**REGIONE CAMPANIA**

**Istituto Zooprofilattico
Sperimentale
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# ATTIVITÀ DI MEDICINA TRASLAZIONALE PER IL BIOMONITORAGGIO IN REGIONE CAMPANIA

Il Biomonitoraggio Umano (HB) rappresenta un utile strumento per indagare la complessa relazione esistente tra ambiente e salute.

Valutare l'esposizione umana a stressors ambientali significa osservare profondamente, con un approccio olistico, tutti i fattori che contribuiscono a generare l'esposizione stessa. Considerando il caso studio "Regione Campania (Italia)", la presente relazione introduce e descrive un innovativo protocollo di HB, che tenta di sopperire alla carenza di integrazione di dati tra discipline scientifiche complementari.

Il modello di biomonitoraggio si basa su una valutazione analitica e sistemica del contesto ambientale. Il paradigma del protocollo considera tre elementi: l'individuazione delle sorgenti di contaminazione, delle vie di migrazione dei contaminanti e dell'effetto sugli organismi target. Persegue tale scopo arruolando coorti di popolazione sana, al fine di identificare potenziali rischi di esposizione ai contaminanti e predire possibili conseguenze cliniche correlate. Un'analisi multilivello integra insieme il monitoraggio delle matrici ambientali, l'individuazione di biomarcatori di esposizione e di effetto in fluidi biologici umani, le valutazioni epidemiologiche, al fine di delineare una fotografia dello stato di contaminazione sul territorio regionale.

Un particolare focus nella dissertazione è dedicato ai profili epigenetici ottenuti dall'analisi degli array di metilazione del DNA, il marcatore molecolare più ampiamente utilizzato in studi di epigenetica ambientale. La modulazione dell'espressione genica in risposta all'esposizione ambientale rappresenta una chiave di comprensione delle interazioni gene-ambiente, utile per approfondire possibili effetti fenotipici e clinici sulle popolazioni suscettibili, così come l'eziologia delle neoplasie e delle patologie cronico-degenerative. Ciò permette di traslare le conoscenze acquisite in azioni di sanità pubblica e sviluppare strategie di prevenzione. Al di là della presente relazione, difatti, l'obiettivo a lungo termine dello studio è di creare una mappa dinamica ed integrata dei livelli di contaminazione ambientale campani, al fine di supportare l'implementazione di interventi di sanità pubblica e fornire un modello scientifico di riferimento per la valutazione del rischio da esposizione. In accordo con una prospettiva di tipo "One Health", il modello aspira ad essere traslato in differenti contesti e per varie applicazioni, e a diventare uno strumento utile per l'analisi comparativa, multi-criteriale e multidisciplinare.

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# 1. INTRODUCTION

## 1.1 Environment and Health

The environment, meaning as the whole physical, chemical, and biological factors external to a person (Pruss-Ustun et al., 2016), is an actual determinant for human life.

According to this omni-comprehensive definition, a fine and breakable balance governs the relationships between its biotic and abiotic compartments, and any perturbation affecting the system could be able to move this balance towards new arrangements. A combination of actions and reactions tries to restore the system balance, moved away from its "optimum", triggering mechanisms of positive (enhancing) or negative (regulatory) feedback of the phenomena. This type of regulation results in different condition of healthiness or risk for the unsettled ecosystem and its components.

Humans, as part of the environment and of the biotic compartment, are influenced by the succession of these events and by regulatory mechanisms during their lifespan. They are exposed to environmental events and contribute through their activities to the changes occurring in the environment itself. The main difference between natural and human-induced ecosystem changes is in the spatial and time scale of events.

Human activities, including population growth, industrialization, economic development, and urbanization, cause disturbances over time scales of decades to a few hundreds of years. Such environmental impacts and pollution processes could occur at smaller (local) spatial scale (e.g. point-source pollution associated with a hazardous waste site) or at a much larger scale, encompassing entire regions, until the entire planet (Maximilian et al., 2019).

It is well-known that, as dark side of the anthropization, the widespread of contaminants in the environmental matrices define a heavy threat on ecosystems health and particularly on target organisms, highlighting the environmental pollution as a growing concern for global health, and representing it as one of the great existential challenges of the Anthropocene epoch (Landrigan et al., 2018).

As stated by the World Health Organization (WHO), in 2012 approximately 24% of deaths recorded worldwide have been linked to environment, which is roughly 13.7 million deaths a year (this data includes infectious diseases related death). Moreover, the Global Burden of Disease (GBD) study estimates that the pollution-related disease was responsible for 9 million premature deaths in 2015 (16% of total global mortality) (Forouzanfar et al., 2016). Especially air, water, and soil contamination, together with electromagnetic pollution, industrial activities, in-door and occupational exposure, represent factors potentially correlated to chronic and degenerative diseases onset (Pruss-Ustun et al., 2016).

