jean-Baptiste Fini): CNRS will assess **thyroid hormone disruption exerted** by mixtures of compounds present in alpha-fractions from the placentas using the Xenopus Laevis assay. CNRS has a lot of experience working with this bioassay and different chemical families.

CNRS will test the effect of alpha fractions containing chemical mixtures from the placentas on the gene expression (BDNF, GDNF), behavior, and brain structure of Xenopus Laevis.

UH (presented by Tim Nawrot): Telomere length is a potential biomarker of biological age that can be influenced by environmental contaminants and can be easily measured in leukocytes. This biomarker is an important predictor of adult lifespan and has been used as a predictor of mortality.

P53 and mitochondria function are key factors for mitochondria damage and ageing, which constitutes the so called ''Molecular core axis for Ageing''. It is known that air pollution influences this core axis for ageing, moreover, air pollution is an important predictor of telomere length at birth. In addition, children from obese mothers had 5% shorter telomeres. As a result, telomere length is a potential biomarker for children's developmental health.

UH presented preliminary results of telomere length measured in placenta biopsies. These results are fully described in D14.4.

UGR (presented by Jose Manuel Molina): Preliminary results from UGR team. Hormonal activity in the alpha extracts from placenta samples was assessed by UGR team for the proof of concept. Two different bioassays were tested:

* Proliferation based analysis: **E-screen**. Total Xenoestrogen Burden (TEXB) to estimate the total xenoestrogenic activity of the placenta samples. Calculation of the estradiol equivalents.

Main finding: 100% of alpha fractions tested showed evidence of xenoestrogenic activity in all the placenta samples, at varying degrees.

***** Luciferase reported gene assay: Androgen receptor within PALM cells. No agonistic neither antagonistic activities were found.

MU (presented by Zuzana Novakova): They will test oxidative stress effect biomarkers in the placentas: MDA, GSH, GSSG, 8-OHdG, Total 8-isoprostane E2 and F2a Interlaboratory comparison. Brief literature searches about biomarkers implemented in placenta and urine.

DTU (presented by Anne Marie Vinggaard): DTU has a lot of experience using different bioassays of endocrine activity. They will test the effect of alpha-fractions derived from placentas on the Aryl Hydrocarbon receptor expression (AhR) assay.

5.1 Summary of the Proof of Concept

Several partners involved in the Deliverable D14.4 presented a brief presentation about their developed bioassay and the effect biomarker they will focus on. Future results obtained from this proof of concept will determine not just effect biomarkers, but also their mechanism of action, which will play a key role in the interaction with WP13, thus increasing their evidence when linking environmental contaminants exposure and adverse effects on human health.

Further and more detailed content on the proof of concept will be covered in the Deliverable D14.4.

6 Interaction between WP14-WP13

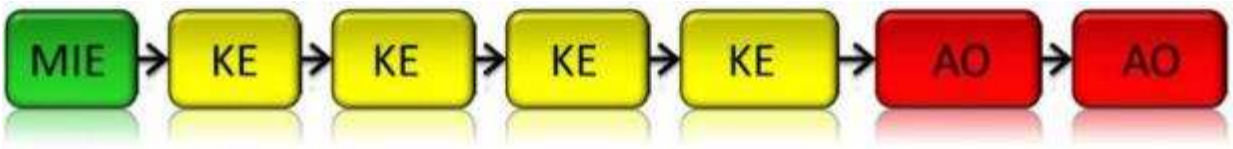
6.1 Training in AOPs, by Mirjam Luijten (RIVM)

During the first Granada workshop, an interaction between work packages 14 and 13 was made. One session was a training on adverse outcome pathways (AOPs) by Mirjam Luijten (RIVM), which could be useful to identify and select relevant biomarkers of effect for HBM purposes.

An Adverse Outcome Pathway (AOP) is ''a conceptual framework that portrays existing knowledge concerning the linkage between a direct molecular initiating event and an adverse outcome, at a level of biological organisation relevant to risk assessment'' (Ankley et al. 2010, Environ. Toxicol. Chem., 29(3): 730-741). A better understanding of AOPs, could help WP13 and WP14 to use the information gathered to **support risk-based decision-making.**

Building an AOP: The main structure of an adverse outcome pathway is the following:

- Molecular initiating event (MIE) – A specialised type of KE that represents the initial point of chemical interaction, on the molecular level, within an organism, that results in a perturbation that starts the AOP.
- Adverse Outcome (AO) – A specialised type of KE that is generally accepted as being of regulatory significance on the basis of correspondence to an established protection goal or equivalence to an apical endpoint in an accepted regulatory guideline toxicity test.



At the end of the training, we could summarise AOPs in the following five points:

(1) **AOPs are not specific**, they do not try to describe what a single chemical does, but try to describe what any chemical is able to perturb from the MIE, with sufficient potency and duration to produce a Biological reason of failure. Thus, describing AOP does not require chemical-specific information, just to apply those motifs in a predictive context, which requires understanding chemical-specific properties (e.g., potency, ADME) that dictate the magnitude and duration of perturbation at the MIE.

(2) **AOPs are modular:** Between two Key events we found which is called 'Key Events Relationships (KER)', they consist in a change from one KE to another in which an alteration in the first will determine the next key event in a positive direction or in a negative direction.

(3) AOPs are a pragmatic functional unit of development and evaluation.

(4) For most real-world applications, AOP networks are the functional unit of prediction. By building modular AOPs, we gradually describe the complexity of potential interactions while meeting system biology, enhancing the prediction of its possible behavior.

(5) AOPs are living documents.

AOPs are a way of organising existing knowledge. As methods for observing biology evolve we could:

i. establish new possibilities for KEs;

ii. ability to measure KEs with greater precision/accuracy.

The main conclusion was that, as new experiments are published the weight of evidence supporting (or rejecting) KERs will grow and new AOPs and new branches in AOP networks will be discovered, so we are in front of an evolving concept that will grow up with time and research.

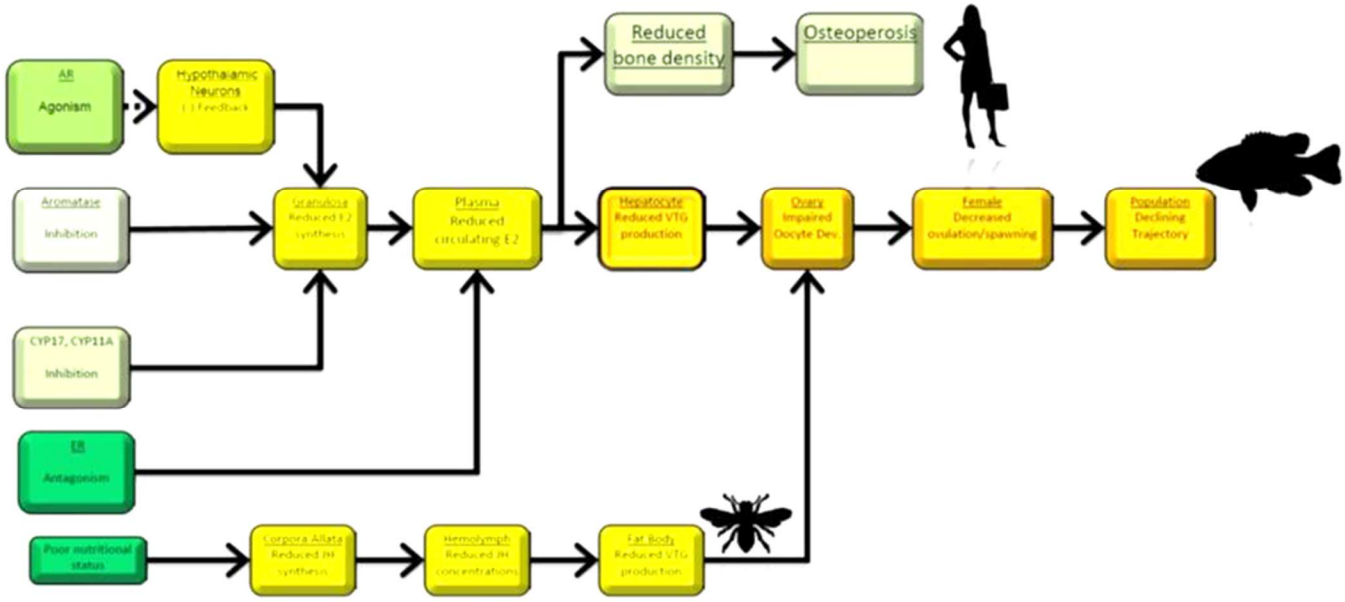


Figure 7: AOP network taken from Dan Villeneuve (EPA) presentation, used during Mirjam Luijten's training session

6.2 Proposal of interaction between WP13 and WP14 (by MU)

AOPs provide a conceptual framework for the linkage between a direct molecular initiating event (MIE) and an adverse outcome, at a level of biological organisation relevant to risk assessment. In this line, some epidemiologic effect biomarkers could provide relevant information on MIEs and **Key Events (KEs)** that take part in a particular adverse outcome pathway (AOP), thus constituting the best integration of experimental and epidemiologic knowledge.

The future interaction between WP13 and WP14 will focus on this new avenue in order to advance our understanding of exposure and health relationships with the aim to facilitate risk assessment and policy actions. It was agreed that several working groups from both WP13 and WP14, will work together to choose the best epidemiologic effect biomarkers obtained from all the WP14 literature searches, in conjunction with WP13 knowledge on AOPs. These decisions will be based on each chemical group, the adverse outcomes of highest interest, and the critical window of exposure. All this work will help to implement the best effect biomarkers in the new HBM4EU recruitments presented in the Workshop by Greet Schoeters (VITO).

6.3 Short overview about new recruitments/cohorts for HBM4EU, by Greet Schoeters (VITO)

A brief overview of the planning of HBM4EU recruitments/cohorts was outlined. The WP14- WP13 interaction will be based on these HBM4EU objectives, taking into account, each chemical group, the most important or suspected adverse outcomes, and the critical window of development (See Annex at the end of the document).

Table 2: Specific substance groups for each age category to achieve Europe wide coverage

Children	Adolescents	Adults
Phthalates and DINCH	Phthalates and DINCH	Bisphenols
Flame retardants	Per/poly-fluorinated compounds	Cadmium and PAH Occupational exposures Cr IV and anilines.

7 Preliminary conclusions

At the end of the Granada Workshop, the goals previously established were reached:

- A preliminary list of biomarkers of effect that could be of interest for HBM programs (Deliverable **D14.2**) and deadline for the communication of results to WP14 by **April 25th**.
- Roles from each partner taking part in the Deliverable **D14.4** 'Proof of concept' and deadline for the communication of results to WP14 leaders by **30th April 2018**.
- Successful interaction between WP14 and WP13, which will play a key role in the **selection** of the best effect biomarkers for HBM.
- Future interactions were accorded with Ludek Blaha (WP13) in order to contribute to Pillar 2 and the new HBM4EU recruitments/cohorts, providing the best up to date and relevant information concerning AOPs and epidemiologic effect biomarkers for the first list of prioritised substances.
- Next to implementation in surveys of pillar II, novel effect biomarkers will be further studied in longitudinal cohorts. Cross talk between WP14 and W13.2 has been reinforced since WP13.2 has made an overview of longitudinal cohorts with focus on exposures to HBM4EU priority compounds and related health outcomes. In addition, UGR has access to different longitudinal cohorts (EPIC, INMA, etc.) which could be used for these purposes.

