HBM4EU

science and policy
for a healthy future

# WP14:

# Applying Translatable and Accessible

# Effect biomarkers

## HBM4EU Training workshop 2021

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Epidemiología y Salud Pública

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# *Biomarker definitions*

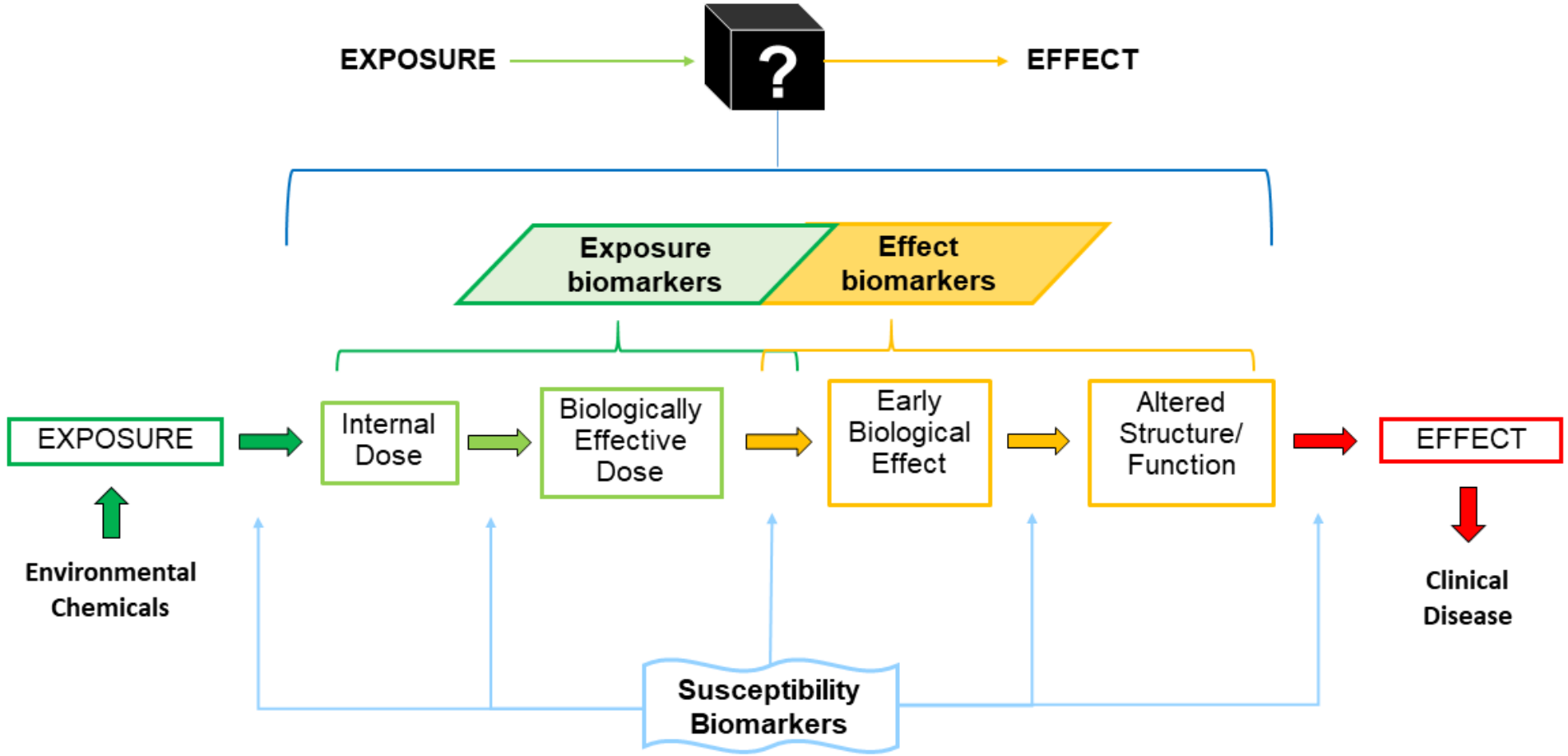
## Types of Biomarkers for Environmental Health Research (WHO, 2001)

***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 recognized as associated with an established or possible health impairment or disease.

***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.

# *The exposure-health continuum seen through the eyes of biomarkers*




**Mustieles et al., 2020**. Bisphenol A and its analogues: a comprehensive review to identify and prioritize effect biomarkers for human biomonitoring. Environment International.

# *What is the overarching goal of effect biomarkers in HBM?*

"*Human biomonitoring (HBM) is a tool of **health-related** environmental monitoring with which **populations are examined for their exposure to pollutants from the environment**. The results are also intended to provide information as to whether (further) pollutant reduction measures are needed and on the effects of existing measures."*

The committee acknowledges that there has been substantial research developing biomarkers further along the exposure-effect continuum, including prominent work by Gan et al. (2004); Hecht (2003); Joseph et al. (2005); Kensler et al. (2005); Qian et al. (1994); Rappaport et al. (2005); and Yu et al. (1995). 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.

→ **HBM4EU** → **Policy strategies**

Human Biomonitoring for Environmental Chemicals. Committee on Human Biomonitoring for Environmental Toxicants. National Research Council, Washington DC, 2006.

# *Biomarkers to evaluate risk*

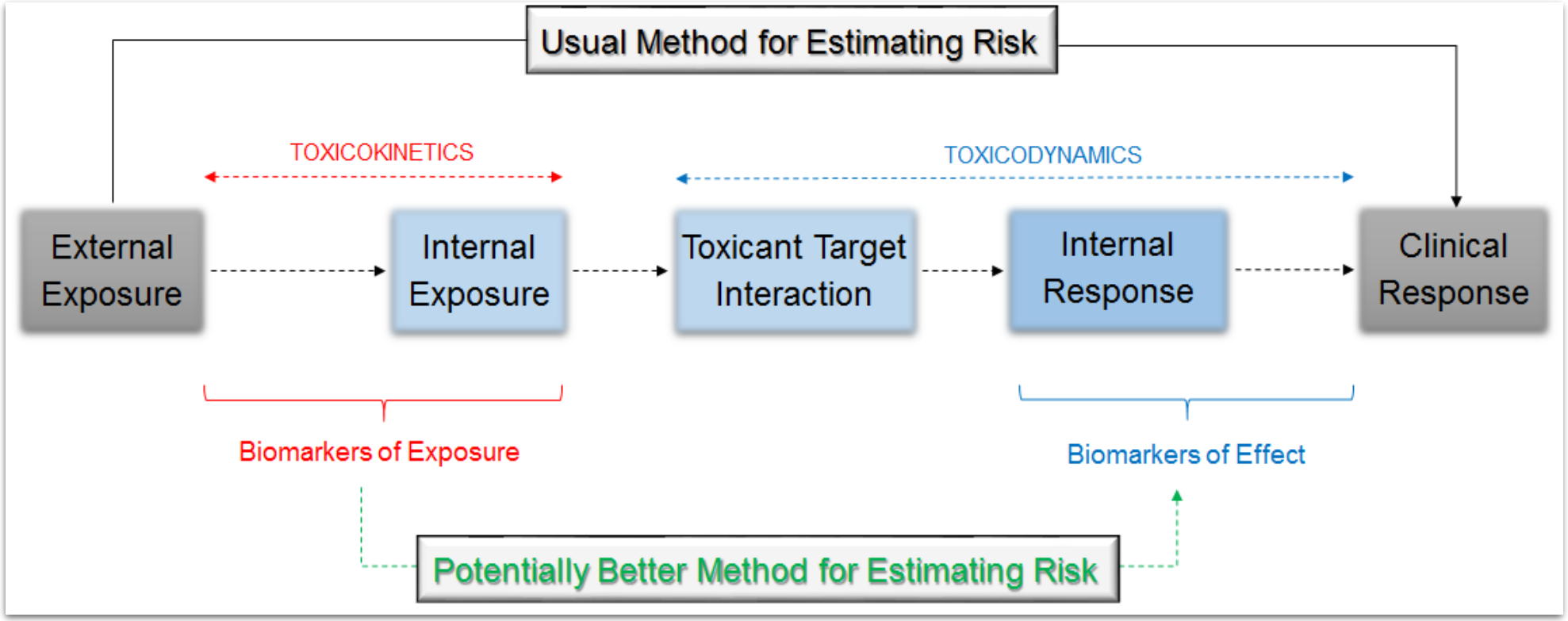


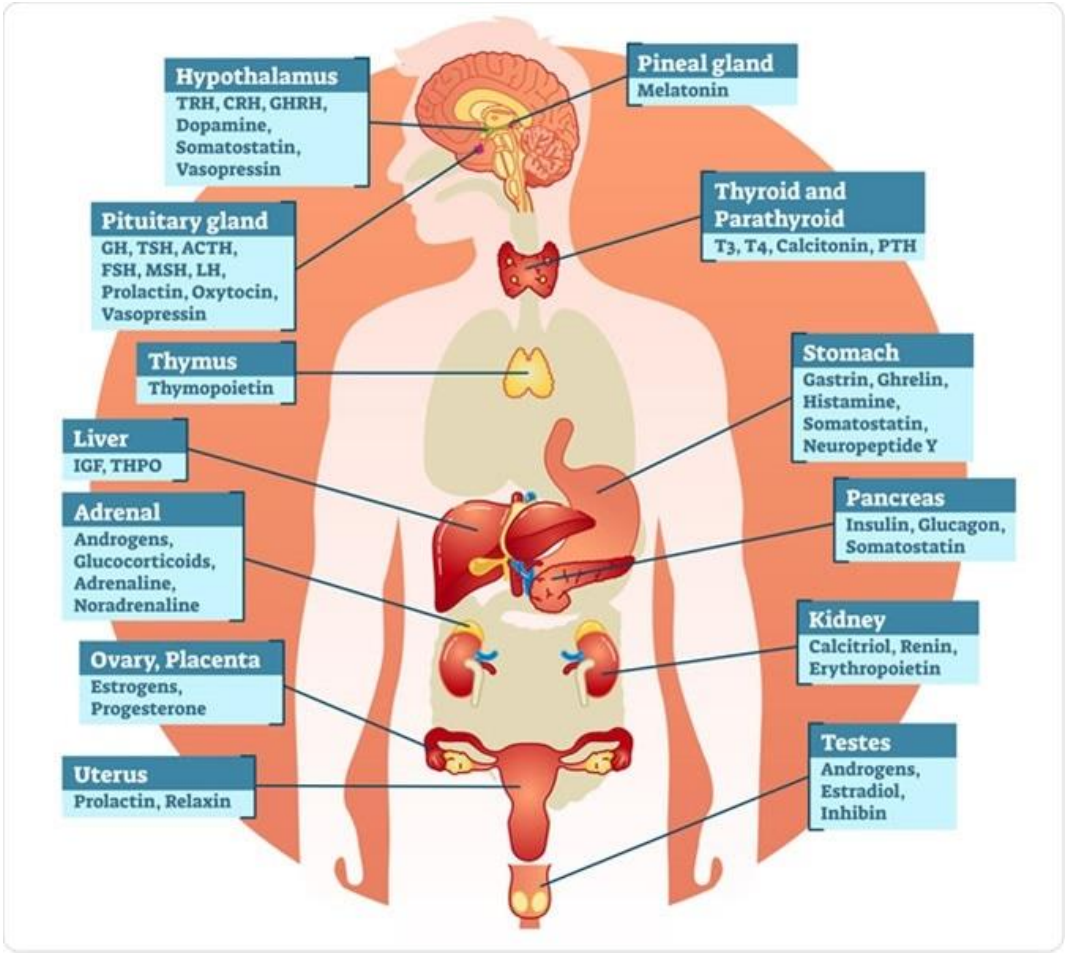

Fig. 1.Rationale for using biomarkers to assess risk (adapted from Schulte and Waters, 1999). Louro et al., 2019. International Journal of Hygiene and Environmental Health.

# Advantages of using effect biomarkers in addition to health endpoints

1. Provide an “early warning” signal for any type of disease (e.g., effect biomarker measured in cord blood at birth that may indicate a greater risk of a later disease).
2. Provide an estimation of risk for health outcomes with a very long latency period such as cancer (e.g., micronuclei assay).
3. Provide objective information on health outcomes that are not easy to evaluate, such as behavior and cognitive function (e.g., neurotrophins such as BDNF to complement neurodevelopmental tests in children, or to predict the risk of long-term cognitive decline in adults).
4. Allow the evaluation of exposure-effect relationships in healthy populations with no apparent disease (e.g., metabolic disease in children)
5. Allow the evaluation of potential mechanisms and dose-response relationships (e.g., type 2 diabetes vs. HOMA-IR)
6. Allow the design of mediation analyses, which can importantly support the biological plausibility of exposure-outcome associations investigated in human populations (e.g., BDNF in INMA-Granada).