Over the past decades scientific research has supposed a link between exposure to environmental stressors and human diseases (Ziech et al., 2010). On the basis of these solid scientific evidences a lot of substances derived from pollution phenomena were classified

as carcinogenic or mutagenic due to their dangerousness (https://monographs.iarc.fr/agents-classified-by-the-iarc/). Most harmful compounds have been identified in occupational health studies as characteristics of professional exposure. For example, one of the most studied occupational carcinogens, the asbestos, had a terrible history of proved correlations between professional exposure and adverse clinical outcomes (Siemiatycki 2007; Frank et al., 2014).

Nevertheless, the sources of pollution are increasingly widespread and heterogeneous and, above all, follow diverse time of action in terms of exposure. This means that the phenomena do not affect only workers or cohorts of people with acute events, but the whole general population and, in a holistic point of view, affect the entire biotic compartment. Hence, humans are exposed to many environmental stressors during life, but the real balance is often represented by the addition of a series of environmental factors not linked to pollution, called confounders, which contribute to generate the final consequence in term of health or diseases onset (e.g. individual life or food habits).

The interpretations as to the extent of environmental or confounders remains unclear own to the multifactorial nature of diseases. Some observations suggest the minor role of environmental pollutants in chronic diseases incidence rather than lifestyle factors, others underline contaminants as the major contributing factors (Belpomme et al., 2007a; Belpomme et al., 2007 b; Irigaray et al., 2007; Clapp et al., 2008; Miller et al., 2012, Joseph et al., 2013).

In 2018, The Lancet Commission on pollution and health has developed the concept of "Pollutome", as the totally of all forms of pollution that have the potential to harm human health causing illnesses (Fig. 1A). In the opinion of the Commission it could be viewed as a fully contained (nested) subset of the "Exposome", the term coined in 2005 by Wild to identify every exposure to which an individual is subjected from conception to death. The Exposome complements the genome by providing a comprehensive description of lifelong exposure history (Wild 2005 and 2011), and its multidisciplinary measurement could be considered a useful way to improve prevention strategies from a public health perspective (Siroux et al., 2016) (Fig. 1B).
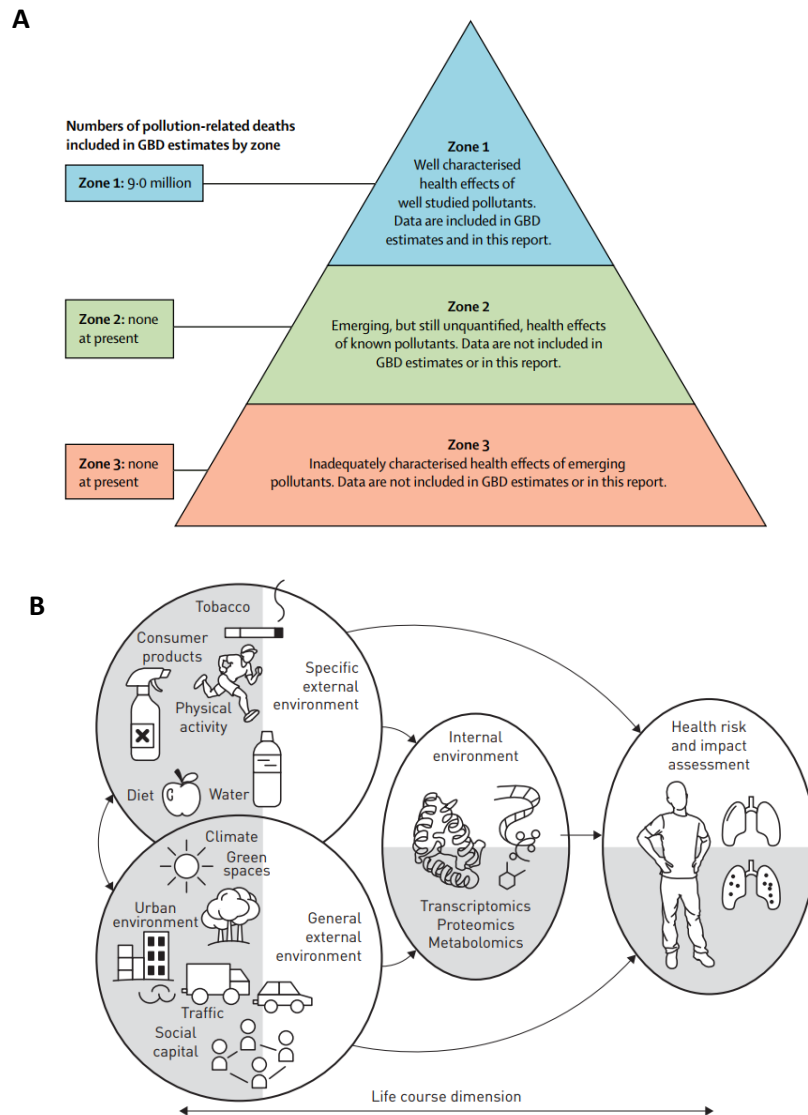
**Figure 1**. **(A)** The Pollutome. The totally of all forms of pollution that have the potential to harm human health. The Lancet Commission on pollution and health divides the Pollutome into three zones. Zone 1: well-established pollution-disease pairs, with robust estimate contribution to the global burden of disease; zone 2: emerging effects of known pollutants, where evidence of causation is building but associations are not fully characterized; zone 3: new and emerging pollutants, whose effects are unknown and not yet quantified (source Landrigan et al., 2018). **(B)** The Exposome. Effects and interactions between the three different domains of the Exposome: the general external, the specific external and the internal. The internal exposome comprises processes as metabolism, inflammation, aging etc.…The specific external includes for example lifestyle and food habits. The general external is the wider climatic, social, economic and psychological influences of the environment (source Siroux et al., 2016).