8 Future steps: selection process of effect biomarkers for their implementation in HBM4EU aligned studies

The next step for WP14 is the progressive selection of those effect biomarkers of highest interest for HBM4EU aligned studies, established by Pillar 1 and 2. Therefore, a close interaction between pillars is needed to reach this purpose. In this line, D14.3 (Report on available biomarkers of effect of utility in human epidemiological studies for the first set of prioritised substances) will explore this process more in depth. A preliminary structured process for this selection is briefly described:

(1) Inventory of effect biomarkers found in the literature search based on the health outcomes of highest relevance for the first set of prioritised chemical substances (WP14).

(2) Identification of the adverse outcomes of highest concern for the first set of prioritised substances, based on the experimental literature and on available Adverse Outcomes Pathways (AOPs) (WP13).

(3) Once the Adverse Outcomes of highest concern for a specific chemical family have been identified based on the experimental knowledge (WP13), the corresponding available effect biomarkers related to these adverse outcomes obtained from the literature searches will be listed. This will provide a list of effect biomarkers (HBM) related to the most important adverse outcomes observed for a given chemical family in animal studies (WP13).

(4) With this information, groups of participants from both WP14 and WP13 will be created for each chemical family, to discuss through telephone calls and videoconferences the final proposal of effect biomarkers to be implemented in HBM4EU aligned studies for each chemical family of interest.

(5) The window of development in which each chemical family will be assessed in HBM4EU aligned studies (children, adolescents and/or adults), as well as the biological matrix available (urine, serum, etc.) will also be taken into account (VITO presentation).

(6) Finally, the convergence of other non-scientific variables such as the availability of biological samples, as well as technical and economic resources will also influence the final selection of effect biomarkers to be implemented in HBM4EU aligned studies.

9 Annex

9.1 Literature search

9.1.1 Bisphenols (coordinated by UGR)

9.1.1.1 UGR

UGR: Most physiologic and anthropometric effect biomarkers were classic

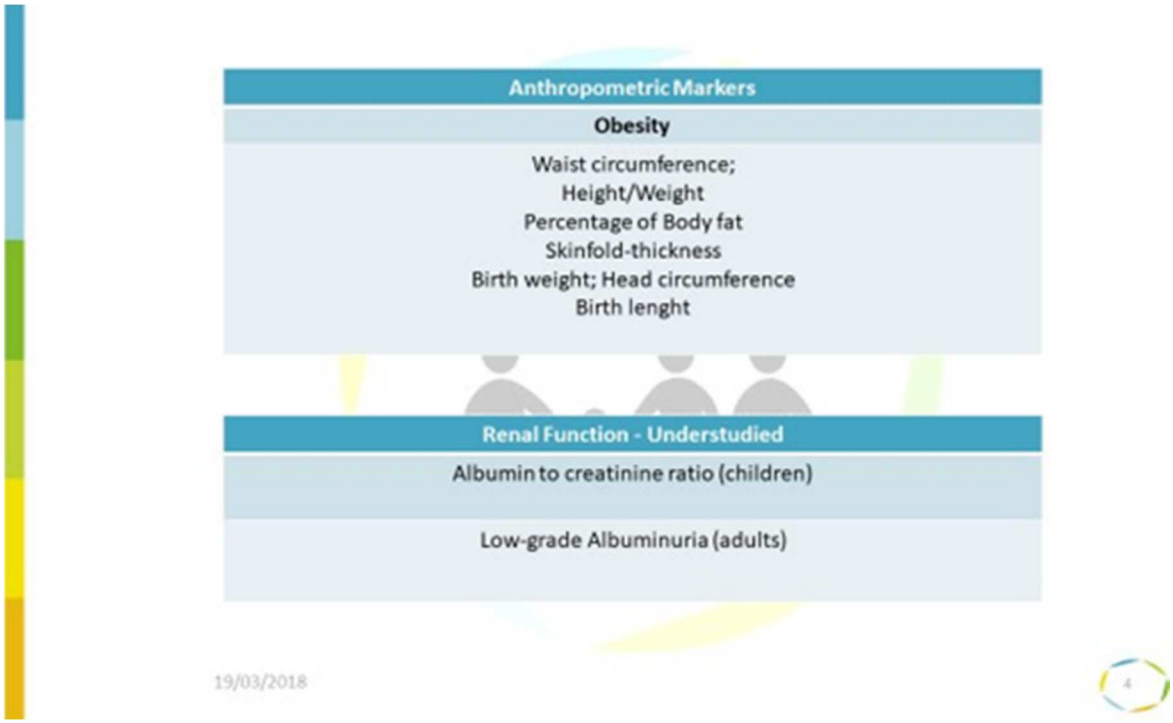
Reproductive and Endocrine effect biomarkers		
Classical and Studied	Classical and Understudied	Novel
TSH, FT3, FT4, TT, T/E, FSH, E2, LH SHBG	PRL, PROG, **Cortisol, ACTH** **DHEA-S** Androstenedione Pregnenolone	**Kisspeptin** sFlt-1 sFlt-1- PlGF Nuclear receptors expression in serum Urinary estrogen metabolism: 2-MeO-E1; 2-MeO-E2 4-MeO-E1; 4-MeO-E2
LH/FSH ratio; T/E2 ratio TT/LH ratio FAI (=TTx100/SHBG) TT: Cortisol ratio		

TSH: Thyroid stimulating hormone; FT4:Free Thyroxine; FT3: Free Triiodothyronine; TT: Total testosterone; T/E: ratio testosterone estrogen; FSH: Follicle stimulating hormone; E2: Estradiol; LH: Luteinizing hormone; SHBG: Sex hormone-binding globuline; FAI: Free androgen index; PRL: Prolactine; PROG: Progesterone; ACTH: Adrenocorticotropic hormone; DHEA-S: Dehydroepiandrosterone sulfate; sFlt-1: Soluble fms-like tyrosine kinase 1; PlGF: Placental growing factor; Nuclear receptors expression in serum

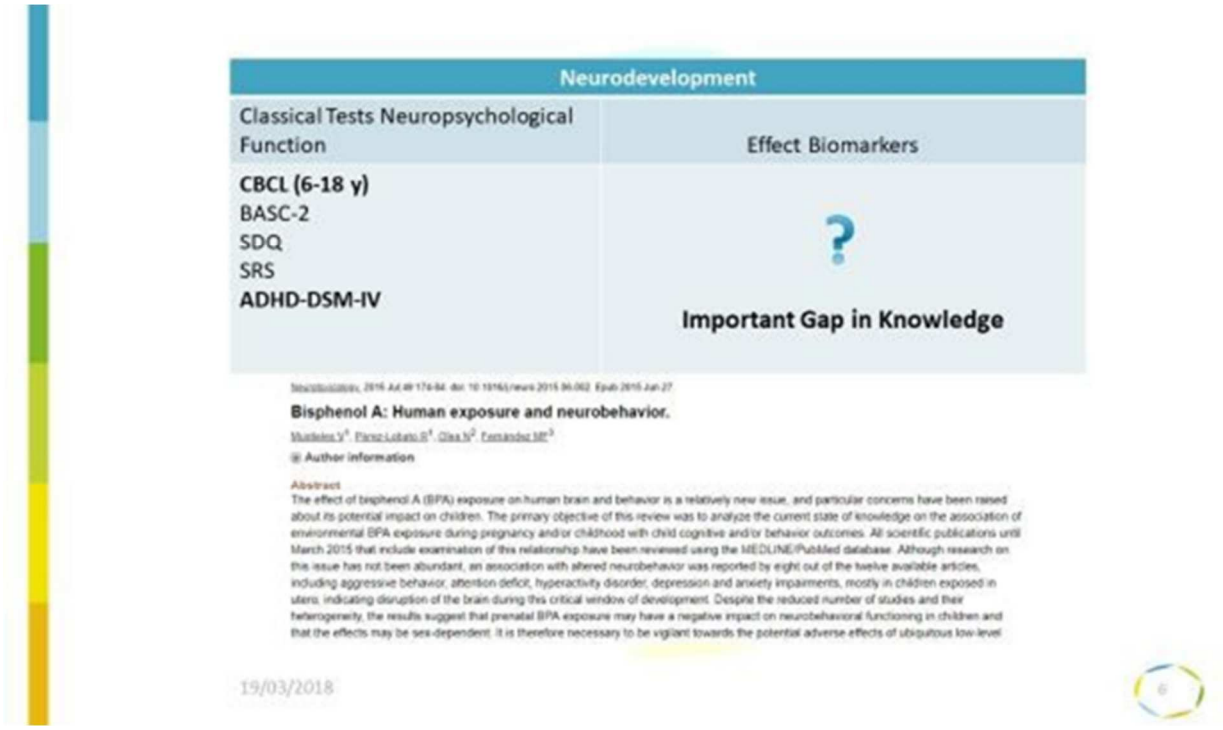
19/03/2018

Cardio-Metabolic effect biomarkers		
Classical and Studied	Classical and understudied	Novel
Cholesterol (total); LDL; HDL; TG; HbAc1; FSG; Insulin HOMA-IR	**Leptin; Adiponectin**; γ-glutamyl transferase (GGT); Alkaline phosphatasa; ALT (Alanine amino transferase); AST (Asparate amino transferase); **Vitamin D**; hs-CRP; IL-6; **Blood Pressure (children)**	-
Blood Pressure		

19/03/2018


Anthropometric Markers
Obesity
Waist circumference;
Height/Weight
Percentage of Body fat
Skinfold-thickness
Birth weight; Head circumference
Birth lenght
Renal Function - Understudied
Albumin to creatinine ratio (children)
Low-grade Albuminuria (adults)
19/03/2018


HBM4EU
Allergy/Immune System
IgE (eosinophyl count)
IL-33; TSLP; IgE
IL-6;IL-10;TNF-α