# *Main characteristics of effect biomarkers*

1. ***Predictive*** *potential for a* ***future*** *adverse health outcome.*
2. ***Valid*** *for a given chemical or family of chemicals. How to evaluate this? Ideally through AOPs (e.g., KEs).*
3. ***Heterogeneous:*** *There may be as many effect biomarkers as signaling routes in humans (but of course not all parameters are effect biomarkers). Different levels of biological organization.*

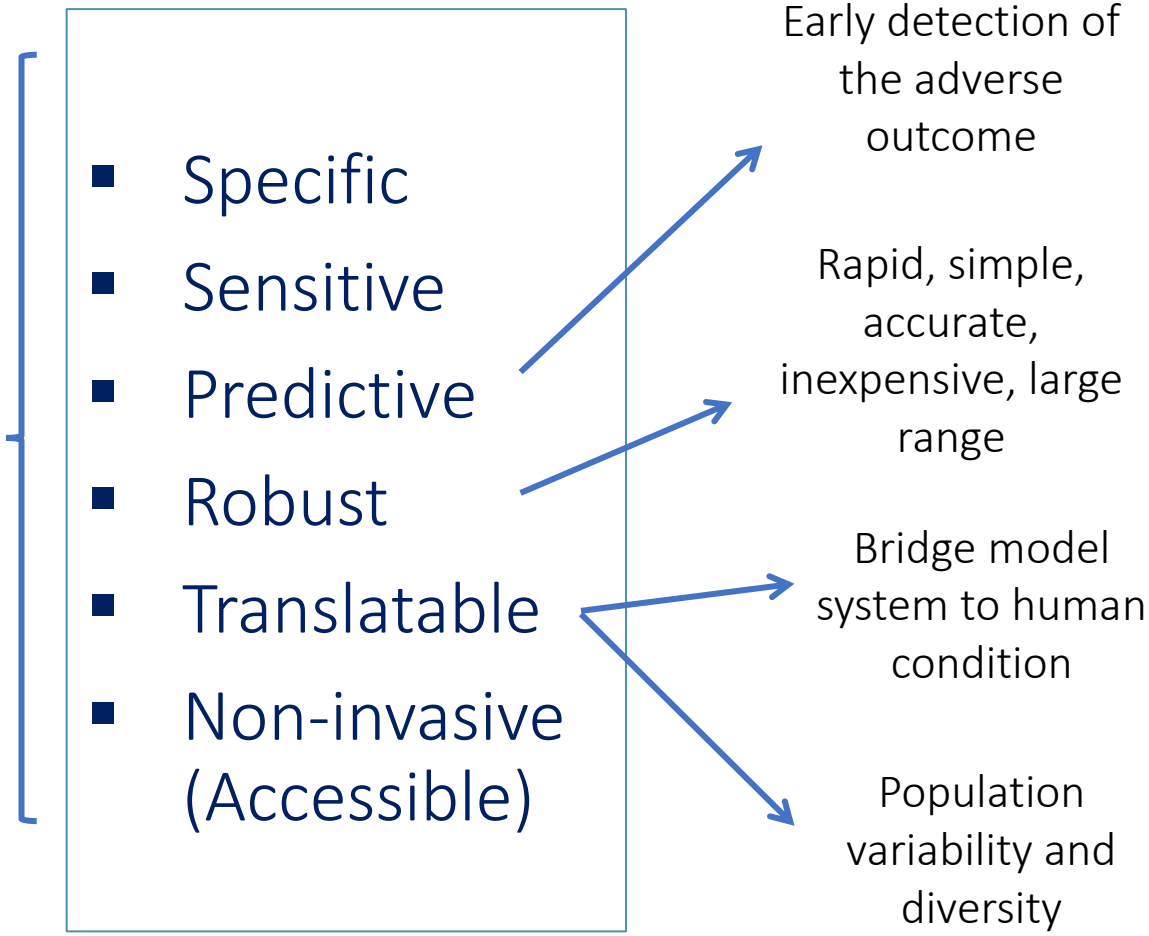



But there is much more…

# *Characteristics of useful effect biomarkers*

Context to use for regulatory decision making for humans: it is **still a big challenge!!**

**Given that there is no perfect effect biomarker**, combining several effect biomarkers at different biological levels of organization seems a good strategy:
- Serum/urine protein
- DNA methylation
- Gene expression
- Novel and classic

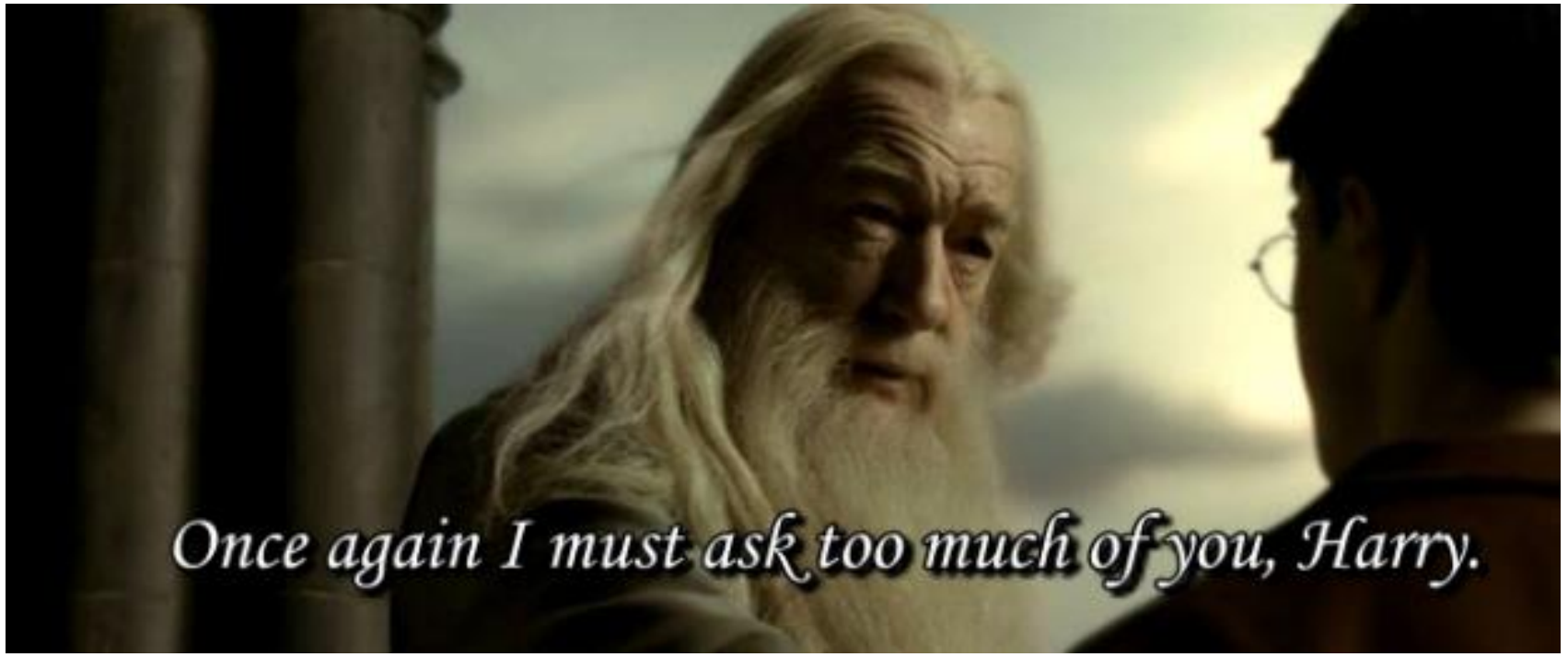


# *Attributes of a useful effect biomarker*

Some considerations to define a valid biomarker to detect chemically-induced adverse outcomes in animals and humans:

1. It should demonstrate the correlation with the response which is trying to predict (with the truth)
2. Acceptable selectivity and specificity
3. Adequate sensitivity
4. The biology and performance of the biomarker are aligned with its use:
   - ✓ Acceptable magnitude of changes in response to the environmental compound
   - ✓ Cover an appropriate measurement range
   - ✓ Acceptable intra- and inter-subject variability associated with the biomarker's baseline and response
5. The availability of reliable and reproducible measurement methodology
6. Analytical and clinical validation should show that the biomarker is appropriated for its proposed use






**Search, Research, Search, Research…**

# WP14 “Effect biomarkers” General Objectives

**1. To create an inventory of effect biomarkers for each chemical family and prioritize its use.**

**2. Identification of gaps in knowledge**

**3. Validation, implementation and development of novel or understudied effect biomarkers useful for HBM**

**4. Contribute to the understanding of exposure-health relationships and mixture effects**

## HBM4EU PRIORITY SUBSTANCES

The first list of HBM4EU priority substances was identified in 2016 and includes:

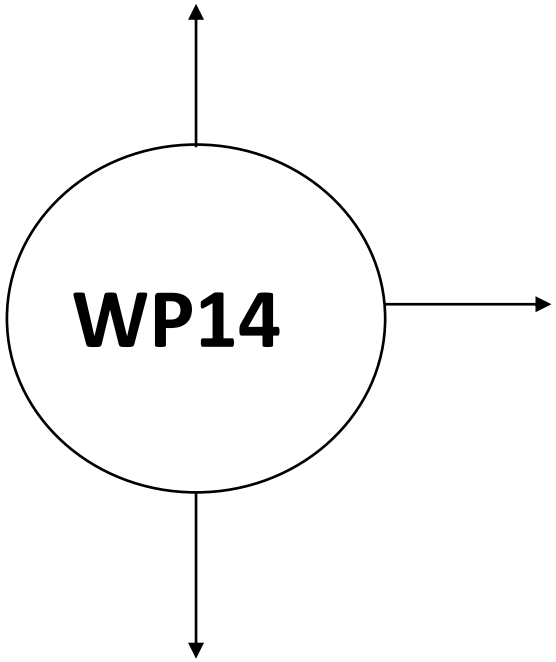
- Aniline family
- Bisphenols
- Cadmium and chromium VI
- Chemical mixtures
- Emerging substances
- Flame retardants
- Polycyclic Aromatic Hydrocarbons (PAHs)
- Per-/poly-fluorinated compounds
- Phthalates and Hexamoll® DINCH

A second round of prioritisation was conducted from 2017 to 2018. The second list of HBM4EU priority substances includes:

- Acrylamide
- Aprotic solvents
- Arsenic
- Diisocyanates
- Lead
- Mercury
- Mycotoxins
- Pesticides
- Benzophenones

1. Literature searches to advance the **KNOWLEDGE**

Scientific corpus → New paradigm→ Inventory → Systematic implementation of effect biomarkers → Regulatory Uptake




**2. TECHNICAL AND PHYSIOLOGICAL VALIDATION & IMPLEMENTATION** of novel effect biomarkers

Evaluate the **REALIABILITY OF MEASURES & BIOLOGICAL PLAUSIBILITY** of human exposure-outcome associations