Up to this short overview, environmental pollution problems could be considered as a multilevel sum of past and present events, which could or not affect people at the same time, and nor to the same intensity or way (Trevors, 2010). Notwithstanding in the last decades there was a raise attention and a higher awareness for this hot issue, the complexity and the interconnection of the problems require likewise complex and interconnected solutions.

Only through effective methods and models it could be possible to identify the variables and the causal links that feed the phenomena and influence each other's parts. Observational studies focusing on these phenomena, on ecosystem alterations and on related biological modifications, allow to go back to the source of the alteration itself and to identify mechanisms of cause-effect. Therefore, the state of well-being or disease will be determined by the correlations between all the variables that contribute to generate the exposure. Actually, the "exposure" represents the key concept that could give, by its measurement, the chance to define the relationships between environment and health.

## 1.2 Environmental Risk Assessment and Human Biomonitoring

The complex investigation of the causal link between environment and health could be interpreted through a multi-level analysis that starts from the definition of environmental exposure, through its evaluation, up to the assessment of the associated risk for health.
Thus, exposure measurement becomes a useful tool in understanding, characterizing, and reducing associated health risks (U.S. EPA 2019).
In a general meaning, the "Risk" could be defined as an expectation determined by the observation of phenomena and variables, with a percentage of uncertainty depending on the field of application. From a mathematical point of view, the risk is defined as a combination of the probability of an event occurring and the severity of its damage, therefore the extent of the consequences determined. Accordingly, the risk becomes a two-dimensional assessment expressed by the two variables: probability and magnitude. Thanks to these two factors, it permits to identify, predict or prevent, a series of phenomena whose effects could be deleterious. The system investigated must be screened, analyzed, deepened, in its entire structure, through protocols and methods that allow to generate a predictive definition and verification model. This model should be a tool at the service of authorized decision-making levels.
To measure the exposure means to assess the "Environmental Exposure Risk".
In general, it is carried out to examine the effects of an agent on humans (Health Risk Assessment) and on ecosystems (Ecological Risk Assessment) (Dinis et al., 2009).
The European Environment Agency in 1998 described a series of key task in general Risk Assessment to carry out especially for the environmental ones as guideline:
    i.   Problem formulation: pollutants characteristics and chemistry;
    ii.  Hazard identification: presence and toxicity of chemicals;
    iii. Release assessment: sources and rate releases;
    iv.  Exposure assessment: target of exposure and type of exposure;
    v.   Consequence or effect assessment: how is it toxic and at what exposure levels and what is the effect on the receptors;

vi. Risk estimation and characterization: what does the risk assessment tell us about the situation and what are the risks, quantitative or qualitative;

vii. Risk evaluation: how important is the risk to those affected, those who create it, and those who control it.

It follows that the risk characterization and/or risk evaluation are used as input for risk management in order to come up with an answer to which actions should be taken and how should the remaining risks be handled (Fairman et al., 1998).

The scientific and methodological approach aimed to estimate exposure importance and extension is the Human Biomonitoring (HB).

According to Centre of Disease Control and Prevention (CDC) definition, HB is a method for assessing human exposure to pollutants or their effects, by measuring these chemicals, their metabolites or reaction products in human specimens (CDC, 2005).This means a repeated and controlled quantification of these markers, on susceptible subjects (or exposed in the past) in a general environment or in a workplace (Zielhuis and Henderson 1986; Manno et al. 2010).

Exposure to environmental pollutants occurs through different routes, such as inhalation, ingestion, and dermal absorption and the amount of pollutant uptake is named as the "absorbed dose." Thus, the body burden of a specific pollutant is determined by factors, such as the pollutant's concentration in a specific environmental medium, its physical and chemical properties, and timing of exposure, as well as individual factors, such as uptake, metabolism and excretion rates. HB considers all these factors by measuring the concentrations of a chemical or its metabolites in human matrices (WHO, 2015).

It was firstly used in occupational medicine and then transferred to life habits or territorial emergencies.