Neurodevelopment
Classical Tests Neuropsychological Function
Effect Biomarkers
CBCL (6-18 y)
BASC-2
SDQ
SRS
ADHD-DSM-IV
?
Important Gap in Knowledge
Bisphenol A: Human exposure and neurobehavior.
Author information
Abstract
The effect of bisphenol A (BPA) exposure on human brain and behavior is a relatively new issue, and particular concerns have been raised about its potential impact on children. The primary objective of this review was to analyze the current state of knowledge on the association of environmental BPA exposure during pregnancy and/or childhood with child cognitive and/or behavior outcomes. All scientific publications until March 2015 that include examination of this relationship have been reviewed using the MEDLINE/PubMed database. Although research on this issue has not been abundant, an association with altered neurobehavior was reported by eight out of the twelve available articles, including aggressive behavior, attention deficit, hyperactivity disorder, depression and anxiety impairments, mostly in children exposed in utero, indicating disruption of the brain during this critical window of development. Despite the reduced number of studies and their heterogeneity, the results suggest that prenatal BPA exposure may have a negative impact on neurobehavioral functioning in children and that the effects may be sex-dependent. It is therefore necessary to be vigilant towards the potential adverse effects of ubiquitous low-level
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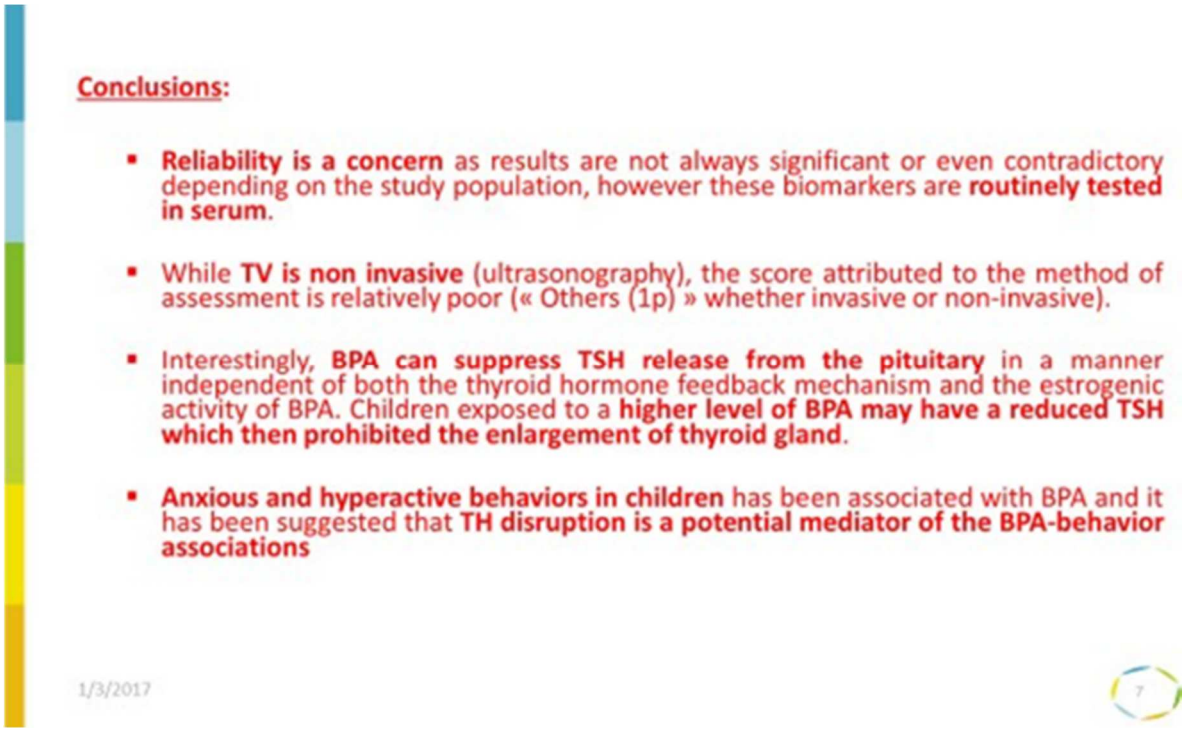
9.1.1.2 CNRS


Conclusions:
Reliability is a concern as results are not always significant or even contradictory depending on the study population, however these biomarkers are routinely tested in serum.
While TV is non invasive (ultrasonography), the score attributed to the method of assessment is relatively poor (« Others (1p) » whether invasive or non-invasive).
Interestingly, BPA can suppress TSH release from the pituitary in a manner independent of both the thyroid hormone feedback mechanism and the estrogenic activity of BPA. Children exposed to a higher level of BPA may have a reduced TSH which then prohibited the enlargement of thyroid gland.
Anxious and hyperactive behaviors in children has been associated with BPA and it has been suggested that TH disruption is a potential mediator of the BPA-behavior associations
1/3/2017
7

Potential biomarkers of effect for human biomonitoring

In experimental studies, a surprisingly wide range of effects of bisphenols are documented, mediated by numerous pathways through tissue or cell specific alterations and leading to a diverse range of endpoints.

The majority of these biomarkers are invasive or conducted post-mortem in human studies.

Non-invasive biomarkers of effect for « neurobehaviour » from *in vivo* studies:

- Brain-derived neurotrophic factor (*BDNF*) has roles in various stages of neural circuit development, and regulates neural circuit structure and synaptic plasticity in the adult brain.

  BDNF expression and DNA methylation are altered in several psychiatric disorders that are associated with early-life adversity, including depression, schizophrenia, bipolar disorder, and autism,

  - In mice, *BDNF* DNA methylation in the blood may be used as a predictor of *BDNF* DNA methylation and gene expression in the hippocampus as well as behavioural deficits (Kundakovic M et al., 2015)

- Glial cell line-derived neurotrophic factor (GDNF) major roles implicate the maintenance of neurons and glial cells, as well as protecting them against oxidative stress.

  Reports suggest increased **plasma GDNF levels** in ADHD patients, and decreased levels associated with depression.

  In Alzheimer's disease, these levels might inform neuronal degradation.

  - Interestingly, in rats exposed to BPA, transcript levels of Gdnf in the prefrontal cortex were decreased by BPA in both juvenile and adult rats (Castro B et al.,2015).

- The zinc finger transcription factor Sp4 (specificity protein 4) plays an important role in the transcriptional coupling of energy generation and energy consumption in neurons;

  A significantly increased **SP4 relative expression in peripheral blood mononuclear cells** (PBMC) has been observed in AD patients (Villa C et al., 2012)

  - A study on zebrafish and mice revealed that sp4 expression is a sensitive target of early-life exposure to BPA toxicity (SH Lam et al.,2011).

Potential biomarkers of effect for human biomonitoring

Non-invasive biomarkers of effect for « reproductive disease » from *in vivo* studies:

The human placenta provides a critical interface between the maternal and fetal circulations during pregnancy.

- Placental corticotrophin-releasing hormone (CRH) is a peptide hormone which is involved in fetal development.

  Increased plasma CRH is associated with elevated risk of premature delivery

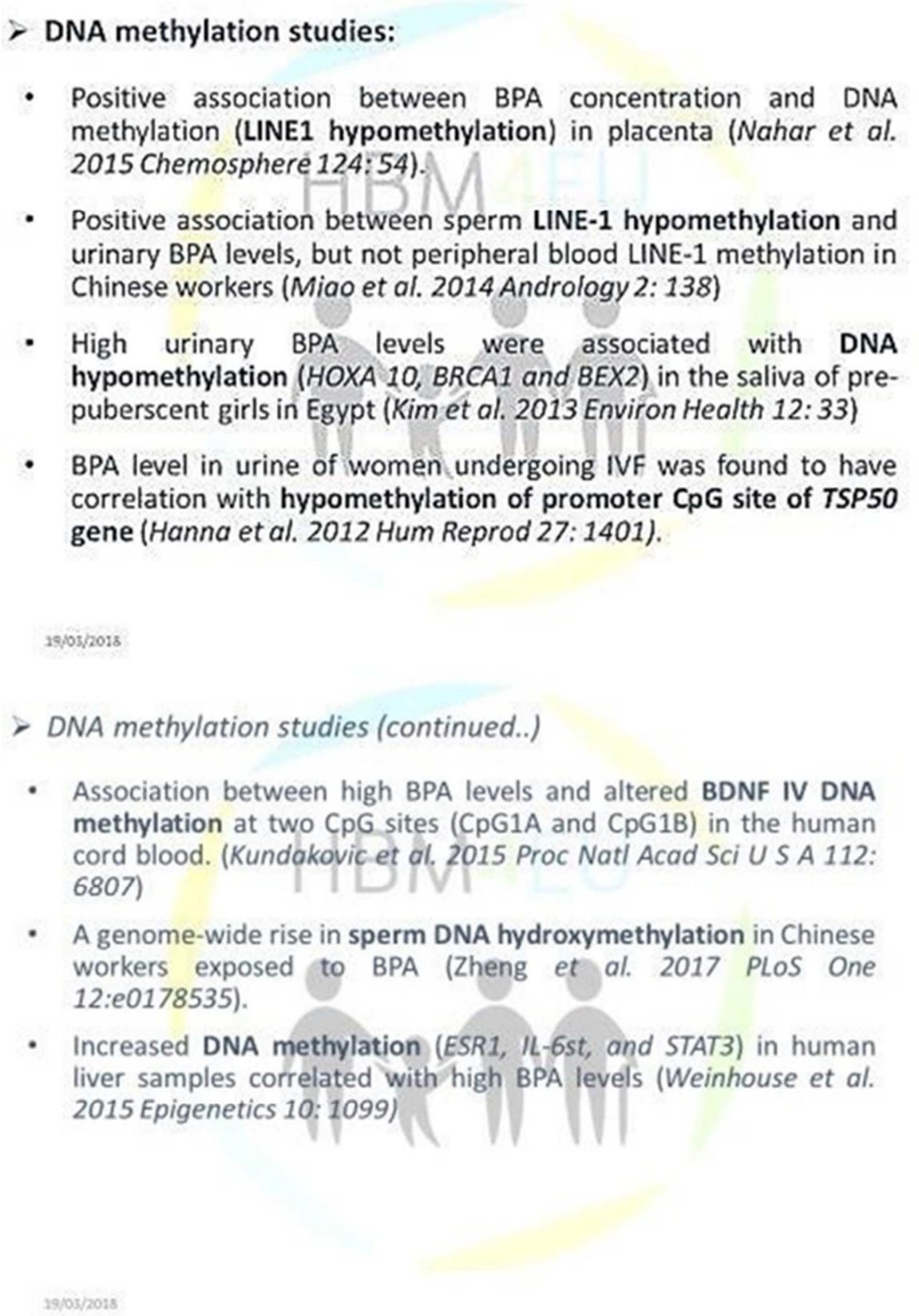
  - bisphenol A **increased CRH mRNA expression in the placental cells** *in vitro (H Huang et al,.2012)* & was induced in mice treated with BPA, possibly by **activating atypical placental protein kinase C isoforms** (phospho-PKC ζ/λ and δ) in mice (W Tan et al,.2013).

- Homeobox gene *Hoxa10* controls uterine organogenesis.

  DNA methylation is one mechanism by which HOX gene expression is modulated during normal placental development.

  - Exposure to BPA leads to aberrant methylation in the promotor and intron of Hoxa10, persisting after birth in CD-1 mice. Hoxa10 mRNA and protein expression were increased in the reproductive tract of mice exposed in utero via hypomethylation. Altered methylation affects ER binding to the Hoxa10 ERE and increases estrogen responsiveness (Bromer J.G et al.,2010).