3. In vitro/in vivo **DEVELOPMENT** of novel biomarkers

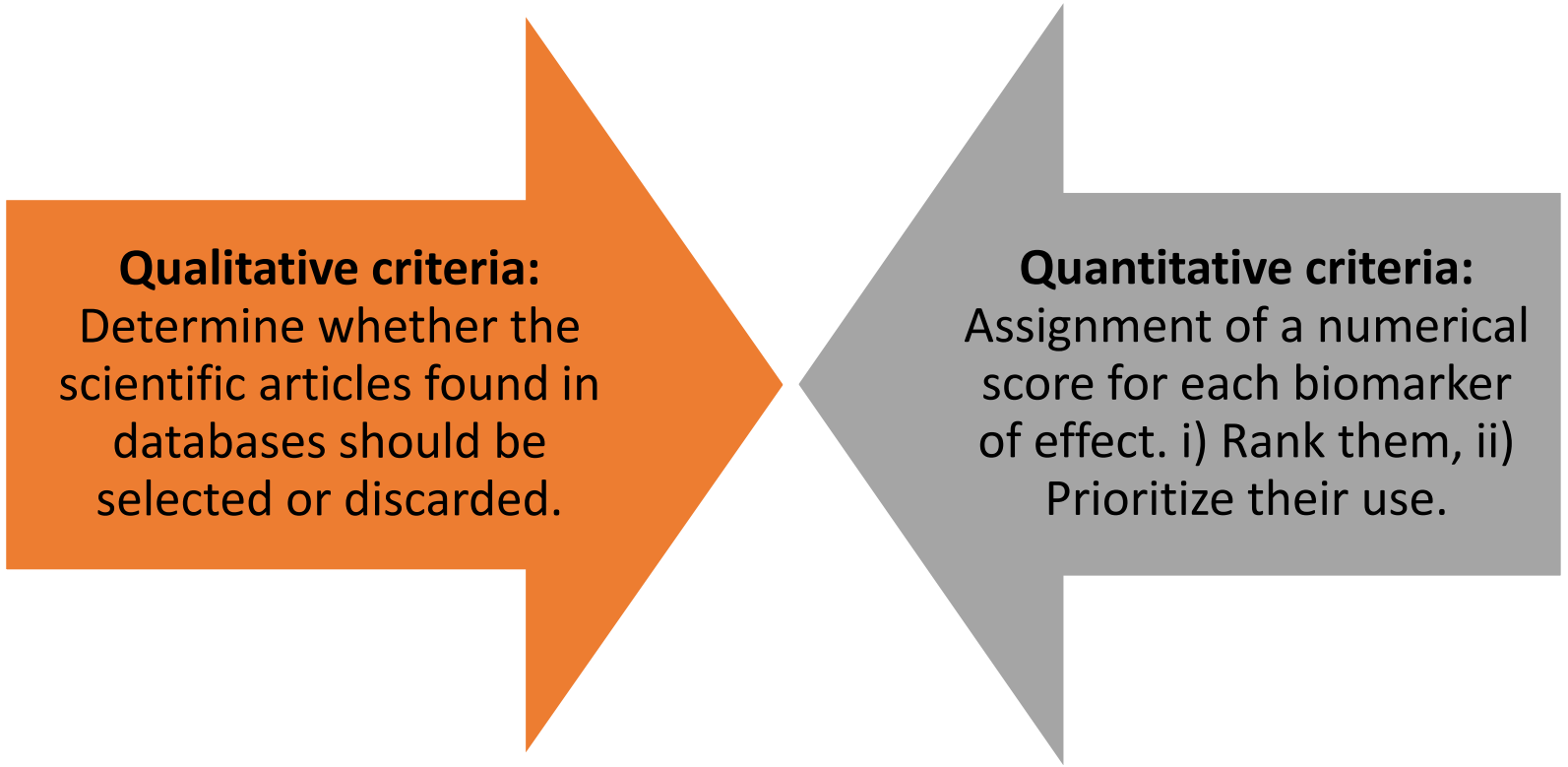
Biomarkers of combined effect to chemical mixtures
Biomarker discovery

# 1. Scientific Corpus: Literature searches, Inventory, Prioritization

## D14.1. ‘’Criteria for prioritization of biomarkers of effect’’

*“To set up relevant criteria for prioritization of biomarkers of effect that will be searched in the scientific literature, related to the 1st set of prioritized substances in the HBM4EU project’’*

- Qualitative and Quantitative Criteria: Scoring tables



# QUANTITATIVE CRITERIA

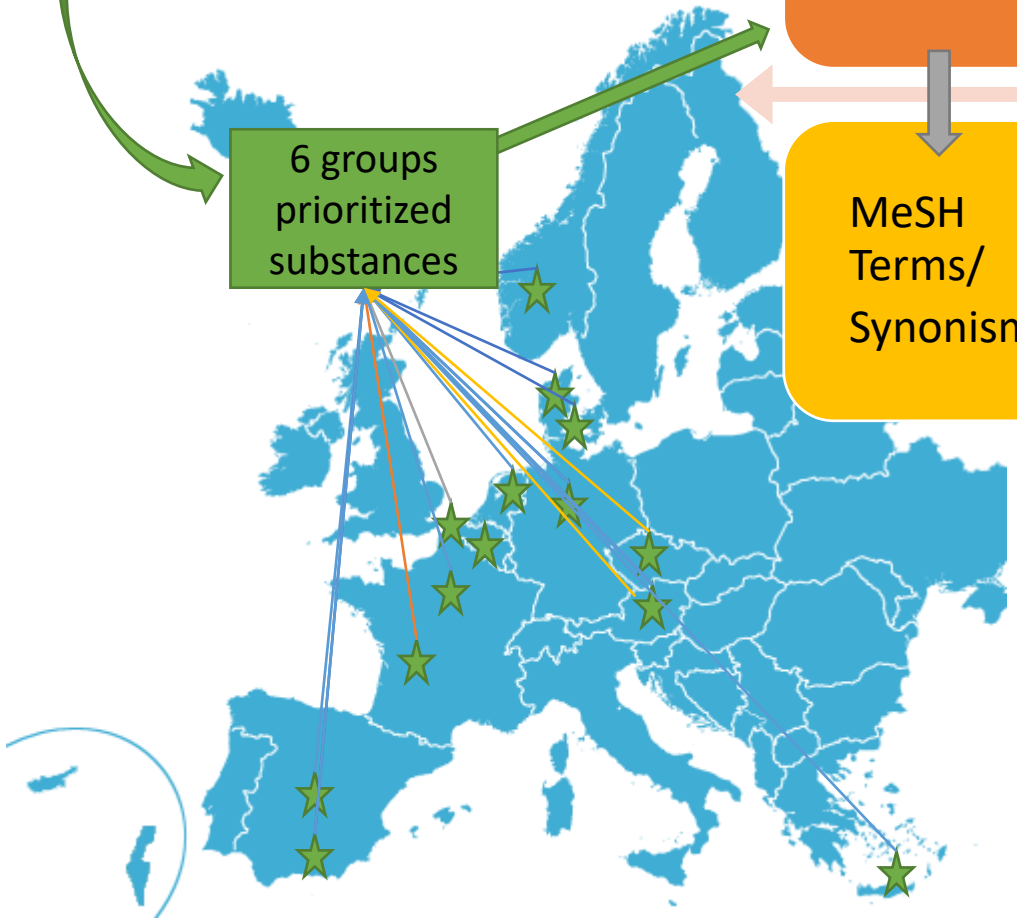
| WP14 TASK 14.1 LITERATURE SEARCH. QUANTITATIVE CRITERA FOR THE PRIORITIZATION OF EFFECT BIOMARKERS | | | Score |
|---|---|---|---|
| Has the biomarker been assessed in human matrices? | Non-invasive | Urine(5p) | |
| | | Saliva(4p) | |
| | | Placenta(2p) | |
| | Invasive | Serum(3p) | |
| | | Others(1p) | |
| Is there a plausible mechanism of action (MoA)? | Yes(2p) please, report | | |
| | No (0p) | | |
| Is there a described AOP for this biomarker of effect? | Yes; report (3p)/email link | | |
| | No (0) | | |
| Has the biomarker been implemented in epidemiologic studies? | YES (Please, proporcionate the DOI; 5p) | | |
| | NO(0p) | | |
| How would you define the feasibility, based on cost, efficacy, specificity, sensitivity and reliability of the biomarker? | Unsure(0p); Indicate your concerns | | |
| | Low(0p) | | |
| | Middle(2p) | | |
| | High (5p) | | |
| TOTAL Score (Max. 20) | | | |

# D14.2 METHODOLOGY

Wide (>**66.000 references**) and comprehensive Literature Searches
→ PubMed Database

16 Partners

6 groups prioritized substances



**Exposure:** Chemicals Families

**Effect:** Health Endpoints

MeSH Terms/ Synonisms

**References** division based on expertise

| Health Outcome |
| --- |
| Neurodevelopment |
| Reproductive Diseases |
| Endocrine Diseases |
| Obesity and Cardio-Metabolic Diseases |
| Allergy-Immunological Diseases |
| Cancer |

**Granada Workshop**

# Overall results of these searches:

1. Inventory of effect biomarkers (D14.3)

2. Specific review works adapted to each chemical family

3. Prioritization of effect biomarkers for the HBM4EU aligned studies

# Inventory of biomarkers D14.3 → Delivered

## Aligned studies: Proposed list of effect biomarkers based on chemical family, outcome (health endpoint) and age subgroup

| Cohort Age / Chemicals | Health Endpoint | Children (6-11 y) 2700-3000 participants | Adolescents (12-19 y) 2700-3000 participants | Adults (20-40 y) 2700-3000 participants |
|---|---|---|---|---|
| **Phthalates, DINCH** | Neurodevelopment | **Neurobehavioral Tests:**<br>CBCL(6-18): Child Behavior Checklist<br>WISC-IV (6-16y) Wechler Intelligence Scale for Children<br>**Neurodevelopment**<br>Brain Derived Neurotrophic Factor (BDNF), GDNF or Sp4 | | *Not measured in adults* |
| | Reproductive | **Reproductive Hormones (Serum):**<br>Luteinizing hormone (LH),<br>Follicle Stimulating Hormone (FSH),<br>Sex hormone-binding globulin (SHBG),<br>Total testosterone (TT), Estradiol (E2).<br>Serum Kisspeptin (KISS)<br>**Gene expression of nuclear receptors in blood** (monocytes, lymphocytes):<br>Estrogen receptors (ER) α and β, Androgen receptor (AR), Arylhydrocarbon Receptor (AhR), Pregnane X receptor (PXR), Peroxisome proliferator-activated receptor (PPAR) γ | | |
| | Endocrine | **Thyroid hormones levels (Serum):**<br>Thyroid Stimulating Hormone (TSH),<br>Triiodothyronine ($T_3$), Thyroxine ($T_4$) | | |
| | Metabolic-Obesity | **Glucose metabolism (Serum):**<br>Fasting blood glucose (FBG); Fasting insulin levels; Glycated haemoglobin (HbA1c); Homeostatic model assessment (HOMA); HOMA-IR= (Fasting blood glucose (FBG) x Insulin) / 450).<br>**Serum Lipids:**<br>Low density lipoprotein (LDL), high density lipoprotein (HDL), total cholesterol (TC) and triglycerides (TG)<br>**Adipokines (Serum):**<br>Adiponectin, Leptin<br>**Anthropometrics:**<br>BMI z-score; Body fat mass; Blood pressure | | |
| | Allergy/Immune | Serum Immunoglobulin E (IgE) | | |

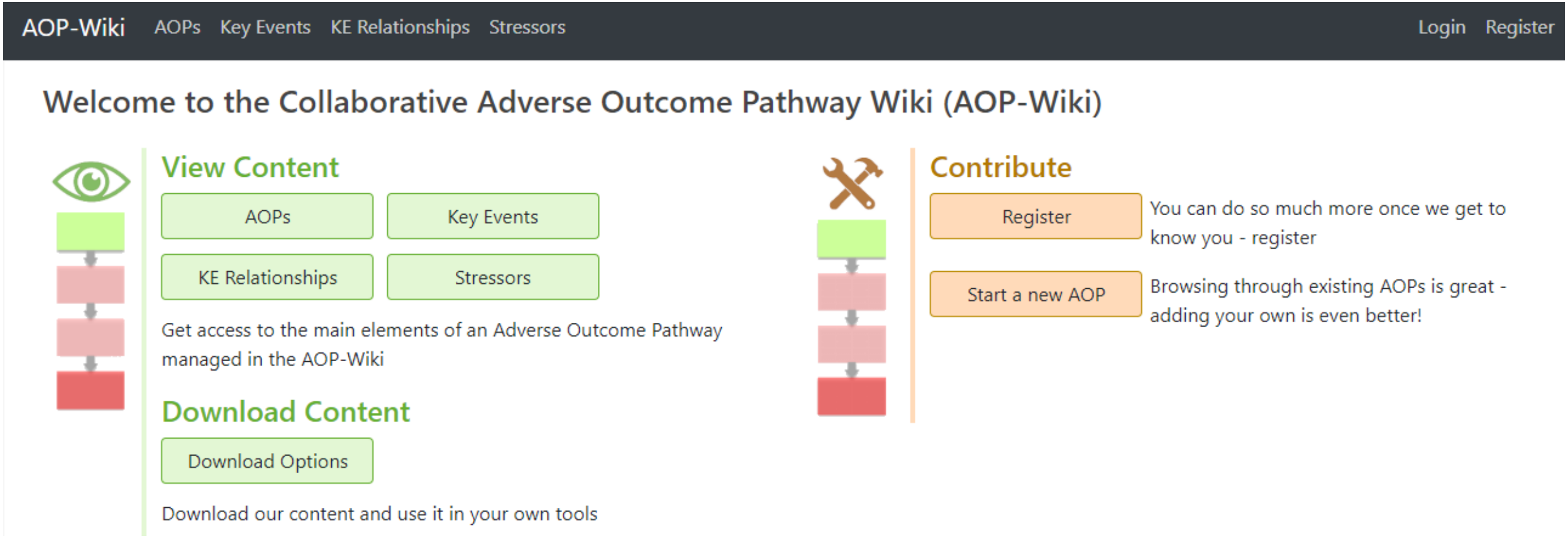
| Cohort Age / Chemicals | Health Endpoint | Children (6-11 y) | Adolescents (12-19 y) | Adults (20-40 y) |
|---|---|---|---|---|
| **Per- poly fluorinated compounds** | Neurodevelopment | *Not measured in children* | **Neurodevelopment Test**: WISC-IV (6-16y)<br>**Neurodevelopment**<br>Brain Derived Neurotrophic Factor (BDNF), GDNF or Sp4 | *Not measured in adults* |
| | Reproductive | | **Reproductive Hormones:**<br>LH, FSH, E2, TT, SHBG | |
| | Endocrine | | **Thyroid Hormones:**<br>TSH, T3, T4 | |
| | Metabolic-Obesity | | **Glucose metabolism**<br>FBG, Fasting insulin levels; HbA1, HOMA-IR<br>**Serum Lipids:**<br>LDL, HDL, TC, TG<br>**Liver Enzymes:**<br>Alanine transaminase (ALT), Aspartate transaminase (AST), Alkaline phosphatase (ALP), Gamma-glutamyl transferase (GGT), Serum bilirubin.<br>**Adipokines:**<br>Adiponectin, leptin<br>**Others:**<br>BMI z-scores; Body fat; Blood pressure.<br>**Gene expression of nuclear receptors in blood** (monocytes or lymphocytes or whole blood):<br>PPAR α, γ, δ,<br>**Genes of cholesterol metabolism:** NR1H2 (LXRB), NR1H3 (LXRA), NCEH1, ABCG1 and NPC1 | |
| | Allergy-Immune | | **Serum IgE**, Absolute eosinophil counts (**AEC**), eosinophilic cationic protein (**ECP**). Basophils count. **Lymphocytes subpopulation (**B cells, CD4-positive T helper cells.<br>**Serum antibody** concentrations against common infectious. | |