Concerning occupational medicine, the human biomonitoring allows to figure out and acquire data about health consequences after exposure to toxic compounds, thanks to its measured time and known type of contact to single elements or to a mixture. Examples are petroleum distribution workers (Heibati et al., 2018) or incinerator workers (Mauriello et al., 2017) exposure assessment.

Different analytical methods to estimate the uptake of occupational chemicals in human body fluids were developed in the early 20th century (e.g. lead and a few other compounds and relative metabolites) (Angerer et al., 2007). Many years later ('80s), HB was included in exposure and health surveillance programs as part of regulatory requirements, for instance for chemical industries. Indeed, when HB is compared to environmental evidence, it shows itself to be an effective tool to improve occupational risk assessment at individual or group level, supporting the same health surveillance plans (Manno et al., 2010). During the health surveillance each worker is usually subjected to imaging, instrument, and biochemical testing to detect any clinically relevant occupation-dependent change. The use

of biomarkers and of HB approach represents not only the method to estimate the internal exposure or to predict potential health effects, but also the chance to assess a causal relationship between health impairment and chemical exposure, when a change is firstly detected in exposed workers.

Therefore, in the field of occupational medicine, the "occupational risk assessment" could be defined as a qualitative and quantitative characterization of an occupational risk; it consists of three fundamental tools: environmental monitoring, health surveillance and biological monitoring. It becomes clear that the advantage of using biomarkers and HB approach in occupational surveillance and study is recognizable when the toxicological significance of the compound object of the analysis is sufficiently known. Some examples are below. Zidek et al in 2017 reported in a review the use of HB data for regulatory risk assessment in "Canada Chemicals Management Program". DEMOCOPHES study (Schwedler et al., 2017) was developed and carried out in Europe, with an attempt of harmonize HB studies on European scale, in order to support regulation. Same purposes for Human Biomonitoring for Europe (HB4EU) project ([www.hbm4eu.eu](www.hbm4eu.eu)), which joints efforts of many countries and environmental agency, coordinating and advancing HB in Europe, to provide scientific evidence for chemical policy making.

Nowadays the application of HB is beyond the limits of the occupational surveillance and is an attractive approach for research activities (Manno et al., 2014).

Despite the impressive potential of the HB, particularly in occupational risk assessment, it is not always correctly used or interpreted. Some ethical issues become evident. As early as in 2002, The Code of Ethics of the International Commission on Occupational Health (ICOH, 2002), highlighted some questions:

1. The aims of the study: if HB is performed for research interest or for health surveillance.
2. The definition of an appropriate study design: depending on the aims, the right choice of biomarkers, of matrices, of sample size (WHO's guidelines suggest a minimum of 120 subjects randomly selected), of reference group, of inclusion and exclusion criteria of enrollment.
3. The scientific approach and methodology.
4. The biomarkers validation and their predictive value.
5. The operative aspects: samples collections and analysis.
6. Interpretation of biomarkers data and management of the results.
7. Data protection and communication.

Beyond the occupational risk assessment, when HB is combined with health data, they together create an epidemiological study, aimed to observe the body burden of pollutants and their health effects. In addition, when HB is surrounded by environmental evidence it gives the possibility to obtain a systemic and full view of the condition investigated. In fact,

HB alone usually does not reveal exposure sources and routes. Thus, environmental monitoring remains crucial for the development of targeted policy actions (WHO, 2015).

Monitoring and analyses on environmental matrices (soil, water, air, food, biosentinel models), gives the overview of territorial context, a state of art of critical issues and of potential contaminated areas, mapping the spatial dimension of compounds and geolocalizing the putative sources of pollution (Balassone et al. 2016; Ducci et al. 2017; Ducci et al. 2019).

Actually, in the systemic overview, the multidimensionality and the complexity of the observations are what gives the chance to divide the phenomenon in several levels of analysis, which, in the final intersection, could provide the global reading and interpretation.

Despite the great potential of the approach, the main problem in the majority of environmental assessment protocols and human biomonitoring studies seems to be exactly the lack of data integration and methodological sharing between different science fields, as well as the lack of a holistic approach through which acquire a real snapshot of the overall degree of contamination. Frequently also the epidemiological analyses are twisted by a unilateral view, led by the observations on mortality and diseases incidence, linked with a single source of pollution, without any consideration for the sum of variables contributing to the condition.

## 1.3 Exposure, Effect and Genetic Susceptibility Biomarkers

HB protocols consider the determination of exposure, effects and susceptibility biomarkers in order to define the internal dose of pollutants and their direct effects on biological and cellular system (Migliore 2018) (Fig.2).