9.1.1.3 INSERM


➢ DNA methylation studies:
• Positive association between BPA concentration and DNA methylation (LINE1 hypomethylation) in placenta (Nahar et al. 2015 Chemosphere 124: 54).
• Positive association between sperm LINE-1 hypomethylation and urinary BPA levels, but not peripheral blood LINE-1 methylation in Chinese workers (Miao et al. 2014 Andrology 2: 138)
• High urinary BPA levels were associated with DNA hypomethylation (HOXA 10, BRCA1 and BEX2) in the saliva of pre-puberscent girls in Egypt (Kim et al. 2013 Environ Health 12: 33)
• BPA level in urine of women undergoing IVF was found to have correlation with hypomethylation of promoter CpG site of TSP50 gene (Hanna et al. 2012 Hum Reprod 27: 1401).
19/01/2018
➢ DNA methylation studies (continued..)
• Association between high BPA levels and altered BDNF IV DNA methylation at two CpG sites (CpG1A and CpG1B) in the human cord blood. (Kundakovic et al. 2015 Proc Natl Acad Sci U S A 112: 6807)
• A genome-wide rise in sperm DNA hydroxymethylation in Chinese workers exposed to BPA (Zheng et al. 2017 PLoS One 12:e0178535).
• Increased DNA methylation (ESR1, IL-6st, and STAT3) in human liver samples correlated with high BPA levels (Weinhouse et al. 2015 Epigenetics 10: 1099)
19/01/2018

➢ MicroRNA expression studies:

- Two studies described miRNA expression in placenta:
  - ✓Study 1: Out of 1349 miRNAs analyzed, 34 miRNAs were upregulated. A strong association between **BPA levels in placenta and miR-146a** was observed (*De Felice et al. 2015 Genomics 8:56)*.
  - ✓Study 2: **No association** was observed between BPA and miRNA expression, but a positive association between miRNA and metals (Hg, Pb) and PCBs (miRNA 1537) (*Li et al. 2015 Epigenetics 10: 793)*.

➢Gene expression studies:

- BPA, cadmium and PCBs showed positive correlation with **KISS1 gene expression** in placental samples. In addition, BPA showed positive correlation with **leptin and leptin receptor gene expression**. (*Xu et al. 2015 Reprod Toxicol 52: 62)*
- Higher BPA levels induced higher expression of two **estrogen-responsive genes, ESR2 (ERβ) and ESRRA (ERRα)** in the blood. (*Melzer et al. 2011 Environ Health Perspect 119: 1788)*

➢Hormone assays:

- Association between pregnancy phthalate and BPA concentrations and peripubertal levels of **male reproductive hormones** (Estradiol, testosterone, inhibin B, and sex hormone-binding globulin (SHBG) and dehydroepiandrosterone sulfate (DHEA-S) (*Watkins et al. 2017 Environ Health 16: 69*)

9.1.1.4 NIPH

By Tim Hofer, Inger-Lise Steffensen, Hubert Dirven

Epidemiological studies last 10 years on bisphenols & oxidative stress effect biomarkers within:

	BP	8-OHdG	MDA/ TBARS	8-isopr ost.	HNE-MA/p -carb	8-NO_2G ua	3-NO_2 Tyr	NO	Antiox. enzymes (GPx, ...)	Total thiols (GSH)	CRP, IL, etc.	Ox stress genetic polymor.
Allergy-Immunological diseases (Clayton EM 2011 was excluded as not ox stress; is covered by UGr)												
Donohue KM 2013	A							exh.				
Erden ES 2014	A		serum							serum	serum	
Yang M 2014	A		urine									
Watkins DJ 2015	A	urine		urine								
Lv Y 2016	A	urine										
Zhang T 2016	A/AF/F/S	urine										
Huang YF 2017	A	urine		urine	urine	urine			plasma		plasma	
Cancer												
Song H 2017	A										serum	
Endocrine diseases (-)												
Obesity and cardiometabolic diseases												
Hong YC 2009	A	urine	urine									
Yi B 2011	A	urine	urine									
Aslimakopoulos AG 2016	A/AF/F/S	urine										
Kim JH 2016	A											DNA
Kim JH 2017	A		urine									DNA
Neurodevelopment (Kaur K 2014 was excluded as considered experimental)												
Kondolot M 2016	A				plasma				red BCs (also Se)	plasma		
Reproductive diseases												
Yang YJ 2009	A	urine	urine								serum	
Veiga-Lopez A 2015							plasma					

By Tim Hofer, Inger-Lise Steffensen, Hubert Dirven

BPA: polycarbonate- & epoxiplastics, effective antioxidant in PVC

Major issues with **8-OHdG, MDA, 8-isoprostane**

- ❑ These markers are not specific for bisphenols, and can originate from inflammation, irradiation, tissues not relevant for the health endpoint, foods, etc.
- ❑ High variable background levels in urine (levels are often normalized to creatinine levels). Prefer urine over serum?
- ❑ MDA/TBARS analyses criticized (heating, unspecific)

Conclusions

- ➢ Overall the studies indicate that BPA exposure is associated with increased levels of 8-OHdG, MDA and 8-isoprostane to some extent
- ➢ Suitable: urinary 8-oxodG, 8-isoprostane, (HNE-MA)
- ➢ Biomarkers are not specific for BPA (=not highest score)

9.1.2 Phtalathes (coordinated by VITO)

9.1.2.1 VITO

Novel effect biomarker	Score
Allergy & immunology	
Genetic polymorphisms of the oxidative-stress related genes	10
Endocrine	
Metabolomics: Amino acids and enzymes in urine	15
11β-HSD2 activity: (THF+allo-THF)/THE with tetrahydrocortisol (THF), allo-tetrahydrocortisol (allo-THF), tetrahydrocortisone (THE) in urine	14
Kisspeptin in serum	12
Metabolism	
DNA methylation (Genes with DMRs involved in inflammatory response (IRAK4 and ESM1), cancer (BRCA1 and LASP1), endocrine function (CNPY1), and male fertility (IFT140, TESC, and PRDM8))	11
Reproductive	
Genotype of CYP17A1, estrogen receptor 1 (ESR1), and 2 (ESR2) in serum peripheral lymphocytes by PCR	12
Expression of metallothioneins (MTs), fatty acid transport protein 1 (FATP1) and heart fatty acid binding protein (HFABP) in umbilical cord blood	11

NRF2 PPARγ GR PPARγ ER NF κβ PPARγ ER PI3K PPARγ

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9.1.2.2 UCY

Chemical Group: Phthalates

Results of Screening

Cancer	
Novel Biomarkers of effect	**Score**
Detection of Polymorphisms of ESR and CYP17A1 genes by DNA sequencing in blood samples	13
Detection of the glutathione S-transferase M1 enzyme (GSTM1; a major detoxification enzyme) by PCR in blood samples	11
Some examples of Classic Biomarkers of effect: histopathological analysis, tissue sections to confirm breast cancer or uterine lesions	

Most common mechanisms of action involved:

- The estrogen receptor pathway is suggested (xenoestrogens bind ERs and mimic estradiol)

Questions/Gaps in knowledge:

- Are these novel markers specific for the health effects and/or chemical group studied?
- How feasible is it to use sequencing/PCR to determine health effects?

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Chemical Group: Phthalates

Results of Screening

Behaviour/Neurobehaviour	
Novel Biomarkers of effect	**Score**
Assessment of the parental attitudes towards sex-atypical play with questionnaires.	13
Measured oxidant-antioxidant status by detecting several markers, including selenium levels, in the serum of autistic children	12
Child visual-spatial abilities were evaluated using the Virtual Morris Water Maze (VMWM), a computerized version of a rodent test of learning and visual-spatial reference memory.	11
Cortical thickness (Whole-brain structural MRI was acquired with a T1-weighted magnetization-prepared rapid acquisition gradient echo (MPRAGE) scan)	9
Some examples of Classic Biomarkers of effect: Common neurobehavioral tests are applied such as the Child Behaviour Checklist (CBCL) and the Strengths and Difficulties Questionnaire (SDQ)	

Most common mechanisms of action involved:

- It is suggested that these phthalates affect testosterone levels which affect brain development and sexual differentiation

Questions/Gaps in knowledge:

- Not sure of the cost and applicability of these methods.
- Is cortical thickness specific for the effects of these phthalates?
- Are questionnaires a valid biomarker of effect?

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Results of Screening

Endocrine	
Novel Biomarkers of effect	**Score**
0	N/A
Some examples of Classic Biomarkers of effect: hormones in serum (adiponectin or insulin for diabetes), weight changes to evaluate endocrine disruptors	

Most common mechanisms of action involved:

- Phthalates act as endocrine disruptors by interacting with nuclear receptors such as peroxisome-proliferator-activated receptors (PPAR's)

Questions/Gaps in knowledge:

- Could not find any novel biomarkers of effect
- The mechanism of action was not established in most cases but suggested

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Chemical Group: Phthalates

Results of Screening

Reproductive	
Novel Biomarkers of effect	**Score**
The biomarker for oxidative stress 8-hydroxy-2'-deoxyguanosine (8-OHdG) was measured in urine	17
Couples completed questionnaires on the time to pregnancy and about contraception.	16
Anogenital distance, a measure of the distance from the anus to a genital landmark within a few days of birth.	13
During the pregnancy, subjects completed an extensive questionnaire including items on demographics, lifestyle, and reproductive history.	13
Some examples of Classic Biomarkers of effect: Hormone levels (such as testosterone, estradiol) in serum, anthropometric measurements (such as body size, female partners' cycle)	

Most common mechanisms of action involved:

- Phthalates act by disrupting estrogen levels thereby affecting sexual activity
- Phthalates act as oxidative agents affecting DNA

Questions/Gaps in knowledge:

- Mechanism of action not quite clear, these effects could lead to a variety of disorders
- Adverse Outcome Pathways are too wide and not specific

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9.1.3 PFAS-PFOA (coordinated by AU)

9.1.3.1 AU

Epidemiology PFAAS: Reproduction-1

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	Fetal growth							
	BW	LBW	BL	HC	PI	PW	AC	BC
n								
No.	1, 3, 4, 7, 12, 16, 17, 22, 24, 31, 34, 35, 36, 37, 39, 40, 42, 43, 49, 50, 51, 54, 55, 59, 61, 63, 64, 66, 73, 79, 80, 81, 84, 86	16, 51, 55, 62, 63, 69, 80	1, 7, 21, 36, 50, 51, 59, 64, 75, 79, 86	7, 12, 21, 42, 43, 51, 59, 73, 79	1, 36, 53, 59, 64, 86	21, 49	21, 42, 43	66*
Score								

Abbreviations: AC = Abdominal circumference; BC = Body composition; BL = Birth length; BW = Birth weight; HC = head circumference; LBW = low birth weight; PB = Placental Weight; PI = Ponderal index.
*** Novel biomarker? / - Air displacement plethysmography**

	Gestational duration			
	GA	SGA	LGA	PB
n				
No.	12, 24, 43, 51, 55, 86	12, 34, 51, 63, 79, 81	81	12, 16, 51, 55, 61, 62, 63, 69, 81, 86
Score				

Abbreviations: GA = Gestational age; LGA = large for gestational age; PB = Preterm birth; SGA = Small for gestational age