| Cohort Age / Chemicals | Health Endpoint | Children (6-11 y) | Adolescents (12-19 y) | Adults (20-40 y) |
|---|---|---|---|---|
| **Bisphenols** | Reproductive | *Not measured in children* | *Not measured in adults* | **Reproductive Hormones (Serum):**<br>LH, FSH, E2, TT, SHBG<br>KiSS<br>**Semen quality**<br>**Gene expression of nuclear receptors in blood** (monocytes or lymphocytes):<br>ERα and β, AR, ESRRα, ESRRβ |
| | Endocrine | | | **Thyroid Hormones:**<br>TSH, T3, T4 |
| | Obesity-Metabolic-Cardiovascular | | | **Glucose Metabolism:**<br>FBG, Fasting insulin levels; HbA1, HOMA-IR<br>**Serum Lipids:**<br>LDL, HDL, TC, TG<br>**Liver Enzymes:**<br>ALT, AST, ALP, GGT, Serum bilirubin.<br>**Anthropometrics:**<br>BMI z-scores; Body fat; Blood pressure<br>**Chronic inflammation:**<br>C reactive protein (CRP)<br>IL-6, TNF-α). |
| | Allergy/Immune | | | Serum IgE |
| | Hormone-dependent cancers | | | **Urinary hydroxyestrogens:**<br>2-metoxyestrone<br>2-metoxyestradiol<br>4-metoxyestrone<br>4-metoxyestradiol<br>16-alpha-hydroxyestrone |

| Cohort Age / Chemicals | Health Endpoint | Children (6-11 y) | Adolescents (12-19 y) | Adults (20-40 y) |
|---|---|---|---|---|
| **Flame retardants** | Neurodevelopment | WISC-IV (6-16y)<br>**Neurodevelopment**<br>Brain Derived Neurotrophic Factor (BDNF), GDNF or Sp4 | *Not measured in adolescents* | *Not measured in adults* |
| | Reproductive | **Reproductive hormones (Serum):**<br>LH, FSH, SHBG, TT, E2 | | |
| | Endocrine | **Thyroid Hormones (Serum):**<br>TSH, $T_3$, $T_4$ | | |
| | Metabolic-Obesity | **Glucose metabolism (Serum):**<br>FBG, Fasting insulin levels; HbA1, HOMA-IR<br>**Serum Lipids:**<br>LDL, HDL, TC, TG<br>**Adipokines (Serum):**<br>Adiponectin, Leptin<br>**Inflammation (Serum):**<br>**CRP** (C-reactive protein)<br>**Anthropometrics:**<br>BMI z-score; Body fat mass | | |
| | Allergy/Immune | Serum IgE | | |

| Cohort Age / Chemicals | Health Endpoint | Children (6-11 y) | Adolescents (12-19 y) | Adults (20-40 y) |
|---|---|---|---|---|
| **PAHs** | Cancer *(Blood or Urine samples)* | *Not measured in children* | *Not measured in adolescents* | **DNA damage**<br>Urinary 8-oxo-deoxyguanosine concentrations (8-OHdG)<br>**DNA damage and genotoxicity (serum):**<br>Comet Assay **or**<br>CBMN assay (Lympochyte Cytokinesis-Block Micronucleous Assay). |
| | Respiratory health *(Blood or Urine sample)* | | | **Pulmonar function:**<br>**CC16**: Club cell secretory protein (Serum and/or urine) |
| **Cohort Age / Chemicals** | **Health Endpoint** | **Children (6-11 y)** | **Adolescents (12-19 y)** | **Adults (20-40 y)** |
| **Cadmium** | Renal Function | *Not measured in children* | *Not measured in adolescents* | **Markers of tubular damage:**<br>Urinary Beta-2 microglobulin (B2MG) concentrations<br>Urinary N-acetyl-β-D glucosaminidase (NAG) concentrations<br>**Markers of glomerular function:** Urinary albumin concentrations<br>**Novel markers:** Urinary levels of Kidney Injury Molecule (KIM-1) and Cystatin C. |
| | Oxidative Stress | | | Urinary levels of 8-OHdG, 8-isoprostane |

# **WP13-WP14 Interaction**: AOPs to help the identification and prioritization of epidemiologic effect biomarkers



# Some examples of the reviews published inside WP14

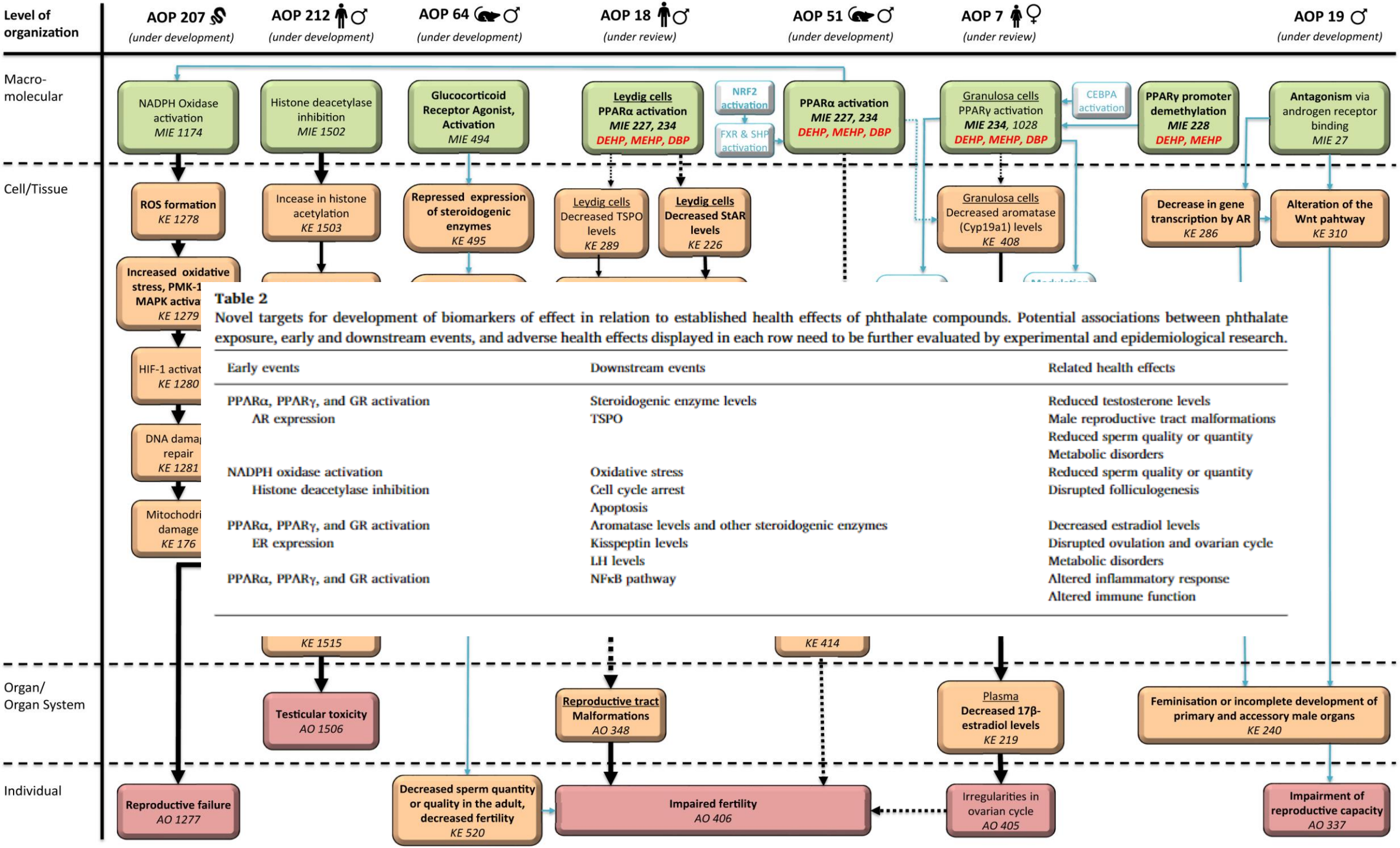



**Table 2**

Novel targets for development of biomarkers of effect in relation to established health effects of phthalate compounds. Potential associations between phthalate exposure, early and downstream events, and adverse health effects displayed in each row need to be further evaluated by experimental and epidemiological research.

| Early events | Downstream events | Related health effects |
|---|---|---|
| PPARα, PPARγ, and GR activation<br>AR expression | Steroidogenic enzyme levels<br>TSPO | Reduced testosterone levels<br>Male reproductive tract malformations<br>Reduced sperm quality or quantity<br>Metabolic disorders |
| NADPH oxidase activation<br>Histone deacetylase inhibition | Oxidative stress<br>Cell cycle arrest<br>Apoptosis | Reduced sperm quality or quantity<br>Disrupted folliculogenesis |
| PPARα, PPARγ, and GR activation<br>ER expression | Aromatase levels and other steroidogenic enzymes<br>Kisspeptin levels<br>LH levels | Decreased estradiol levels<br>Disrupted ovulation and ovarian cycle<br>Metabolic disorders |
| PPARα, PPARγ, and GR activation | NFκB pathway | Altered inflammatory response<br>Altered immune function |

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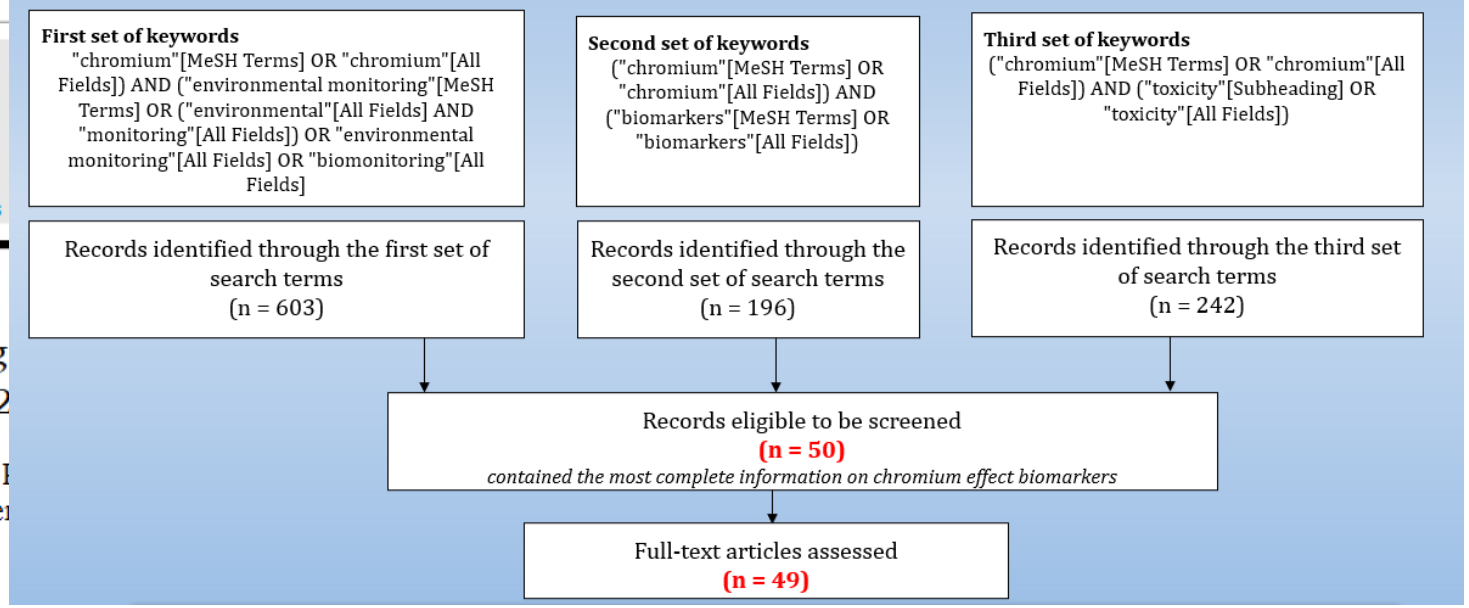
Review article