Biomarkers of Exposure are the results of exposure itself and estimate chemicals or metabolites, in body fluids or in tissues, reflecting the internal dose and the level of pollutants absorbed. Inorganic and organic compounds, such as heavy metals, dioxins, dioxins-like and polychlorinated biphenyl (PCBs), and some of their derivatives, are among them. Biomarkers of Effect represent the biochemical or molecular reaction of the system to the xenobiotic compounds. They identify early and generally reversible biological effects: the measure of DNA damages, chromosome aberrations, sister chromatid exchange, DNA adducts and proteins, inflammatory cytokines, epigenetic changes. Biomarkers of Susceptibility reflect the individuality, that is the predisposition to be susceptible to adverse effects related to exposure. For example, polymorphisms of relevant xenobiotic metabolizing enzymes.
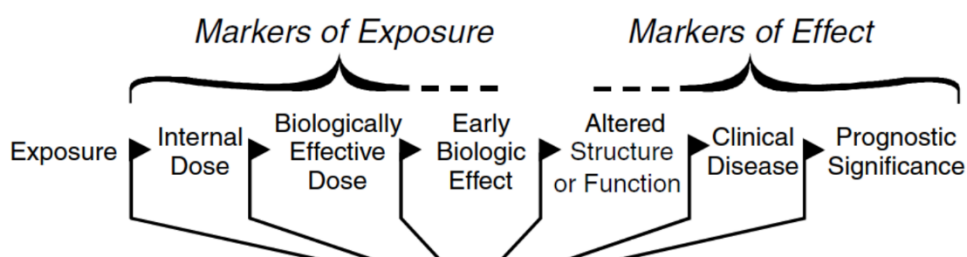
**Figure 2**. The continuum from exposure to disease. During different life stages biomarkers allow to evaluate the way and the magnitude of chemical interferences on biological processes, up to the alteration of structure and function, which means disease onset (source De Bord et al., 2015).

An important issue, in a correct HB study design, is the appropriate choice of the sample matrix to analyze. It could depend on the study design, on the aims and on the type of exposure investigated in the HB. In the last decades, the improvement in technologies and methodological approaches allow to have very low limits of quantification (LOQ) and, in consequence, have expanded the use of non-invasive matrices even if with low concentration of xenobiotics investigated.

As reported in Table 1, blood is one of the most suitable matrices for a series of investigations, given the half-life of its cellular components and the balance at systemic levels with other organs and tissues, even if it is limited by the invasiveness of the collection procedures (Smolders et al. 2009). A lot of heavy metals, such as lead and cadmium, are measured in blood. Dioxins and dioxins-like compounds, given their lipophilic nature, were originally evaluated in adipose tissue, but from '80s they are successfully estimated in serum, even if its low percentage of fats (WHO, 2015). Over the recent years, because of the great amount of serum required for high resolution analysis methods for these organic compounds (Brasseur et al., 2014), a lot of HB considers alternative specimens. For instance, breast milk of new mums is as a good reference matrix for this type of measurement given its lipophilic nature and given the level of information obtained on mother and child at the same time (Fang et al., 2013; Schuhmacher et al., 2019).

Urine is probably the most frequently used matrix in HB to quantify the degree of environmental or occupational exposure to pollutants, especially for substances with short biological half-life. The collection and analysis of urine samples carries no associated risk, and large volumes can at once be gathered per individual (Esteban et al., 2009). Urinary samples are useful for the analysis of inorganic compounds and metabolites of organic ones, for instance volatile organic compounds, like vinyl chloride, or inorganic mercury, which reflects a recent exposure to this pollutant.

Other less common matrices, like hair, nails, saliva and teeth are practical for HB on children cohorts, given their non-invasiveness. These alternatives, with derivative of the most common ones, are generally used for in-depth analysis and particular tests. For example erythrocytes, a blood derivative, are the more appropriate for the Hexavalent Chromium Cr(VI) speciation; in fact Cr(VI) ions, after uptake by inhalation or

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percutaneously are carried in the blood plasma and penetrate, depending on the concentration, into the erythrocytes, which represent the golden standard target for its quantitative determination (Lewalter et al., 1985). Similar consideration for hair samples, used for Mercury (Hg) speciation. The growth rate of hair (1 cm per month) and the tendency of Hg to accumulate in hair make it possible to estimate long-term exposure. In addition, tracing the dynamic changes along hair growing direction provides an insight into average exposure on timescale ranging from weeks to months (Li et al., 2019a). Generally, more than the 80% of Hg in hair is as Metyl-Hg, which is taken up by hair follicles as MeHg-cysteine complexes (Pino et al., 2018). The semen is useful for the evaluation of Lead exposure; because of the evidence of structural alteration of spermatozoa in workers exposed to the element (Naha et al., 2005), there is a growing use of this specimen for the evaluation of exposure and the correlation with male infertility (Fatima et al., 2010 and 2015; Onul et al., 2018).