	Postnatal growth						
	Weight	Weight gain	Height	WC	PI	BMI	PWi
n							
No.	1, 2, 50, 79	129	1, 2, 50, 79	129	1	2, 129	42, 43
Score							

Abbreviations: BMI = Body-mass index; PI = Ponderal index; PWi = Penile width; WC = Waist circumference

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Aarhus University, Denmark

Epidemiology PFAAS: Reproduction-2

	Gestational Weight Gain
n	
No.	5, 30
Score	

	Anogenital distance
n	
No.	42, 43
Score	

	Stillbirth / Miscarriage	
	Stillbirth	Miscarriage
n		
No.	62, 63, 86	15, 26, 46, 62, 69
Score		

	Sex ratio
n	
No.	10, 40
Score	

	Birth defects			
	Unspecified	Chryptorchidism	Hypospadias	Cerebral Palsy
n				
No.	56, 62, 69	71, 77	71	41
Score				

	Preeclampsia
n	
No.	6, 62, 67, 69
Score	

	Delivery complications
n	
No.	56
Score	

	Duration of breastfeeding
n	
No.	20, 60, 70
Score	

Abbreviations: E2 = estradiol; T = testosterone	Onset of puberty		
	Girls		Boys
	Age of menarche	High E2	High T
n			
No.	13, 32, 44	44	44
Score			

	Fertility									
	Women			Men					Couples	
	IvF	MCC	ERI	Semen quality	SCI	SGF	Y:X	Test vol.	TTP	FR
n										
No.	23, 52	47, 48, 87	78	18, 28, 38, 45, 58, 72, 75	38	38	33, 38	75	8, 9, 11, 19, 29, 74, 76, 82, 83	8, 9, 11, 19, 29, 74, 76, 82
Score										

Abbreviations: ERI = endometriosis-related infertility; FR = Fecundability ratio; ivF = in vitro fertilization biomarkers; MCC = menstrual cycle characteristics; SCI = sperm chromatin integrity; SGF = sex gland function; TTP = Time to pregnancy; Y:X = Y:X chromosome ratio

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Epidemiological PFAAS: Metabolic / Cardiovascular

	Diabetes										
	Diabetes	Gestational diabetes	Pre-diabetes	FPG	Ins	Pro-Ins	Insulin resistance	B-cell function	HbA1c	IGT	CRP
n											
No.	91, 92, 110, 114, 123, 127, 142, 143, 144, 151, 172, 233, 234	66, 130, 140, 150	92	66, 94, 99, 102, 110, 117, 122, 127, 135, 145	99, 108, 110, 122, 135, 145	91	91, 99, 103, 110, 119, 122, 135, 145	91, 99, 125, 145	91, 125	130, 140, 144	94, 122, 130
Score											

Abbreviations: CRP =C-reactive protein; FPG = fasting plasma/blood glucose; HbA1c = glycated hemoglobin; IGT = Impaired glucose tolerance; Ins = (Fasting) insulin;; Pro-Ins = (Fasting) proinsulin

	Anthropometry										
	BMI	Overweight / Obesity	WC	BC	BF	FFM	Weight	Height	Skinfolds	WHtR	WHR
n											
No.	88, 89, 90, 94, 99, 108, 113, 119, 120, 134, 135, 145	88, 89, 108, 111, 113, 120, 149	88, 90, 99, 108, 134, 135, 145	109	90, 109, 134	134	99	99	99, 120, 134, 145	111	134
Score											

Abbreviations: BC = Body composition; BF = body fat; BMI = Body-mass index; FFM = fat-free mass; WC = Waist Circumference, WHtR = Waist-to-height ratio; WHR = Waist-to-hip ratio;

	Metabolic syndrome
n	
No.	102, 125, 149
Score	

	Hypertension (high blood pressure)	
	Pregnancy-induced	Non-pregnancy induced
n		
No.	16, 63	92, 132, 143, 147, 149
Score		

	Anemia
n	
No.	56
Score	

	Blood pressure
n	
No.	110, 117, 129, 132
Score	

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Epidemiological PFAAS: Metabolic / Cardiovascular-2

	Cerebrovascular disease
n	
No.	126, 228, 233, 234
Score	

	Coronary heart disease
n	
No.	92, 131, 139, 142, 143, 147, 234
Score	

	Stroke
n	
No.	138, 141, 142, 143
Score	

	Peripheral arterial disease
n	
No.	139
Score	

	Myocardial infarction (heart attack)
n	
No.	233
Score	

	Cardiometabolic risk score
n	
No.	129
Score	

Novel biomarker? / - Cardiometabolic risk score

	Arterial wall stiffness
n	
No.	119
Score	

	Artheroschlerosis		
	IMT	IM-GSM	Carotid plaques
n			
No.	124	124	124
Score			

	Hypertriglyceridemia
n	
No.	125, 149
Score	

	Hypercholesterolemia
n	
No.	102, 143, 147
Score	

	Dyslipidemia
n	
No.	105
Score	

	Arthritis	
	Osteoarthritis	Rheumatoid
n		
No.	112, 143	143, 172
Score		

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Epidemiological PFAAS: Metabolic / Cardiovascular-3

	Renal function		
	eGFR	Serum uric acid	Urea nitrogen
n			
No.	98, 115, 146	94, 107, 115	94
Score			

	Chronic kidney disease
n	
No.	97, 138, 142, 143, 234
Score	

	Hyperuricemia
n	
No.	106, 137
Score	

Abbreviations: eGFR = Estimated glomerular filtration rate

	Liver disease				
	Unspecified	Hepatitis	Fatty liver	Enlarged liver	Cirrhosis
n					
No.	142	96	96, 143	96, 143	96, 126, 143
Score					

	Gilbert Syndrome
n	
No.	101
Score	

Gilbert's syndrome (GS) is a mild liver disorder in which the liver does not properly process bilirubin

	Bone health					
	BUA	SOS	TFBMD	FNBMD	LSBMD	Osteoporosis
n						
No.	117	117	116	116	116	116
Score						

Abbreviations: BUA = Broadband ultrasound attenuation; FNBMD = femoral neck bone mineral density; LSBMD = lumbar spine bone mineral density; SOS = Speed of sound; TFBMD = total femur bone mineral density

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Epidemiological PFAAs: Endocrine-1

	Polycystic ovary Syndrome
n	
No.	213
Score	

	Menopause
n	
No.	98, 198
Score	

	Thyroid disease
n	
No.	143, 186, 195, 206, 207, 219
Score	

	Thyroid stress	
	High TPOAb	Low Iodine
n		
No.	216	216
Score		

Abbreviations: TPOAb = thyroid peroxidase antibody

	Steroids									
	Cholesterol	Sex hormones							Glucocorticoids	
		Test	E2	Prog	17OH-P	Andro	DHEA	DHEAS	FAI	Cortisol	Cortisone
n											
No.	65, 68, 92, 94, 100, 102, 105, 110, 117, 119, 125, 128, 129, 130, 133, 135, 136	38, 71, 75, 94, 179, 190, 193, 199, 204, 205, 212, 221	38, 75, 94, 179, 182, 190, 193, 198, 204, 212, 221	71, 182, 190	71	71, 189	189	71	193	71, 189	189
Score											

Gap: No studies about E1(estrone), E3(estriol); other hormones)

Abbreviations: Andro = Androstenedione; DHEAS = dehydroepiandrosterone sulfate; E2 = estradiol; FAI = free androgen index; Prog = Progesterone; Test = testosterone; 17OH-P = 17-OH-Progesterone; **Gap: No studies about E1 (and other hormones)**

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Epidemiological PFAAS: Endocrine-2

	Thyroid hormones									
	fT3	tT3	T3u	fT4	tT4	TSH	T4-TTR	TGAb	TMAb	TSI
n										
No.	183, 184, 191, 199, 200, 211, 216, 218, 220	183, 184, 187, 191, 195, 196, 199, 208, 210, 211, 214, 216, 218, 220	197	183, 184, 185, 187, 191, 194, 195, 199, 200, 201, 202, 209, 210, 211, 214, 216, 217, 218, 220	181, 183, 184, 188, 191, 192, 196, 197, 199, 203, 208, 209, 210, 211, 214, 216, 217, 218, 220,	117, 183, 184, 185, 187, 191, 192, 194, 195, 196, 197, 199, 200, 201, 203, 208, 210, 211, 214, 215, 216, 217, 218, 220	181	200	195, 200	195
Score										

Gap:
No studies about 3,3'-T2, 3,5-T2, rT3

Abbreviations: fT3 = free triiodothyronine; fT4 = free thyroxine; TGAb = thyroglobulin antibodies; TMAb = Thyroid microsomal antibody; TSH = Thyroid stimulating hormone; TSI = thyroid stimulating immunoglobulin; tT3 = total triiodothyronine; tT4 = total thyroxine; T3u = triiodothyronine uptake; T4-TTR = transthyretin-bound ; Thyroxine; **Gap: No studies about 3,3'-T2, 3,5-T2, rT3**

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Epidemiological PFAAS: Endocrine-3

	Peptide & protein hormones							
	LH	Inh-B	FSH	Leptin	Adiponectin	Globulin	PRL	IGF-1
n								
No.	38, 75, 190, 193, 212	38, 75, 190, 193	38, 75, 190, 193, 212	4, 53, 103, 108, 145	4, 53, 103, 108, 122	94, 125	190	204
Score								

Abbreviations: IGF-1 = insulin-like growth factor 1; Inh-B = inhibin B; LH = luteinizing hormone; PRL = prolactin

	Cytokines					
	IFN-γ	IL-2	IL-4	IL-5	IL-33	TSLP
n						
No.	180	180	180	180	152	152
Score						

Abbreviations: IFN = interferon, IL = interleukin; TSLP = thymic stromal lymphopoietin

	Proteins											
	Total	SHBG	INSL3	Albumin	Haemoglobin	Apo-A	Apo-B	CC16	ECP	TTR	TBG	TG
n												
No.	94	38, 75, 190, 193, 205, 212	71, 190	27, 94, 125	94	94	94	178	156	181	181, 187	191, 218
Score												

Novel biomarker?/ Clara Cell protein 16

Abbreviations: Apo = apolipoprotein; CC16 = 16-kDa club cell secretory protein; ECP = eosinophilic cationic protein (ribonuclease 3); INSL3 = insulin-like factor 3; SHBG = sex hormone-binding globulin; TBG = thyroxin-binding globulin; TG = thyroglobulin; TTR = transthyretin; **Novel biomarker?/- Clara Cell protein 16**

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Epidemiological PFAAS: Endocrine-4

	Enzymes								
	α-glucosidase	ALT	AST	GGT	ALP	γ-GTP	GOT	GPT	PSA
n									
No.	38	94, 96, 104, 107, 117, 121, 133, 136	94, 107, 117, 121, 136	94, 96, 104, 107, 121	94, 107, 121, 136	148	148	148	94
Score									

Abbreviations: ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; GGT = γ-glutamyltransferase; GOT = glutamic-oxaloacetic transaminase; GPT = glutamic-pyruvic transaminase; γ-GTP = γ-glutamyl transpeptidase; PSA = prostate specific antigen