# Biomarkers of effect as determined in human biomonitoring hexavalent chromium and cadmium in the period 2008–202

Célia Ventura [a], Bruno Costa Gomes [a], Axel Oberemm [b], Henriqueta Louro [a], I Vicente Mustieles [d,e,f], Mariana F. Fernández [d,e,f], Sophie Ndaw [g], Marcel Mer Mirjam Luijten [i], Claudia Gundacker [j,*,1], Maria João Silva [a,**,1]




## Most frequent effect biomarkers – oxidative stress b

| | # of studies in the search | Hazard/ H |
|---|---|---|
| 8-OHdG (oxidized DNA) | 11 | Mutagenicity |
| MDA (malondialdehyde) | 8 | Oxidative stress, Muta Immu |
| GSH (glutathione) | 3 | Oxidative stress, Muta |
| SOD (superoxide dismutase) | 3 | Oxidative stress, Muta Immu |
| LPO (lipid peroxidation) | 3 | Oxidative stress, Muta Immu |

## Most frequent effect biomarkers – Genotoxicity

| | # of studies in the search | Hazard/ Health Outcome |
|---|---|---|
| Comet assay (ss/dsDNA breaks) | 10 | Genotoxicity |
| Micronucleus test (chromosome breaks/ loss) | 8 | Genotoxicity/Carcinogenicity |

# Novel effect biomarkers –pros & cons

| Novel effect biomarker | Brief description of the effect biomarker |
|---|---|
| **Gene expression**<br>- DNA repair<br>- detoxifying genes<br>**(Pizzino et al. 2014)** | Related with the **Cr(VI) MoA** (oxidative stress induct<br>**Strengths**:<br>- low invasiveness: blood samples<br>- low cost, depending of the number of genes studied<br>**Limitations**:<br>- low specificity |
| **Epigenetics**<br>**DNA methylation levels**<br>**(Linqing et al. 2016)** | Related with the **Cr(VI) MoA** (epigenetic effects)<br>**Strengths**:<br>- low invasiveness: blood samples<br>**Limitations**:<br>- low specificity<br>- costs are still high. |

- Plausible AOPs for the main health adverse outcomes reported in animal and/or human studies for Cr(VI) relate to:

✓Cancer
✓Respiratory tract sensitization
✓Sensitization of the skin

- **Cancer:** AOP 139 (Alkylation of DNA leading to cancer 1, under development)

Events: Molecular Initiating Events (MIE) Key Events (KE) Adverse Outcomes (AO)

| | Sequence | Type | Event ID | Title | Short name |
|---|---|---|---|---|---|
| Molecular initiating event | 1 | MIE | 97 | Alkylation, DNA | Alkylation, DNA |
| Key events | 2 | KE | 155 | N/A, Inadequate DNA repair | N/A, Inadequate DNA repair |
| | 3 | KE | 185 | Increase, Mutations | Increase, Mutations |
| Adverse outcome | 4 | AO | 885 | Increase, Cancer | Increase, Cancer |

**Genotoxicity**

https://aopwiki.org/wiki/index.php/Aop:139

# Application inside HBM4EU

Setting up a collaborative European human biological monitoring study on occupational exposure to hexavalent chromium

Tiina Santonen[1], Alessandro Alimonti[2], Beatrice Bocca[2], Radu Corneliu Duca[3], Karen S. Galea[4], Lode Godderis[3,5], Thomas Göen[6], Bruno Gomes[7], Ogier Hanser[8], Ivo Iavicoli[9], Beata Janasik[10], Kate Jones[11], Mirja Kiilunen[1], Holger M. Koch[12], Elizabeth Leese[11], Veruscka Leso[9], Henriqueta Lauro[7], Sophie Ndaw[8], Simo P. Porras[1], Alain Robert[8], Flavia Ruggieri[2], Paul T.J. Scheepers[13], Maria J. Silva[7], Susana Viegas[14], Wojciech Wasowicz[10], Argelia Castano[15], Ovnair Sepai[16]

- **Exposure biomarkers + Effect biomarkers,** namely:
  - Comet assay in leuko cytes (INSA)
  - Micronucleus in PBL (INSA) + in reticulocytes (FIOH)
  - Oxidative stress in urine (INRS)
  - Epigenetic markers: Global methylation (KuLeuven) + Gene-specific methylation (KuLeuven, INSA)
  - Telomere lenght (NIOM)

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Review article

# Bisphenol A and its analogues: A comprehensive review to identify and prioritize effect biomarkers for human biomonitoring

Vicente Mustieles[a,b,c,1,*], Shereen Cynthia D'Cruz[d,1], Stephan Couderq[e,1], Andrea Rodríguez-Carrillo[a], Jean-Baptiste Fini[e], Tim Hofer[f], Inger-Lise Steffensen[f], Hubert Dirven[f], Robert Barouki[g], Nicolás Olea[a,b,c], Mariana F. Fernández[a,b,c,2,*], Arthur David[d,2,*]

More than 5000 references screened. More than 100 epidemiologic studies tabulated in detail. An inventory of molecular and biochemical effect biomakers. BDNF and Kisspeptin prioritized using AOP and toxicological data.

# Effect biomarkers obtained from the literature searches related to bisphenols.

## Traditional (and studied) Effect biomarkers

**Reproductive Hormones:** LH, FSH, TT, E2, SHBG

**Thyroid Hormones:** TSH, T3, T4

**Glucose metabolism:** (FBG + Insulin = HOMA-IR) + HbA1c

**Serum lipids:** Total cholesterol, LDL, HDL, TG

**Blood pressure**

**Anthropometric measurements:**
Anogenital distance (AGD);
Waist circumference; Height/Weight;
Percentage of Body fat; Skinfold-thickness;
Birth weight; Head circumference; Birth lenght

## Traditional (less studied) Effect biomarkers

**HPAdrenal-Axis:** CRH - ACTH - Cortisol + Adrenal Androgens (DEAH-S)

**Adipokines:** Leptin and Adiponectin

**Inflammatory markers:** hsCRP, IL-6…

**Liver enzymes:** AST, ALT, ALP, GGT, Bilirrubin

**Renal function:** Urinary albumin, β2-microglobulin, NAG

**Urinary 8-OHdG + 8-isoprostane**

**Others:** IgE, vitamin D (25-OH-D)

## Novel Effect biomarkers

**Kisspeptin**

**Gene expression of nuclear receptors:** ERα, ERβ, AR, ESRRA, ESRRB, PPAR-γ, AhR, TR, GR, ABCG1, NPC1, Genes of cholesterol pathways
**BDNF,GDNF, Sp4**

**OMICS-Epigenetic markers,** such as DNA methylation and micro-RNAs, among others

**Genetic polymorphisms:** CYP17A1, ESR CYP17A1

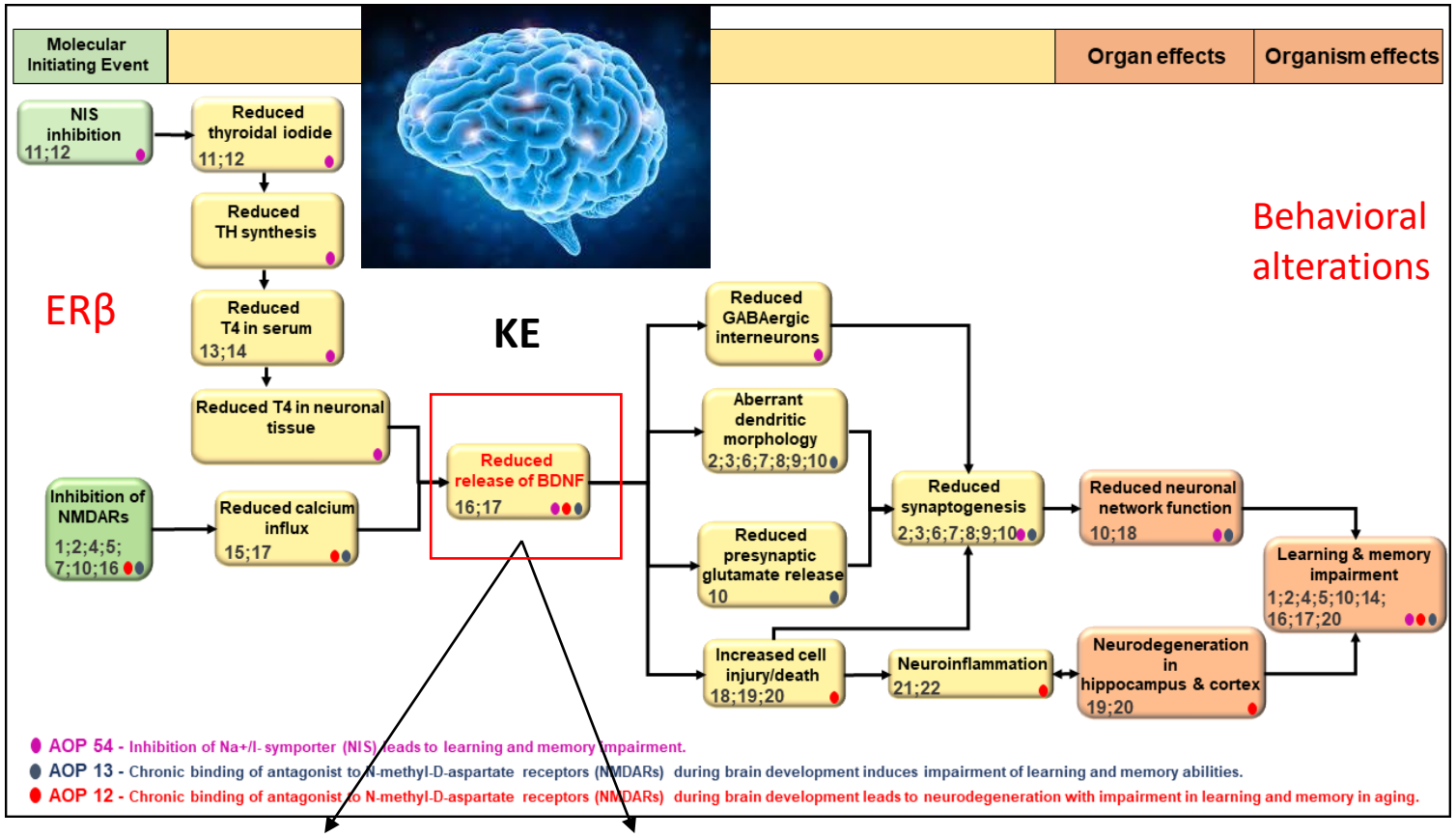
**Important knowledge gaps:** neurodevelopment (BDNF)

**Table 1**
Inventory of bisphenol-related epigenetic and oxidative stress effect biomarkers identified in HBM studies.

| Biomarkers | Matrix | Health endpoint | Number of studies | Strengths | Limitations | Conclusions |
|---|---|---|---|---|---|---|
| | | | | **Epigenetic/Gene expression Biomarkers** [a] | | |
| DNA methylation of BDNF Region IV | Blood | Neurodevelopmental disorders | 1 (Table S2) | DNA methylation is stable over time compared to gene expression or circulating protein levels, which are subjected to short-term variations. BDNF pathway alteration can affect long-term memory, learning, and depression and anxiety disorders. In mice, DNA methylation of BDNF in hippocampus is correlated with blood methylation. | DNA methylation regions should be carefully selected, mainly the promoter regions, so the status of DNA methylation is related with its gene expression.Although the DNA methylation status of BDNF in blood is a promising biomarker for brain function, its predictive potential and role is not fully understood. | Neurotrophins like BDNF constitute potential effect biomarkers of brain function for bisphenols. Molecular/biochemical biomarkers of brain function constitute an important knowledge gap. The potential of this novel biomarker warrants further research at different biological levels (DNA, RNA, protein…) in HBM studies. |
| Gene expression of nuclear receptors (ERs, ERRs, AR, TRs, AhR, PPARs) | Blood and Semen | Reproduction Metabolic disorders | 2 (Table S2) | Gene expression of nuclear receptors and other targets in PBMCs could be a surrogate of their gene expression in target organs, providing relevant data on potential mechanisms of action. | In most cases, the predictive potential for a given disease is unknown. Notwithstanding, emerging data is supporting their suitability for specific health endpoints. | Although their predictive potential is uncertain, when combined with other related molecular or biochemical effect biomarkers, gene expression markers in PBMCs can help to identify potential mechanisms and increase the biological plausibility of epidemiologic associations. |
| KiSS gene expression | Placenta | Pregnancy adverse outcomes/ Reproduction Disorders | 1(Table S2) | *KISS1* is a major regulator of puberty onset and other reproductive functions. Kisspeptin neuron stimulation is an essential event upstream of GnRH pulse release from the HPG axis, and BPA has been shown to adversely affect kisspeptin neuronal system. Therefore, *KISS1* expression could serve as an early indicator of reproductive dysfunctions associated with BPA exposure. | Kisspeptin carries out a variety of physiological functions from reproduction to metabolism. So precisely identifying the health issue associated with *KiSS* deregulation may be difficult. | Kisspeptin gene dysregulation could be a very early indicator of HPG axis dysfunction and its downstream hormonal events associated with reproduction. Since BPA is a recognized reprotoxicant, assessment of *KISS1* in combination with other biomarkers could help to map the key events underlying BPA's adverse reproductive effects. |
| Sperm epigenetic marks (LINE-1 methylation and 5-hydroxy-methylcytosine) | Sperm | Reproduction Disorders | 1(Table S2)<br>1(Table S2) | LINES are a group of long terminal repeats and their methylation status could serve as a surrogate measure of global DNA methylation. 5-hydroxymethylcytosine (5hmC), also called as DNA hydroxymethylation, is an intermediate | A limitation of assessing LINEs is their lack of specificity. LINEs are repeat elements, and mapping their genome location would be difficult. Although a global loss of 5hmC has been observed in some cancers, its | Semen constitutes a non-invasive sample that can provide effect data at different levels of organization: from cell counts and functional aspects, to seminal hormones, and sperm epigenetic and gene expression |