**Table 1**. Biological matrices used in HB studies (source WHO, 2015).

| Matrix | Population | Advantages | Limitations | Compounds measured in the matrix |
|---|---|---|---|---|
| Blood, serum, plasma | General | In equilibrium with all organs and tissues. Well established standard operating procedures (SOPs) for sampling. | Invasive; trained staff and special materials required. Volume limitation. Special conditions for transport and shipment. | POPs, metals/trace elements, organic compounds, tobacco smoke. e.g.: alkylphenols, mercury, lead, BFRs, dioxins, water disinfection byproducts, fluorinated compounds, organochlorine pesticides, organophosphate pesticides, phthalates, PCBs, dioxins. |
| Urine | General | Non-invasive, easy collection, no volume limitation. Allows analysis of metabolite | Composition of urine varies over time. | Metals/trace elements, organic compounds, tobacco smoke. Metabolites of environmental pollutants. e.g.: mercury, cadmium, arsenic, organochlorine compounds, BPA, organophosphate pesticides, parabens, phthalates, PAHs, benzene. |
| Hair | General, with few exceptions (i.e. neonates) | Non-invasive; minimum training required for sampling. No special requirements for transport and storage. Information about cumulative exposure during previous months. Segmental analysis is possible. | Hair is exposed to the environment and can be contaminated. Potential variations with subject's hair colour, hair care or race. | Metals/trace elements, POPs e.g.: total mercury, methylmercury, arsenic, cadmium, parabens, organochlorine compounds |

| Matrix | Population | Advantages | Limitations | Compounds measured in the matrix |
|---|---|---|---|---|
| Cord blood | Specific | Non-invasive; provides information about mother and child. Well defined SOP for peripheral blood can be used for cord blood. | Only available at birth in maternity ward settings. Ethical constraints. Special conditions for transport and storage. | POPs and other organic compounds, metals/trace elements, tobacco smoke, e.g.: alkylphenols, mercury, lead, BFRs, dioxins, water disinfection byproducts, fluorinated compounds, organochlorine compounds, organophosphate pesticides, phthalates, PCBs. |
| Breast milk | Specific | Provides information about mother and child. Enriched with lipophilic compounds. | Somewhat invasive. Restricted period of availability. Depuration of chemicals during lactation should be considered. | POPs, metals/trace elements, organic compounds, tobacco, e.g.: alkylphenols, BPA, dioxins, BFRs, fluorinated compounds, PCBs, organochlorine pesticides, lead, cadmium, mercury, phthalates |
| Amniotic fluid | Specific | Invasive | Limited to women undergoing amniocentesis or Caesarean section. | POPs, organic compounds, metals/trace elements, e.g.: phthalates, mercury, organochlorine pesticides |
| Placenta | Specific | Non-invasive | Restricted to certain period of life; requires homogenization | Metals/trace elements, POPs, organic compounds, e.g.: mercury, cadmium, lead, organochlorine pesticides, BPA, BFRs, dioxins, PCBs, PAHs, phthalates |

| Matrix | Population | Advantages | Limitations | Compounds measured in the matrix |
|---|---|---|---|---|
| Meconium | Specific | Non-invasive, easy collection. Reflects prenatal exposure. | Only available at birth. | Metals/trace elements, POPs, organic compounds, tobacco, e.g.: mercury, cadmium, organochlorine pesticides, PCBs, phthalates |
| Nails | General | Non-invasive, easy collection. No special storage or transport requirements. Provide information about short and long term exposure. | Exposed to the environment and can be contaminated (toenails are less exposed). Less documented for HBM applications. | Metals/trace elements, tobacco, e.g.: arsenic, mercury, cadmium, lead |
| Deciduous teeth | Specific | Non-invasive. No special requirements for transport and storage. | Low availability; restricted to a certain period of life. Less documented for HBM applications. | Metals/trace elements, organic compounds, tobacco, e.g.: lead, cadmium |
| Sweat | General | Non-invasive | Difficult collection. Less documented for HBM applications. | Metals/trace elements, organic compounds, e.g.: lead, cadmium |

| Matrix | Population | Advantages | Limitations | Compounds measured in the matrix |
|---|---|---|---|---|
| Semen | Specific | Mainly used to measure biomarkers of effect. | Invasive. Only available from males. Less documented for HBM applications | Metals/trace elements, POPs, organic compounds, e.g.: mercury, phthalates, dioxins, PCBs, organochlorine pesticides |
| Exhaled air | General | Non-invasive. Direct assessment of exposure through air | Limited to volatile chemicals. Difficult sampling, transport and storage. | Metals, disinfection byproducts, e.g.: lead, cadmium, trihalomethanes |
| Saliva | General | Non-invasive, easy collection | Lower concentrations of analytes than in blood; requires sensitive analytical techniques. Variation in flow rate and composition. The use of stimulant or absorbent pads can interfere with analysis. Less documented for HBM applications. | Metals/trace elements, organic compounds, POPs, tobacco, e.g.: cadmium, phthalates, BPA, PCBs, dioxins |