	Fatty acids / lipids												
	Unspecified	TG	palmitic	palmitoleic	oleic	linoleic	α-linolenic	arachidonic	DHA	EPA	HDL-C	LDL-C	non-HDL
n													
No.	66	31, 68, 94, 99, 105, 110, 119, 122, 128, 129, 130, 133, 145	31	31	31	31	31	31	148	148	68, 94, 102 105, 110, 119, 122, 125, 128, 129, 133, 135	68, 102, 105, 110, 117, 119, 128, 129, 133, 135, 136	102, 135, 136
Score													

Abbreviations: DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; HDL-C = high density lipoprotein cholesterol; LDL-C = low density lipoprotein cholesterol; TG = triglyceride

	Vitamins
	25-OH vitamin D
N	
No.	117
Score	
Gap: No studies about other vitamins	

	Others		
	Creatinine	Bilirubin	Homocysteine
n			
No.	94, 117	94, 96, 104, 107, 121, 136	132
Score			

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Epidemiological PFAAS: Immunology

	Ulcerative Colitis
n	
No.	143, 172
Score	

	COPD
n	
No.	142, 143
Score	

	Autoimmune diseases			
	Unspecified / combined	Crohn's disease	Lupus	Multiple sclerosis
n				
No.	143	172	172	172
Score				

	Asthma
n	
No.	143, 156, 163, 164, 170, 171, 174, 175, 178, 179, 180
Score	

	Allergy			
	Unspecified	Allergic rhinitis	Food allergies	Allergic Sensization
n				
No.	159, 166, 174, 175	159, 164, 166	154, 167	164, 174
Score				

	Eczema
n	
No.	159, 164, 166, 167, 171
Score	

	Wheeze
n	
No.	159, 163, 164, 166, 167, 171, 177
Score	

	Lung function			
	Unspecified	FVC	FEV_1	FEF_{25-75}
n				
No.	164	170	170	170
Score				

Abbreviations: FEF_{25-75} = Forced expiratory flow; FEV_1 = Forced expired volume; FVC = Forced vital capacity

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Epidemiological PFAAS: Immunology/Infections

	Infections								
	Unspecified	Otitis media	Chicken Pox	RSV disease	Rhinitis / common cold	Lower respiratory tract infections			Skin infection
						Unspecified	Bronchitis	Pneumonia	
n									
No.	157, 167	158, 167	158	158	159, 162	164		158	
Score									

Gap: No of studies about Bronchitis (However, other lower respiratory tract infections are covered)

Gap: No of studies about skin infections

	Infection symptoms				
	Fever	Cough	Nasal discharge	Diarrhea	Vomiting
n					
No.	155	155	155	155, 162	155
Score					

	Vaccine antibodies					
	Tetanus	Diphteria	Influenza	Measles	Mumps	Rubella
n						
No.	160, 161, 162	160, 161	162, 173	162, 174	174	162, 174
Score						

	Blood components							
	WBC	RBC	Platelets	Eusinophiles	Neutrophils	basophils	lymphocytes	Monocytes
n								
No.	94, 169	94	94	156	169	169	169	169
Score								

	Immunoglobulins			
	IgA	IgE	IgG	IgM
n				
No.	94	152, 156, 167, 175, 177	94, 168	94, 168
Score				

Gap: No studies about IgD

Epidemiological PFAAS: Cancer

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		Cancer diagnosis (part 1)										
	Unspecified / total	Bladder	Brain	Breast	Cervix	Colon	Esophagus	Kidney	Leukemia	Liver	Lung	Lymphoma
n												
No.	228	142, 143, 222, 223, 227, 233, 235	223, 235	142, 223, 224, 225, 233, 234, 235, 236	223, 235	143, 223, 230, 235	223	142, 223, 233, 234, 235	142, 223, 233, 235	142, 223, 227, 233, 234, 235, 237	142, 223, 235	223
Score												

	Cancer diagnosis (part 2)												
	Melanoma	Myeloma	Meso-thelioma	Non-Hodgkin	Oral	Ovary	Pancreas	Prostate	Soft tissue	Stomach	Testes	Thyroid	Uterus
n													
No.	143, 223, 235	235	142	142, 233, 235	223	223, 235	126, 142, 223, 227, 233, 234, 235	126, 142, 143, 223, 227, 229, 233, 234, 235	223	223	126, 142, 223, 233, 234, 235	223, 235	223, 235
Score													

	Prostate-specific antigen
n	
No.	226
Score	

Gap: Few studies about human cancer biomarkers

Might be covered by RIVM (OMICS/genetics) or VITO (DNA damage / Oxidative stress)

	All-cause mortality
n	
No.	228
Score	

Epidemiological PFAAS: Neurological

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Some of the questionnaires might be novel but it is difficult to determine

	Cognition
n	
No.	209, 240, 242, 244, 245, 246, 250, 253, 256, 259, 262, 264, 265, 266, 267
Score	

	Attention and behavior
n	
No.	209, 240, 241, 242, 243, 244, 245, 248, 249, 250, 251, 255, 257, 263, 265
Score	

	ADHD
n	
No.	247, 251, 252, 254, 261, 262, 263, 264
Score	

	Autism
n	
No.	239, 252, 253
Score	

	Motor function
n	
No.	209, 240, 241, 242, 243, 245, 249
Score	

	Sleep troubles
n	
No.	260
Score	

	Affective state	
	Depression	Anxiety
n		
No.	209, 238, 258, 264	209
Score		

	Alzheimer's disease
n	
No.	233
Score	

	Parkinson's disease
n	
No.	233
Score	

	Apgar scores
n	
No.	86, 242
Score	

9.1.3.2 VITO

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REPRODUCTIVE: NOVEL EFFECT BIOMARKERS

Novel effect biomarker	Score
Leukocyte telomere length at birth in umbilical cord blood by qPCR	11
Sperm aneuploidy and diploidy rate for chromosomes 18, X and Y by FISH and **sperm DNA fragmentation** by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling technique coupled to flow cytometry	10
Peripheral leukocyte DNA methylation at delivery in umbilical cord blood by methylation profiling microarray	9
Some examples of classic effect biomarkers: semen quality parameters, ROS…	

MoA (human epidemiologic studies):
- DNA damage
- Oxidative stress

Pathways (experimental studies):
- Oxidative stress and activation of the AKT/AMPK pathway in mouse epididymis
- Apoptosis involving the Fas death receptor signaling pathway in rat testis

Questions/Gaps in knowledge:
- How feasible are these DNA techniques?

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METABOLISM: NOVEL EFFECT BIOMARKERS

Novel effect biomarker	Score
Metabolomics: Deoxyarabinohexonic acid, hydroxybutyricn acidD-glucurono-6,3-lactone, α-carboxyethyl hydroxychromanol, arachidonic acid, **hypoxanthine**, oxoglutaric acid, pyroglutamic acid, tetrahydrobiopterin, xanthine in serum, detoxification, antioxidation and nitric oxide (NO) signal pathways	12
Insulin-like growth factor 2 (IGF2)/H19/ long interspersed element 1 (LINE) DNA methylation in umbilical cord blood by pyrosequencing	11
Some examples of classic effect biomarkers: anthropometric parameters (body weight, waist circumference, BMI…)	

MoA (human epidemiologic studies):
- DNA damage
- Oxidative stress

Pathways (experimental studies):
- Oxidative hepatic damage via **mitochondria-dependent and NF-κB/TNF-α-mediated pathway** (rat)
- Impaired glucose homeostasis through affecting adipose **AKT pathway** (mice)

Questions/Gaps in knowledge:
- MDA classic biomarker?
- How feasible is DNA sequencing?

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CARDIOVASCULAR: NOVEL EFFECT BIOMARKERS

Novel effect biomarker	Score
LINE-1 DNA methylation in peripheral blood leukocytes in serum by PCR	10
No classic effect biomarkers	

MoA (human epidemiologic studies):
- DNA damage
- Oxidative stress

Questions/Gaps in knowledge:
- How feasible is PCR?

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9.1.3.3 RIVM

Metabolic/cardiovascular: Cholesterol metabolism & transport

- Epidemiological data: strong association between PFOS, PFOA blood levels & increased total cholesterol.
- Some evidence: correlation between PFOS/PFOA blood levels and expression of genes involved in cholesterol metabolism, cholesterol reverse cholesterol transport (clearance); peripheral blood samples.
- PFOA: *NR1H2* (LXRB), *ABCG1* & *NPC1* altered expression.
- PFOS: *NR1H3* (LXRA), *NCEH1* altered expression.
- Molecular effect biomarker (?): genes linked to cholesterol homeostasis; blood samples; RNA extraction, qPCR, microarray
- **Uncertainties**: e.g. many more genes may influence cholesterol levels; no data on liver gene expression; limited data.

Immunotoxicity

- Epidemiological data: correlation of PFASs with immunosuppression, also in early childhood (National Toxicology Programme, 2016).
- Some evidence: association at transcriptomics level, between prenatal PFASs and number of common cold episodes, and/or rubella antibody levels in early childhood; umbilical cord blood samples.
- Examples*: CYTL1* (cytokine-like protein, specifically expressed in CD34+ cord blood mononuclear cells), IL-27 (immunomodulatory cytokine), PPAR *δ (*peroxisome proliferator-activated receptor- *δ*).
- Molecular effect biomarker(?): genes related to immune functionality; blood samples; qPCR, microarray
- Insights on the mode of action underlying PFASs-mediated immunotoxicity.