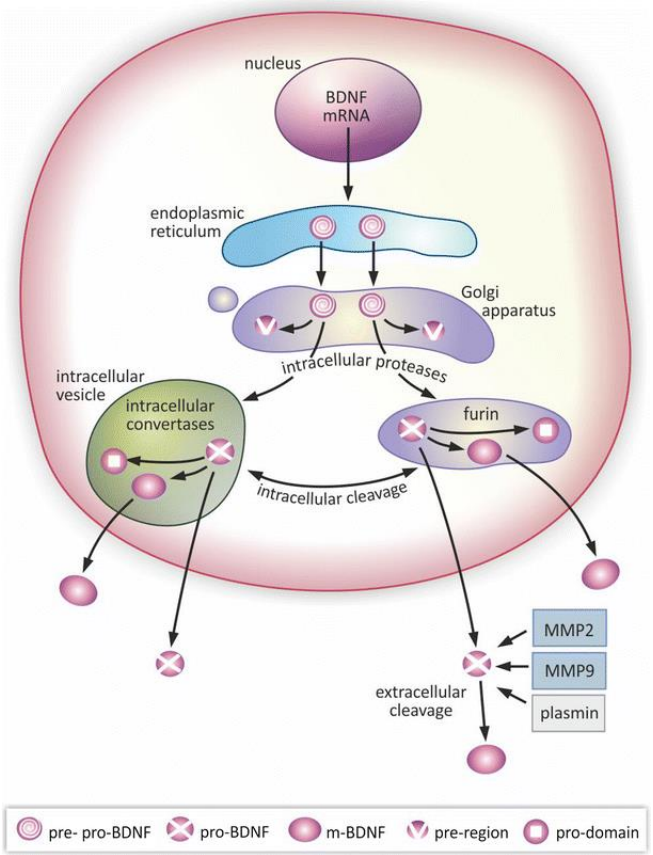
# Why BDNF as a promising effect biomarker?

*Mustieles et al., Bisphenol A and its analogues: A comprehensive review to identify and prioritize effect biomarkers for human biomonitoring. Environ Int. 2020;144:105811.*




**Serum BDNF** **Blood DNA methylation at the IV region (promoter) of the BDNF gene (Kundakovic et al., 2015)**

# Brain derived nuerotrophic factor (BDNF): A key regulator of brain signaling and neuronal plasticity




- Neurotrophin
- Ubiquitous brain functions (hippocampus)
- Age-specific function (fetus, newborn, children, adolescence and adults).
- Alterations linked to cognitive, behavioral and psychiatric conditions.
- WP14 learned lessons: one target, different levels of biological organization

*Kowiański et al., BDNF: A key factor with multipotent impact on brain signaling and synaptic plasticity. Cell Mol Neurobiol. 2018; 38(3):579-593.*

# 2. Technical Validation - Example with BDNF

Can it be measured in human samples?
In which type of samples?
At what levels of biological organization?
What is the temporal stability of each biomarker?
There are comercial ways of measuring it?
Measures are realiable?
What quality control will be followed?

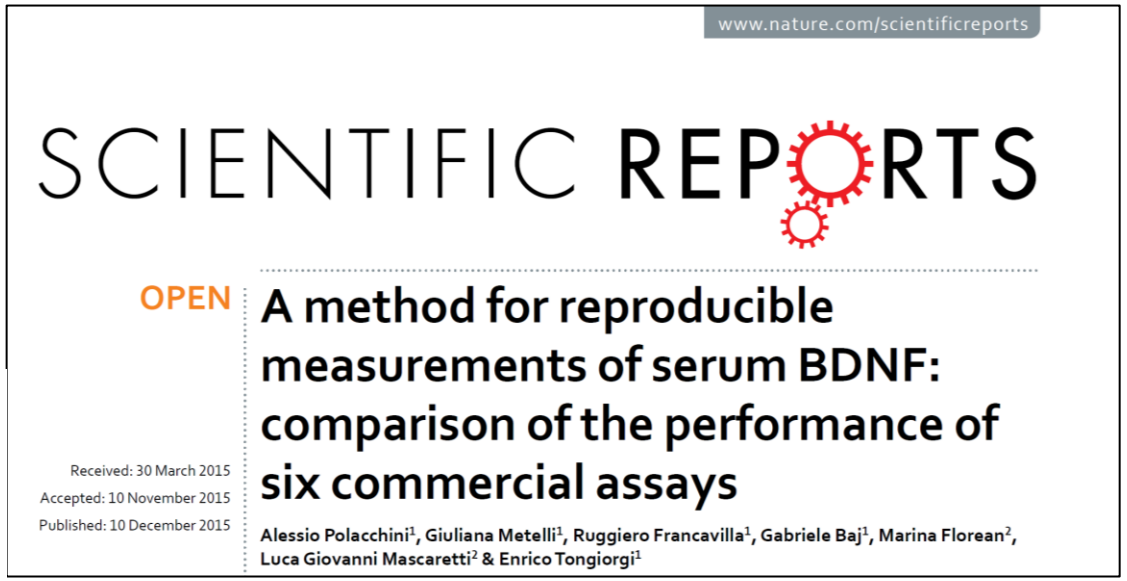
**D14.7 Intra-laboratory quality control measures for effect biomarkers: fine-tuning, precision (intra- and inter-assay variability) and accuracy**

# BDNF measured in serum -Immunosorbent assay

**Quantikine® ELISA**

**Total BDNF Immunoassay**

Catalog Number DBNT00
SBNT00
PDBNT00



www.nature.com/scientificreports

SCIENTIFIC REPORTS

OPEN

**A method for reproducible measurements of serum BDNF: comparison of the performance of six commercial assays**

Received: 30 March 2015
Accepted: 10 November 2015
Published: 10 December 2015

Alessio Polacchini[1], Giuliana Metelli[1], Ruggiero Francavilla[1], Gabriele Baj[1], Marina Florean[2], Luca Giovanni Mascaretti[2] & Enrico Tongiorgi[1]

# *Feasibility & Stability*

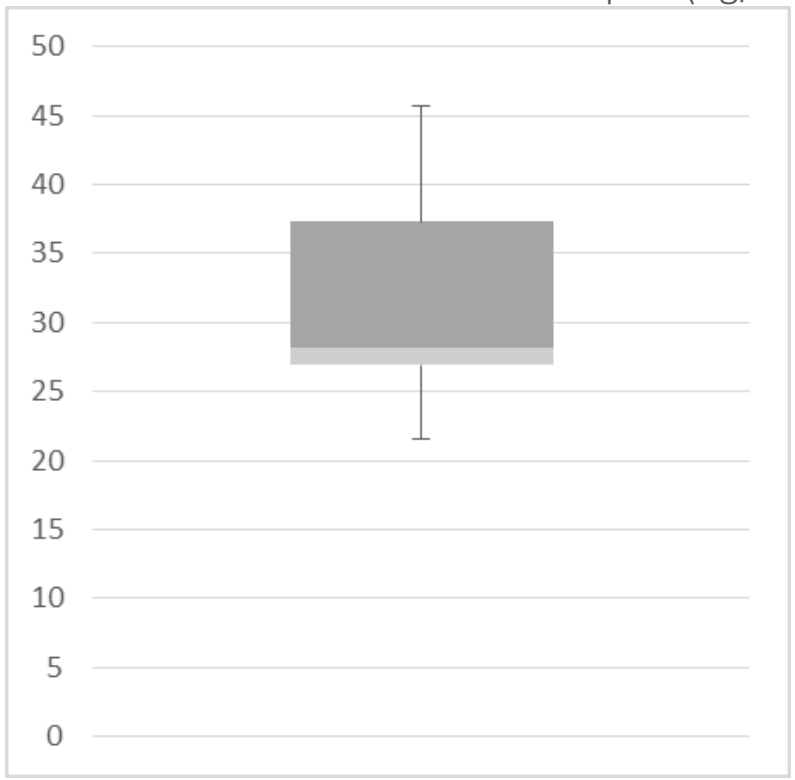


→ BDNF serum levels. **A.** Fresh samples [n=12]; **B.** Old samples, stored at -20ºC from an adult (GRAMO) Spanish cohort [n=12]

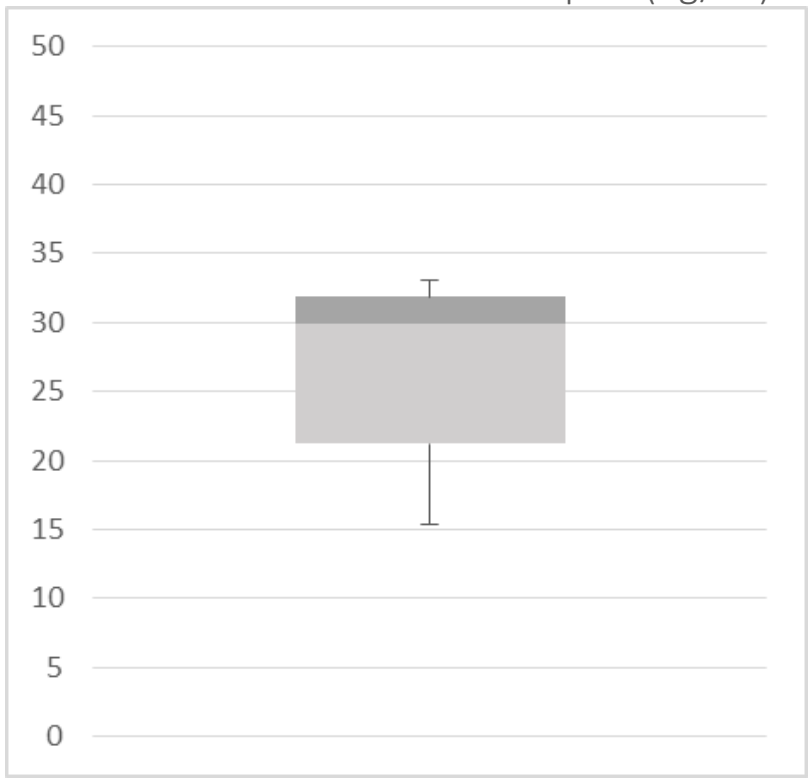
→ Volume: **20 µl** of serum

→ Mean (±SD): **A**= 31.7 (±7.1) ng/ml; **B**=26.9(±6.2) ng/ml

A. BDNF levels in fresh serum samples (ng/ml)




B. BDNF levels in old serum samples (ng/ml)



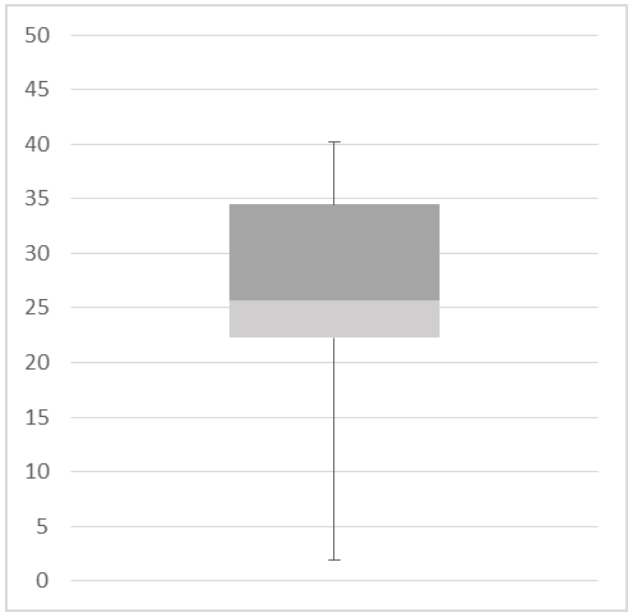
# *Volume optimization*

*A. Data using 20µl of fresh samples (n=12).*

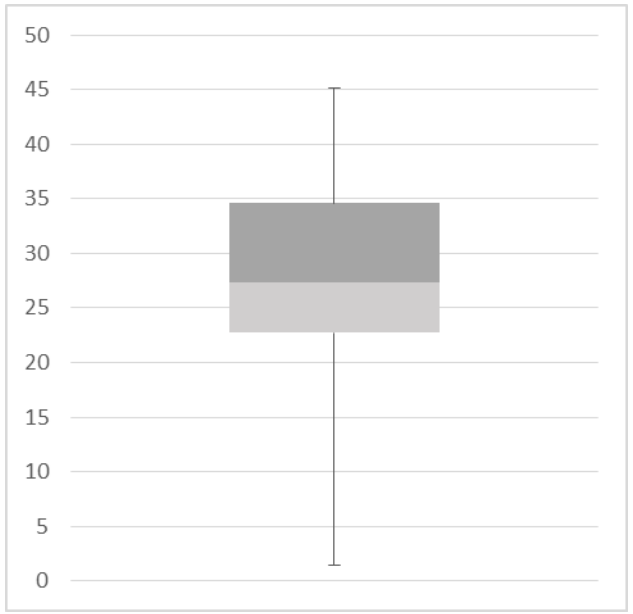
*B. Data using 10µl of same fresh serum samples*

100 fold dilution:
10µl serum + 90µl RD5K
20µl
20µl+180µl
Final dilution 1:100

A.