**1.4 Omics Sciences and Translational Knowledge for Human Biomonitoring**

Beyond exposure, effect and susceptibility biomarkers, the WHO's guidelines underline the role over the last decades of the "omics sciences" for HB, which offered the opportunity to enhance the knowledge of the exposure-response continuum in health risk assessment. According to McHale et al., (2010), the perturbations causing the onset of human diseases can be assessed by measuring the components of the "responsome", by means toxicogenomics technologies. Especially they refer to trascriptome, proteome, miRNome and methylome. In point of fact, the study of genome structure and function and its response to external stressors could integrate some fundamental aspects in HB assessment by toxicogenomic perspective, in order to understand molecular mechanisms subtended to toxicity (National Research Council, 2007; Vineis et al. 2013). A deep insight into gene-environment interaction, through pathways, genetic, protein and metabolic alterations, pointed to predict phenotypic and clinical effects on susceptible populations (Albertini et al. 2000; Krishanu 2011). Noteworthy, these observations have also underlined the involvement of endocrine pathways, immune modulation, inflammatory response, as silent or symptomatic mediators of biological system perturbation associated to environmental exposure (Chighizola  et al. 2012; Gilbert et al. 2016; Almeida et al. 2019; Hassan et al. 2019; Macchi et al. 2019; Zhao et al. 2019).

The toxicogenomics analysis proceeds by two different approaches: one focused mainly on identifying mechanisms of toxicity in animal studies or *in vitro*, another carrying out analysis on well-characterized exposed cohorts of people, in order to directly determine biomarkers of exposure and early effects and assess dose-response (McHale et al., 2010).

The omics techniques (adductomics, transcriptomics, proteomics, epigenomics and metabolomics), provide a molecular "fingerprint" of the exposure or early effects, comparable with profiles generated from already known toxicants.

A series of study have described, with an omics perspective, the relationship between exposure and molecular answer to external stressors (Forrest et al., 2005; Zhai et al., 2005; van Leeuwen et al., 2008; McHale et al., 2009). Reading these studies, it is clear the need of an accurate experimental design and a detailed data collection. Moreover, in the analysis of the advancing in risk assessment (National Research Council, 2008), authors suggest, as recommendation for this approach, the harmonization of cancer and non-cancer  risk observations, to correct the deficiencies in the treatment of uncertainty and variability for this type of quantitative risk assessment. Human toxicogenomics data, if unbiased, could potentially generate biomarkers and enrich the knowledge on the mechanisms underlying several human diseases. In fact, toxicogenomics endpoints not only describe gene-environment interaction and, with an appropriate sample size, could potentially give information on human heterogeneity.

Most human chronic diseases are result of the interactions between inherited genetic factors and modifiable environmental factors (Hunter, 2005). Likewise, most cancer cases are consequence of the interaction of genetic variants and environmental factors. In 1997

Perera estimated that only the 5% of cancers' development is led by genetic factors. For example, genetic susceptibility contributes only for 5-10% on the total incidence of breast cancer cases. The development of sporadic breast cancers might be indeed related to the interaction of genetic variation with environmental exposures (e.g. radiation, organic compounds, carcinogens, xenoestrogens) (Balmain et al., 2003). Molecular epidemiological studies have revealed that many environmental factors may interact with genetic variants to affect the risk of cancer development (Ning et al., 2014). In this context, toxicogenomics has become a fundamental tool for HB, with the primary goal to understand the correlations between environmental stress and human disease susceptibility, exploring the molecular mechanisms of environmental mutagens and/or carcinogens, and identifying potential biomarkers of disease and toxicity, including mutagenicity and carcinogenicity (Waters et al., 2004). Generally, there are two ways to perform toxicogenomics studies: (i) starting with conventional toxicological research and then focusing on omics approaches to detect systematic biological effects, and (ii) starting with the omics study followed by conventional toxicological research to interrogate molecular mechanisms. In this effort, it becomes a challenge to determine the mechanisms of toxicity induced by mixtures containing many chemical components (Ning et al., 2014).

Understanding the interactions between genetic and environmental factors will provide insights into the etiology of cancers and chronic-degenerative diseases. This means the possibility of developing prevention strategy and translate the acquired knowledge into public health actions. Ning et al., supposed that in the next years, powerful infrastructures will be developed to support the storage, access, analysis and management of the huge amount and diversity of data derived from HB and toxicogenomics approach. Powerful bioinformatics tools will be developed for identification of genetic variants and genetic-environmental interactions that contribute to cancer risk. This will hopefully lead to better diagnosis, treatments, and ultimately prevention.