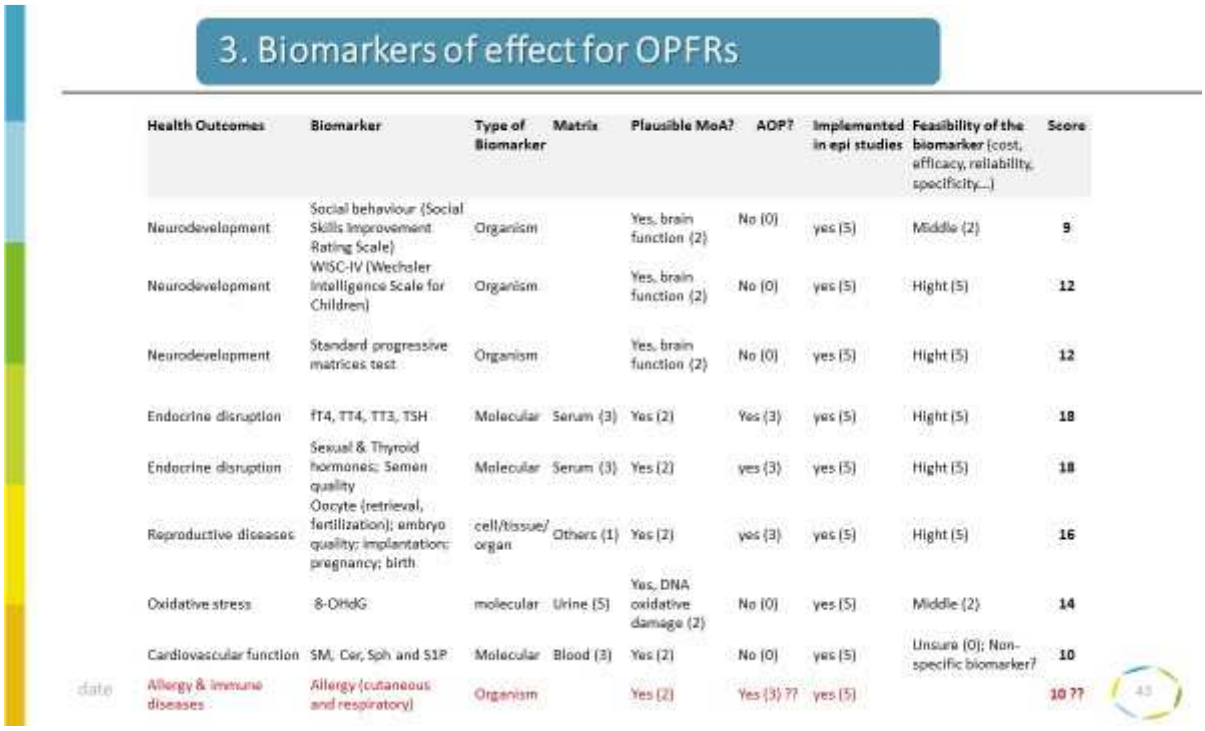
Reproductive system/endocrine disruption

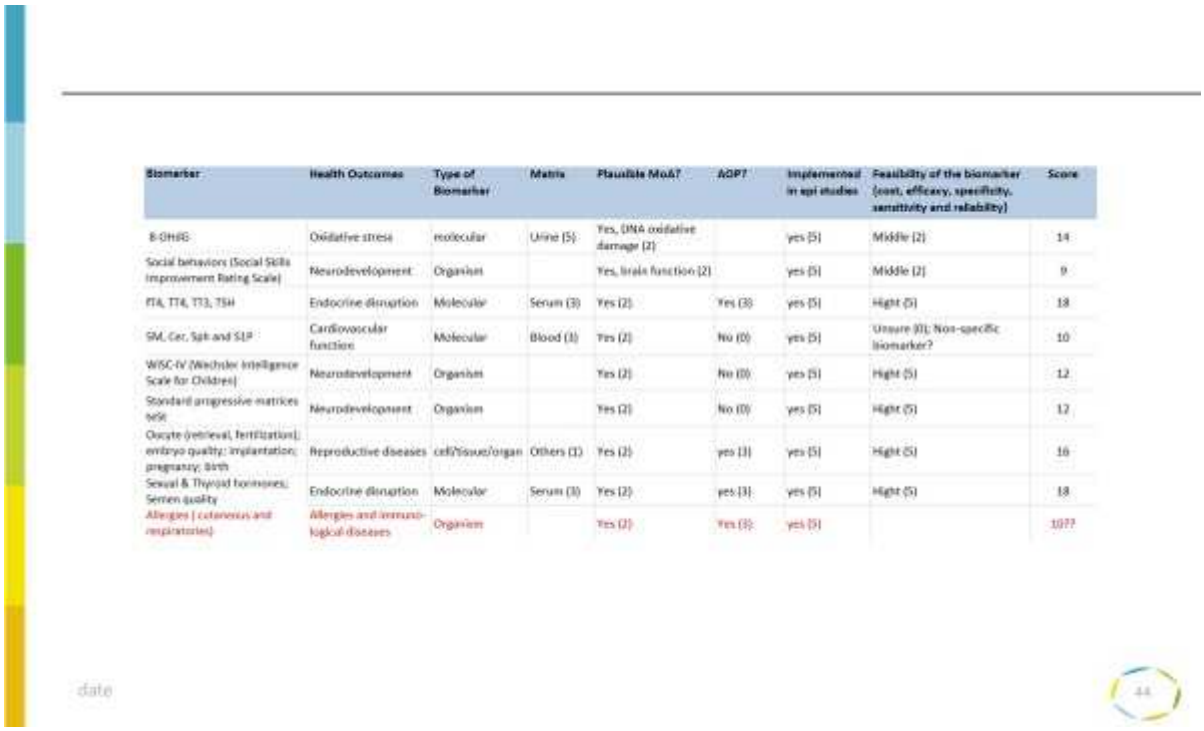
- PFASs are suspected endocrine disrupting chemicals (EDCs).
- Some evidence: PFOS/PFOA serum levels correlated to altered expression of Nuclear Receptors (NRs) main cellular targets of EDCs in women; peripheral blood samples.
- Different behaviour of PFOS and PFOA
  - PFOS: (+) *AR*, *PXR,* infertile women
  - *PFOA:* (-) *PXR* fertile women*;* (-) *AhR*, infertile women
- Molecular effect biomarker(?): specific NRs linked to EDCs, blood samples; RNA extraction, qPCR, microarray.
- Uncertainties: limited amount of data; effects seen mostly in the infertile group; PFOS/PFOA examined together with other EDCs, cannot be solely attributed to PFASs.

41 PFAS | 19.03.2018

9.1.4 Flame Retardans (coordinated by MU and EASP)

9.1.4.1 Organophosphates Flame retardants (EASP)





4. Problems and Doubts

Problems:

- Biomarker definition
- Biomarkers used in *in vitro* studies but not for epidemiology research (OPFR)
- Only a few AOPs have been properly developed (human studies assess AO instead of KE)
- Paucity of data for assessing the feasibility of biomarkers

Doubts:

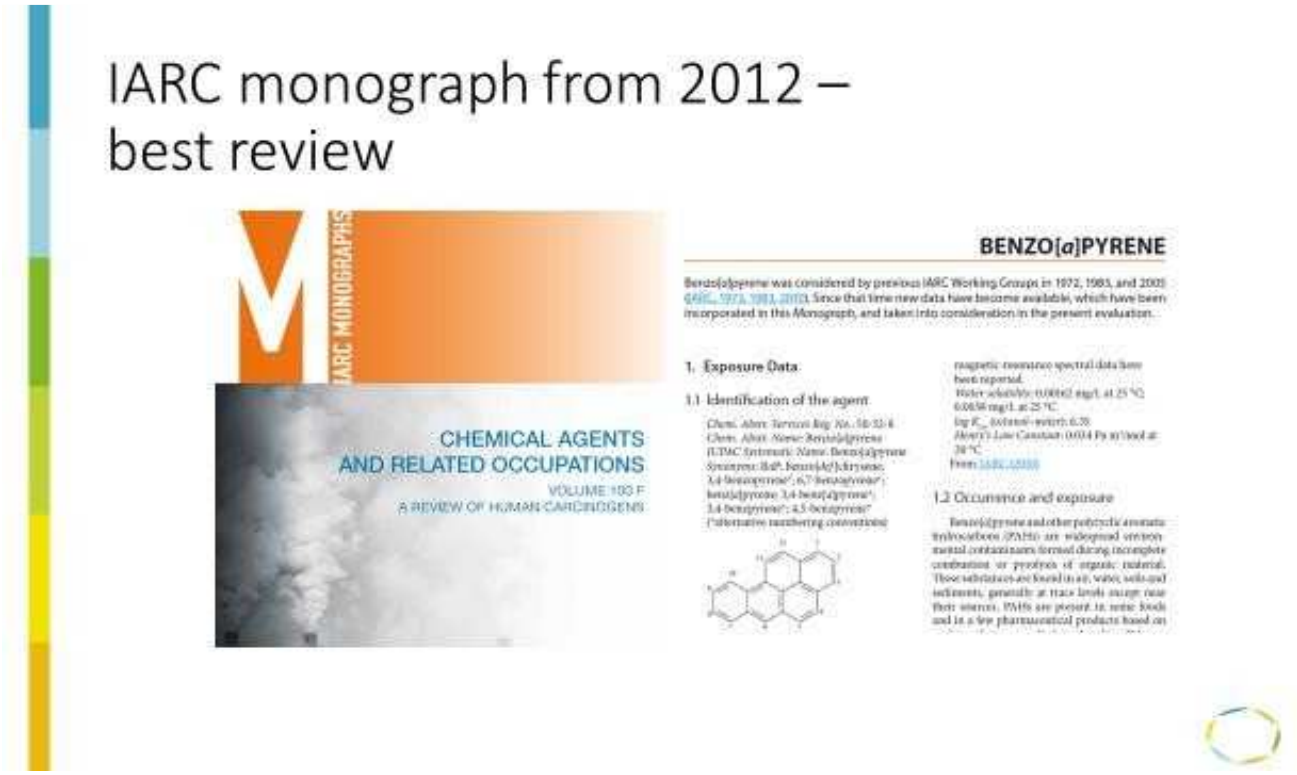
- Are *in vitro* biomarkers amenable for use in epidemiological studies?

5. Conclusions

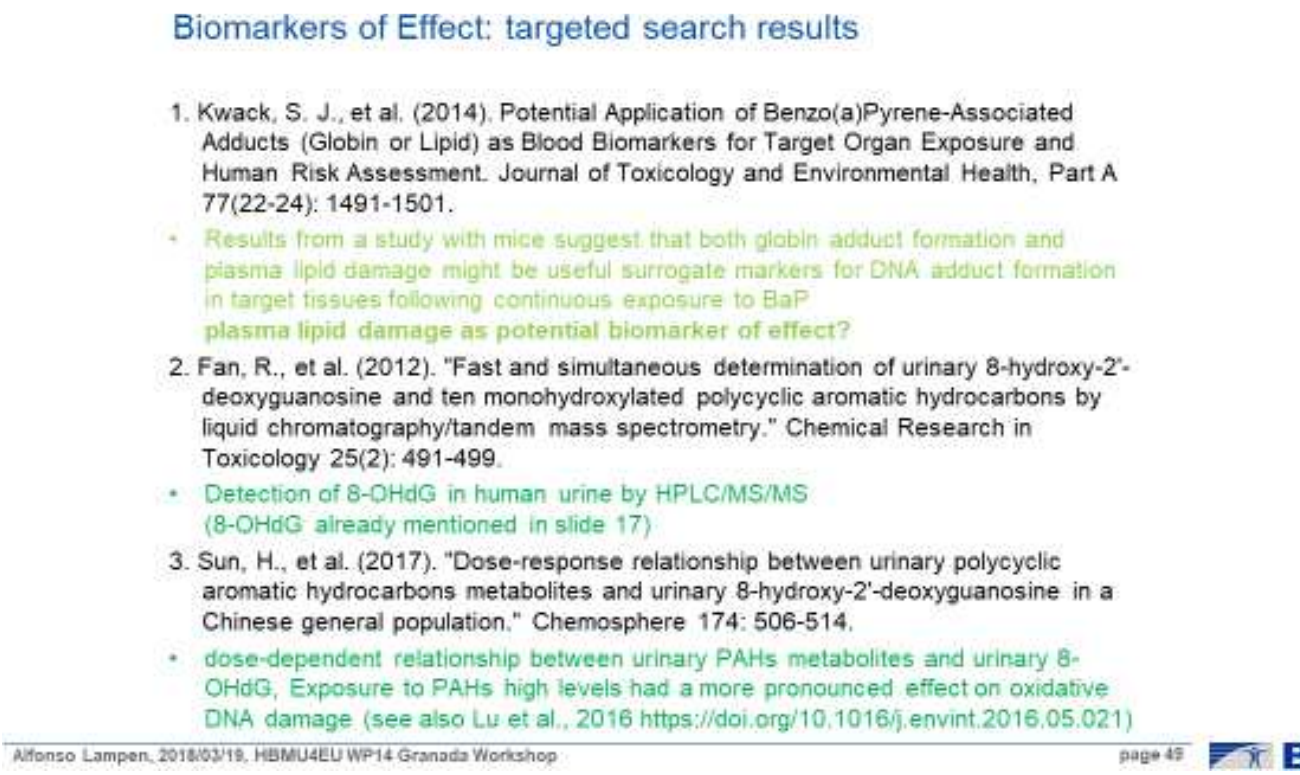
- The available epidemiological information on biomarkers of effect for OPFRs is limited.
- There are HBM studies measuring OPFRs in urine and environmental monitoring (home dust) but not accompanied by biomarkers of effect.
- Novel information is available in *in vitro* studies (animal and human systems) but potential biomarkers have not been translated to human epidemiological research.