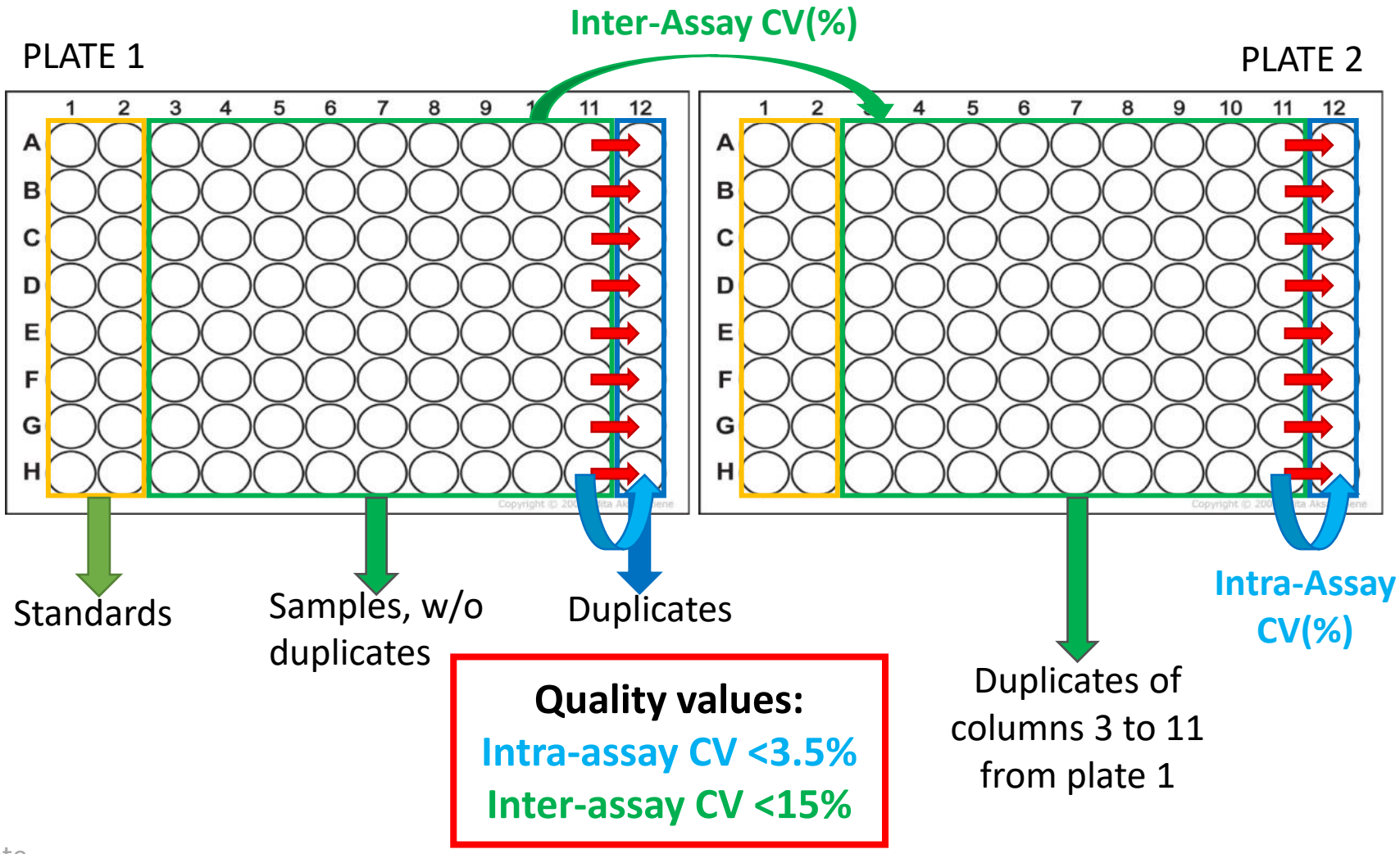

B.



# Quality Control Design

→ Registry of standard curves to control the quality of the plates

→ Intra-Assay and Inter-Assay, variation coefficient CV(%) [2 plates= 72 samples]



# *Data from INMA-Granada Cohort* ***Intra-assay CV(%)***

WP14 (UGR) has analysed serum samples from the INMA-Granada birth cohort (n=134) in their follow-up (teenagers 15-17 yrs. old).

Plates 1 and 2, (n=72 samples); Plates 3 and 4 (n=62 samples)

## Intra-assays CV(%):

| Intra-assay Plate 1 | | | | | |
|---|---|---|---|---|---|
| Samples | pg/ml w/o correction | | Average | SD | CV(%) |
| 1,00 | 484,26 | 512,27 | 498,27 | 19,81 | 3,98 |
| 2,00 | 614,63 | 639,20 | 626,91 | 17,37 | 2,77 |
| 3,00 | 459,61 | 471,72 | 465,67 | 8,57 | 1,84 |
| 4,00 | 469,86 | 488,59 | 479,22 | 13,25 | 2,76 |
| 5,00 | 451,16 | 469,19 | 460,18 | 12,75 | 2,77 |
| 6,00 | 480,63 | 478,21 | 479,42 | 1,71 | 0,36 |
| 7,00 | 554,61 | 581,20 | 567,91 | 18,80 | 3,31 |
| 8,00 | 471,80 | 463,28 | 467,54 | 6,03 | 1,29 |
| | | | | CV(%) | 2,38 |

| Intra-assay Plate 2 | | | | | |
|---|---|---|---|---|---|
| Samples | pg/ml w/o correction | | Average | SD | CV(%) |
| 1,00 | 478,70 | 497,70 | 488,20 | 13,43 | 2,75 |
| 2,00 | 472,47 | 463,24 | 467,85 | 6,53 | 1,40 |
| 3,00 | 443,23 | 434,44 | 438,83 | 6,21 | 1,42 |
| 4,00 | 576,98 | 594,64 | 585,81 | 12,49 | 2,13 |
| 5,00 | 276,27 | 275,37 | 275,82 | 0,63 | 0,23 |
| 6,00 | 338,21 | 362,13 | 350,17 | 16,91 | 4,83 |
| 7,00 | 606,45 | 601,41 | 603,93 | 3,56 | 0,59 |
| 8,00 | 259,52 | 287,98 | 273,75 | 20,12 | 7,35 |
| | | | | CV(%) | 2,59 |

| Intra-assay Plate 3 | | | | | |
|---|---|---|---|---|---|
| Samples | pg/ml w/o correction | | Average | SD | CV(%) |
| 9 | 415,96 | 426,14 | 421,05 | 7,20 | 1,71 |
| 10 | 297,48 | 299,06 | 298,27 | 1,11 | 0,37 |
| 11 | 446,16 | 468,48 | 457,32 | 15,78 | 3,45 |
| 12 | 350,88 | 362,17 | 356,53 | 7,99 | 2,24 |
| 13 | 329,95 | 340,28 | 335,11 | 7,31 | 2,18 |
| 14 | 359,79 | 364,39 | 362,09 | 3,26 | 0,90 |
| 15 | <0,000 | <0,000 | | | |
| 16 | 311,99 | 318,43 | 315,21 | 4,55 | 1,44 |
| | | | | CV(%) | 1,76 |

| Intra-assay Plate 4 | | | | | |
|---|---|---|---|---|---|
| Samples | pg/ml w/o correction | | Average | SD | CV(%) |
| 9 | 582,57 | 632,38 | 607,47 | 35,22 | 5,80 |
| 10 | 381,03 | 378,23 | 379,63 | 1,98 | 0,52 |
| 11 | 582,23 | 593,16 | 587,70 | 7,73 | 1,32 |
| 12 | 470,46 | 509,42 | 489,94 | 27,55 | 5,62 |
| 13 | 400,71 | 416,17 | 408,44 | 10,94 | 2,68 |
| 14 | 478,59 | 499,01 | 488,80 | 14,44 | 2,95 |
| 15 | 6,54 | 7,14 | 6,84 | 0,42 | 6,17 |
| 16 | 122,32 | 127,35 | 124,84 | 3,56 | 2,85 |
| | | | | CV(%) | 3,49 |

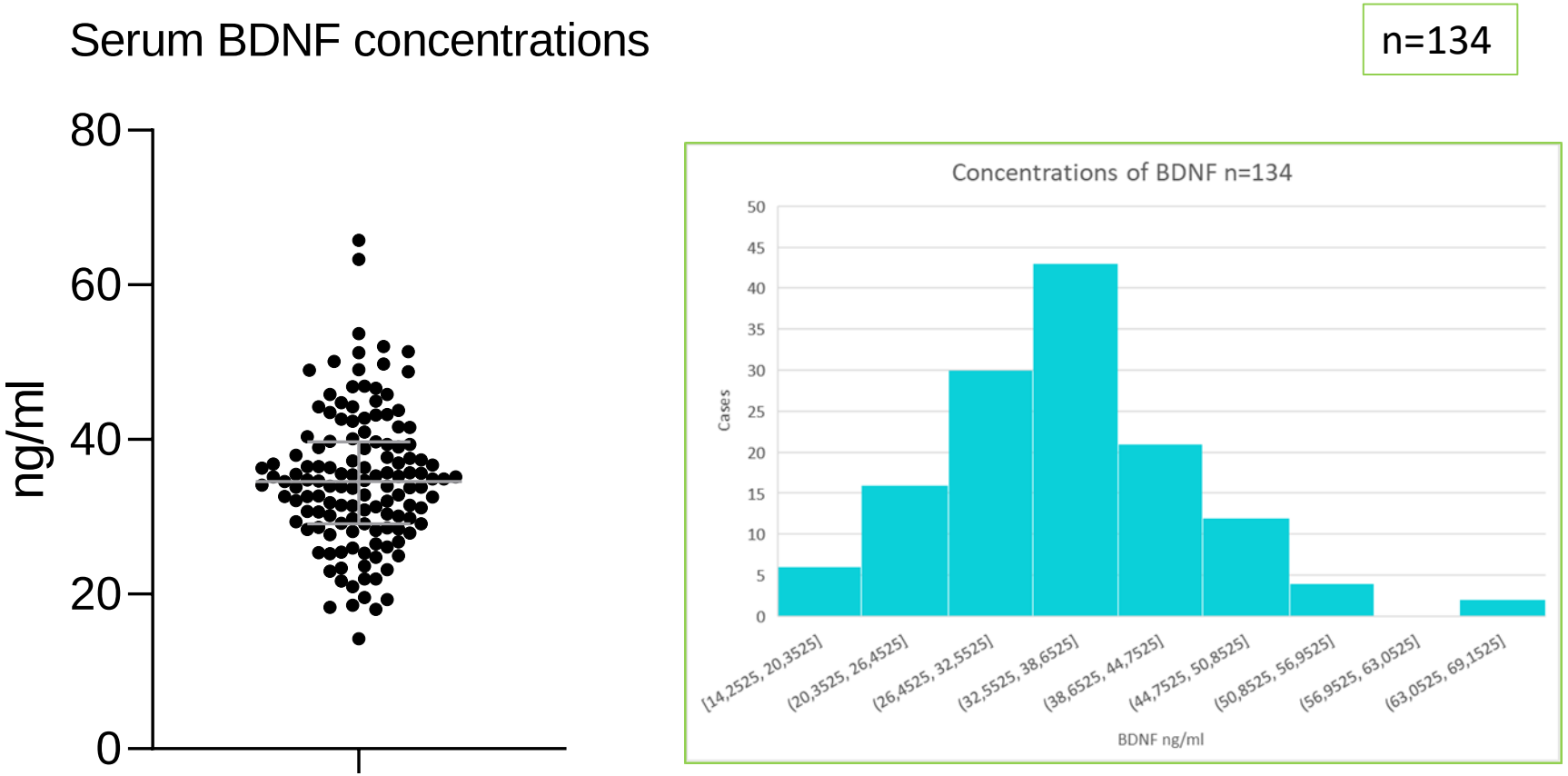
## Inter-assays CV (%) from plates 1-2 and 3-4