9.1.5 PAHs (coordinated by NCRWE)

9.1.5.1 NRCWE



9.1.5.2 BfR



9.1.6 Metals. Cadmium (coordinated by BfR)

9.1.6.1 MUW

Authors	Year	Population	notes	U-Cd	U-Cd Fem	UCd Male	[conc]	Outcome/Biomarker of effect	A1-MG	B2-MG	NAG	RBP	GFR	BP	Bone	DPD	U-Ca	Alb	tot Pr
Eom et al.	2017	Korea			1.4	0.82	µg/g crt	Risk of renal tubule damage significantly associated with urine Cd level		yes	yes		no		no				
								U-Cd was significantly positively correlated with urinary β2-MG, NAG											
								but inversely with eGFR and BMD											
								MDA conc increased with U-Cd in a dose-dependent manner											
Osorio-Yanez et al.	2016	USA			0.21-0.42		µg/g crt	U-Cd associated with reduced third trimester MAP (-1.8; 95 % CI = -3.1,-0.5 mm Hg)						yes					
								and second trimester MAP (-1.1; 95 % CI = -2.3, -0.03 mmHg).											
Zhang et al.	2015	China	exposed	8.3	12.4	5.1	µg/g crt	Age adjusted U-Cd levels contributed most to the alteration of NAG,		yes	yes	ye							
			non-exposed	2.0	1.8	2.2		β2-MG and RBP											
Ke et al.	2015	China	five areas (N=6103)				µg/g crt	positive correlation between UCd and prevalence of Uβ2-MG											
Swaddiwudhipong et al.	2015	Thailand	contam. area	0.7				After adjusting for age,sex, and blood lead levels,		yes				no			yes		no
		594 children	control area	0.4				sign positive associations betw U-Cd urinary β2-MG and urinary Ca											
Nishijo et al.	2014	Thailand	polluted area		7.0	6.3	µg/g crt	U-Cd (B-Cd) sign related to β2-MG and NAG after adjusting for age and smoking habit,		yes	yes								
		>40 yrs	non-polluted area		1.1	0.5		indicating dose-effect relationship between Cd exposure and renal markers											
Hambach et al.	2013	Belgium	122 metallurgic	0.5			µg/g crt	Pb increases the strength of the association between Cd and renal biomarkers.			yes	ye							
			refinery workers					The impact of Cd-B on the urinary excretion of NAG and IAP was only											
								evident among workers with Pb-B concentrations ≥ 75th percentile. The association											
								between Cd-U and the renal markers NAG and RBP was also evidenced when Pb-B ≥											
								75th percentile.											
Nordberg et al.	2012	China	long term observation					Dose-response relationships between UCd and increased levels of biomarkers		yes	yes							yes	
								of renal tubular dysfunction (urinary B2MG, NAG, UAlb)											
								MT mRNA (peripheral blood), autoantibodies against MT (blood plasma)											
Sughis et al.	2011	Pakistan	155 children (8-12 yrs)	0.50	0.65	0.41	nmol/mmol crt	Markers of bone demineralization: U-Ca, DPD increase with U-Cd							yes	yes	yes		
Weaver et al.	2011		712 lead workers	1.0			µg/g crt	Associations between higher U-Cd and creatinine-based kidney function measures					yes						
								Urine crt was associated with serum crt-based but not cystatin-C-based eGFRs.											
Boonprasert et al.	2011	Thailand	case-control study					NAG showed positive correlation with Cd in hypertensive and normotensive group.			yes			no					
Ikeda et al.	2011	Japan	1403 women		1.0		µg/g crt	Cd-U correlated sign with α_1-MG-U, β_2-MG-U, and NAG-U	yes	yes	yes								
								Cd-U is the biomarker of choice compared to Cd-B											
Huang et al.	2009	Korea?	290 adults (gen pop)	0.84			µg/g crt	NAG, MDA, and 8-OHdG were positively correlated with U-Cd			yes								
								The effects of coexposure to Cd and As on biomarkers are more											
								pronounced than for exposure to each metal alone											
Nordberg et al.	2009	China						In addition to Nordberg et al. 2012: changes in glucose metabolism (T2D), exp to As											
Wang et al.	2016	China	N=1235 (2-87 yrs)		0.42	0.38	µg/g crt	U-Cd and B-Cd sign associated with NAG, β2-MG (also in children)		yes	yes								
								NAG was a very sensitive biomarker of cadmium exposure											
Trzcinka-Ochocka et al.	2010	Poland	170 women, 100 men		1.1	0.9		No effect of Cd on bone density, renal markers are more sensitive		yes	yes	ye					yes	yes	
								Urinary NAG, β2-MG, RBP, ALB, Ca were significantly associated with U-Cd											
Swaddiwudhipong et al.	2012	Thailand	2005	9.5				The follow-up examination revealed significant increases in urinary b2-MG,		yes									yes
		N=436	2010	8.8				urinary total protein and serum crt, and a decrease in GFR											

2. Identification of gaps in knowledge for effect biomarkers -> **MoAs?**
3. Consultation of experimental studies and development of original research with the objective to fill these data gaps.

Cadmium AND effect marker AND in vitro AND human (10 years): 10 articles

LDH release

Asis et al. (2014). *Biomed Res Int.* 2014:659358. doi: 10.1155/2014/659358.

Zinc

Mosmer et al. (2009). *Arterioscler Thromb Vasc Biol.* 29(9):1392-8.

Transferrin, serum iron,...

Bernard et al. (1990) *British Journal of Industrial Medicine* 47:559-565

MT expression

Nordberg et al. (2012) *J Trace Elem Med Biol* 26(2-3):197-200.

Oxidative Stress (OS) Biomarker (and others)

Cardiovascular disease serum paraoxonase 1 (PON1)	Almeida Lopes et al., 2017 (marker of susceptibilty)
Biomarkers of renal function and OS MDA, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-OHdG), ALB, NAG	Al-Saleh et al., 2017
Biomarkers of renal function and OS 8-OHdG, cotinine (COT) and creatinine (Cr)	Al-Saleh et al., 2015
Biomarkers of OS + **lipid oxidation** GPx, Selenium, GSH, malondialdehyde (MDA)	Cabral et al., 2015
Biomarkers of OS glutathione peroxidase [GPx], glutathione reductase [GR], protein carbonyls	Castillo-Castaneda et al., 2017
Biomarkers of renal function + OS 8-OHdG, HNE-MA, 8-isoPGF2α, 8-NO2Gua + metabolomics	Chen et al., 2017
Metabolites mitochondrial metabolism (citrate, 3-hydroxyisovalerate, 4-deoxy-erythronic acid) one-carbon metabolism (dimethylglycine, creatinine, creatine)	Ellis et al., 2012
Renal tubule damage β2-microglobulin (β2-MG) and N-acetyl-β-D-glucosaminidase (NAG)	Eom et al., 2017

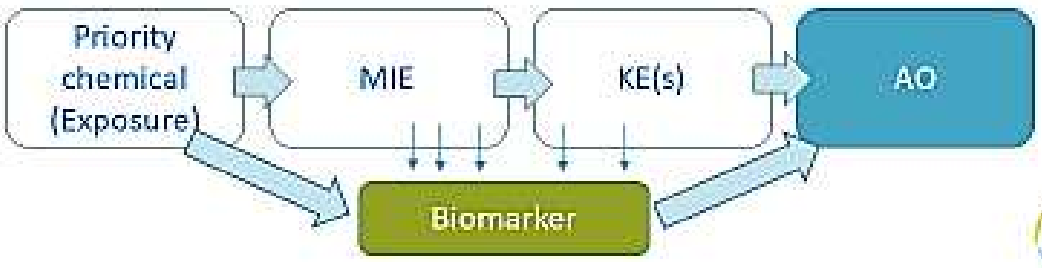
Oxidative Stress (OS) Biomarker (and others) - continued -

renal + oxidat. stress alkaline phosphatase (IAP), superoxide dismutase (SOD)	Hambach et al., 2013
renal function + OS Index malondialdehyde [MDA]	Huang et al., 2011
renal function 8-OHdG 8-oxo-7,8-dihydro-2'-deoxyguanosine	Kippler et al., 2012

9.2 Interaction between WP14-WP13

9.2.1 Proposal of interaction between WP13 and WP14, (by MU)

- **Q3: What biomarkers of effects are of priority** for each chemical group (and also generally) **to indicate/predict relevant prioritized AOs?**
  - What „classical and studied" (established) biomarkers?
  - What classical but understudied?
  - Which novel biomarkers?
- Comprehensive lists must be prioritized → proposal of one or few effect biomarkers to be implemented in European initiative (VITO) or smaller joint initiatives (Task 13.2)
  - Feasibility? Who will measure? QA/QC?
  - Variability of the BM? Sensitivity window (age)?



Work organization → by chemical groups

- **Teams of chemical leaders** established
  - One „team of responsible leaders" for each group of priority chemicals
  - One „partner/person" per each task or WP (see the table)
    - T13.1 AOPs — T13.2 cohorts — WP14 biomarkers — CGLs
  - Responsibilities
    - Coordinate discussions within the chemical group
    - Organize and assign tasks to other partners involved within each task or WP (T13.1, T13.2, WP14) (see following slides)
    - Prepare first „draft" document (1-2 pages) addressing the three main questions by the end of April 2018
    - Elaborate inputs to AWP 2019 (and following) by end of May 2018
    - Identify gaps, work together to address the gaps, propose topics for internal calls, etc.

- **STRUCTURED TEMPLATE with instructions** will be provided
  - will allow to structure the work and efficiently collect info
  - Will be drafted by MU within March 2018

9.2.2 Short Overview about new recruitments/cohorts for HBM4EU, by Greet Schoeters (VITO)