| Inter-assay | | | | | | |
|---|---|---|---|---|---|---|
| Samples | Measure 1 | Measure 2 | Measure 3 | Media | SD | CV(%) |
| Samples 1 to 35 | … | … | … | … | … | … |
| 36 | 36,1 | 36,9 | | 36,5 | 0,6 | 1,7 |
| 37 | | 29,3 | 28,8 | 29,1 | 0,3 | 1,1 |
| 38 | 23,6 | 27,1 | | 25,3 | 2,5 | 9,7 |
| 39 | 29,7 | 35,7 | | 32,7 | 4,2 | 13,0 |
| 40 | 18,7 | 23,2 | | 20,9 | 3,2 | 15,2 |
| 41 | 33,1 | 34,4 | | 33,7 | 0,9 | 2,7 |
| 42 | 32,1 | 39,1 | | 35,6 | 4,9 | 13,9 |
| 43 | 29,1 | 33,9 | | 31,5 | 3,4 | 10,8 |
| 44 | 33,9 | 35,6 | | 34,8 | 1,3 | 3,6 |
| 45 | 26,5 | 30,6 | | 28,6 | 2,9 | 10,1 |
| 46 | 27,5 | 32,8 | | 30,1 | 3,8 | 12,6 |
| 47 | 23,5 | 26,5 | | 25,0 | 2,1 | 8,5 |
| 48 | 38,7 | 46,6 | | 42,6 | 5,6 | 13,1 |
| 49 | 19,5 | 26,5 | | 23,0 | 4,9 | 21,3 |
| 50 | 33,0 | 37,6 | | 35,3 | 3,3 | 9,2 |
| 51 | 30,7 | 37,5 | | 34,1 | 4,8 | 14,1 |
| Samples 52 to 134 | … | … | … | … | … | … |
| | | | | | CV (%) | 14,0 |

**Sample-specific inter-assay CV(%)**

**Total Inter-assay CV(%)=** Media of all sample-specific inter-assays CV

Distribution of concentrations of BDNF (ng/ml) found in 15-17 year-old boys



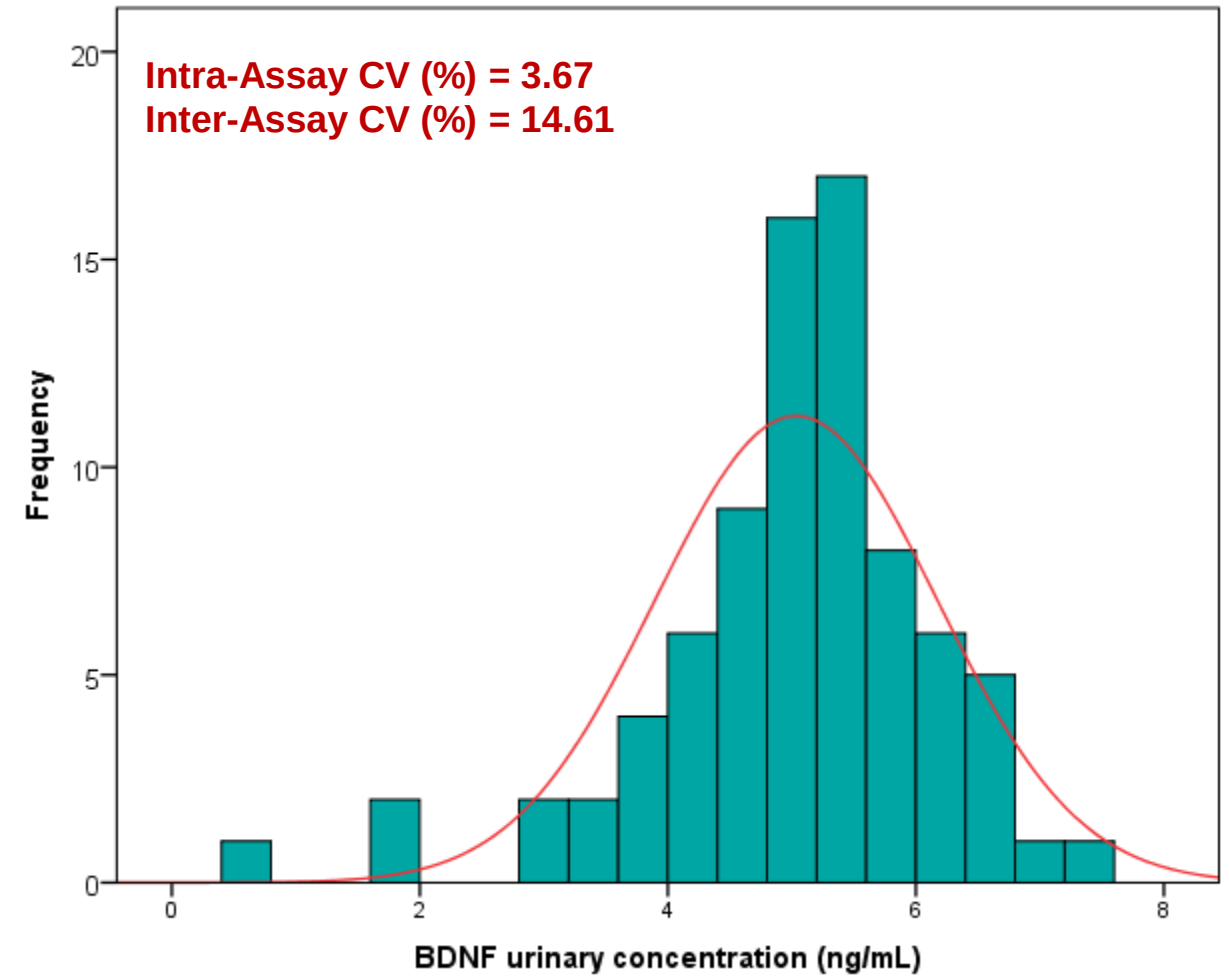

P25= 29.07ng/ml; P50= 34.58 ng/ml; P75= 39,70 ng/ml

# *Example of BDNF in urine samples*



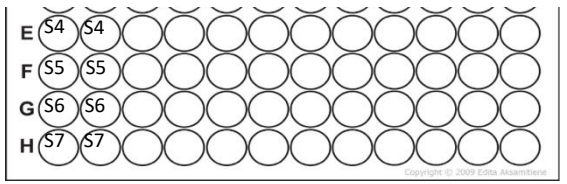
1. Co
   ar
2. Ec
   0.
   0.
   H

5 ml
30%







# *Blood BDNF methylation – Bisulfite pyrosequencing (gold standard)*

**Selection of** promoter re element-bin release of E follows: chr1 2009 (GRCh3

3, 4, 5 & 6) in a cAMP response ivity-dependent d region are as Human February

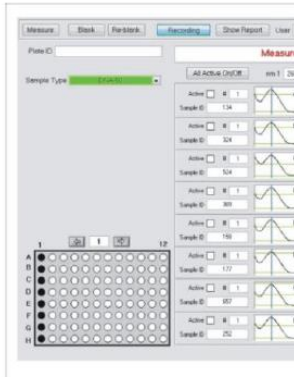
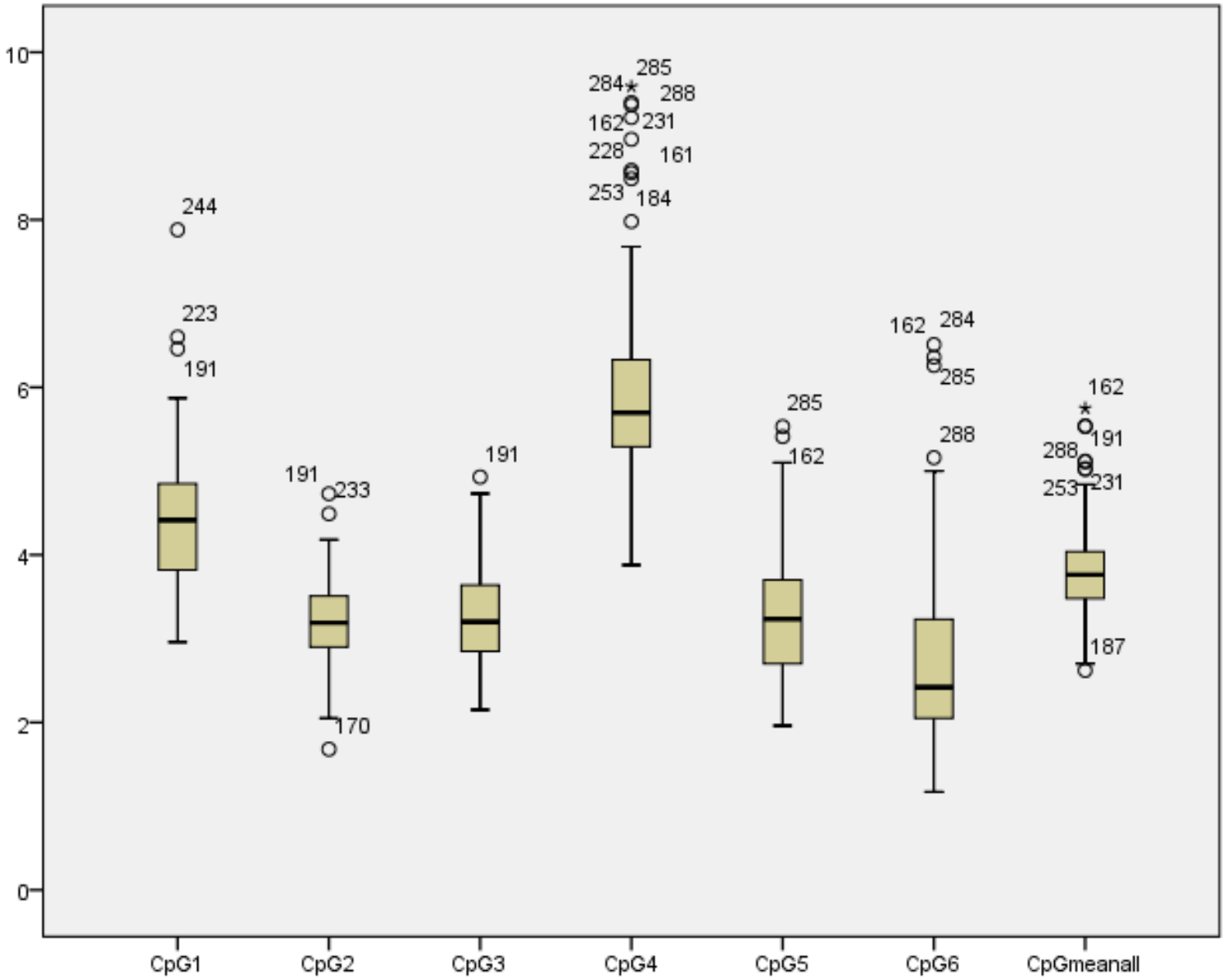
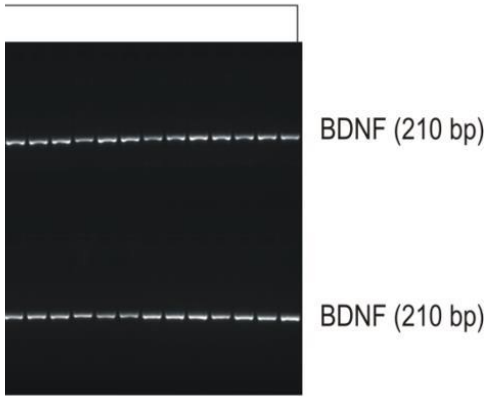


Figure 14: A260/280 ratio (h adolescent DNA samples




es)

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agarose gel

ality check are always

# 3. “Physiological” Validation of BNDF in the INMA-Granada pilot study before implementation in the HBM4EU aligned studies

Are BDNF biomakers predictive of child neurodevelopment?
Is BPA or other chemicals associated with BDNF regulation in humans?
Can BDNF act as a mediator?

# Short bibliography


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https://www.nap.edu/catalog/25962/predicting-human-health-effects-from-environmental-exposures-applying-translatable-and-accessible-biomarkers-of-effect