## 1.5 Environmental epigenetics

Environmental factors can affect the expression of the genome also through alterations in the epigenome, which is intended as the whole epigenetic modifications present along the DNA sequences in a particular cell type (Wild et al., 2013). The term "epigenetic", literally "above the genetics", was firstly used by Conrad Waddington in the 40s to explain "[…] the interactions of genes with their environment which bring the phenotype into being". Epigenetic modifications do not entail change in the genome code, but only in the gene expression. Hence, it provides the link between genes and phenotype. Thanks to the advancing in this research field, it was suggested the role of epigenetic mechanisms in modulation of gene expression programs in response to environmental exposure. Actually, epigenome functions can be considered an interface between the genome and the environment (Hou et al., 2012). Despite the dynamism, due to its reversible changes, an alteration in epigenome may represents a more stable signature of environmental exposure than transcriptomic ones (Wild et al., 2013). Some authors identified epigenetic marks as early changes in the biological system investigated in reaction to exposure to some external stressors. Among them, the tobacco smoking (Vaissere et al., 2009; Wan et al., 2012), the benzene (Bollati et al., 2007), the particulate matters and air pollution (Tarantini et al., 2009; Bollati et al., 2010), the arsenic (Bailey et al., 2013). It is clear that epigenetic plays a mechanistic role in the consequent disease processes. Since epigenetic regulates cellular plasticity, that is the ability of a cell to rapidly respond to its environment, and is a key regulator of critical cell processes including genome stability and gene transcription, it offers an attractive biological mechanism to explain how environmental exposures can cause disease (Ladd-Acosta and Fallin, 2016).

Epigenetic modifications include DNA methylation, histone modifications and long non-coding RNA activity. They are physiologically involved in the regulation of many cellular processes, including DNA-protein interactions, suppression of transposable elements mobility, cellular differentiation, embryogenesis, X-chromosome inactivation and genomic imprinting, regulating chromatin remodeling and spatial and temporal gene expression (Meissner et al., 2008; Portela and Esteller, 2010).

### 1.5.1 DNA Methylation mechanisms

DNA methylation is the most deepened epigenetic mechanism in relation to environment stressors. It occurs when a methyl group ($-CH_3$) is covalently bonded to the carbon 5 (5C) position of a cytosine and this happens exclusively in the context of CpG dinucleotides (Clark et al., 1995). The CpG dinucleotides tend to cluster in some regions, even if they are distributed in the whole human genome, representing about 1% (Varriale and Bernardi, 2010). High-density regions called "CpG islands" have more than 500 bases, with more than 55% GC (guanidine and cytosine) content and an expected/observed CpG ratio of >

0.65. Approximately 40-60% of human gene promoters contains CpG islands and are usually unmethylated in physiological conditions; about 6% of them become methylated in a spatial- (tissue) and temporal- (lifespan) specific manner (Fig. 3a). Up to 2 Kb (kilobases) upstream the CpG islands, are located the "CpG shores", with more than 75% of tissue-specific differentially methylated regions. Methylation in shores shows higher correlation with gene expression than CpG islands (Fig. 3b). Finally, "CpG shelves" are located about at 4 Kb from islands. Occasionally some CpG clusters are located within the body of the gene (Fig. 3c), or even in the UTR region (untranslated region), where they are normally more susceptible to methylation (Esteller, 2002). Considerable amount of CpG are also found in repetitive DNA elements such as transposons and retrotransposon-like elements. Highly methylated CpG in these regions are necessary to prevent transposition, chromosome and genome instability (Ehrlich et al., 1982) (Fig. 3d).
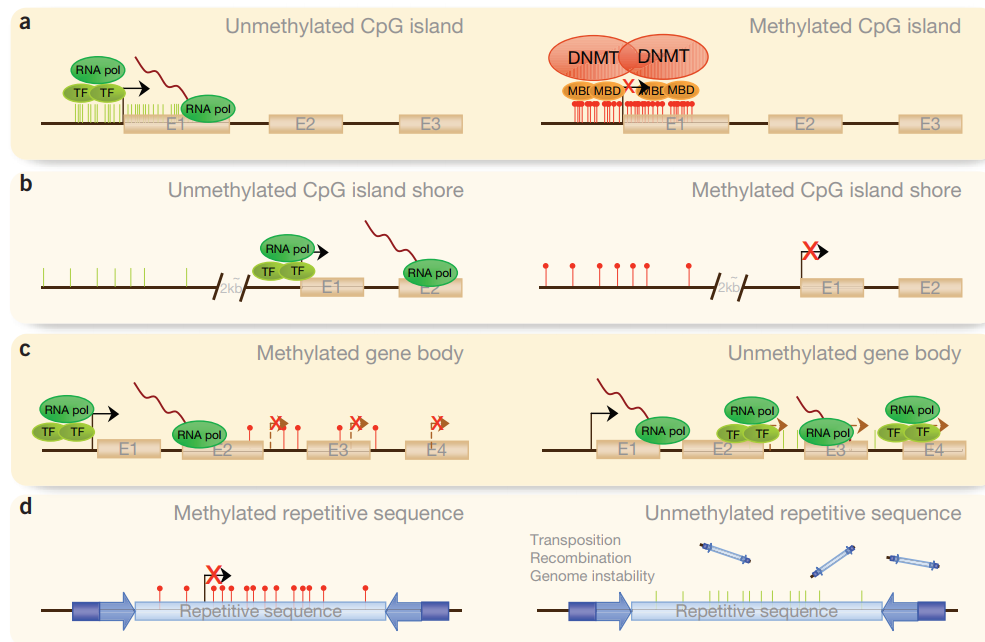


**Figure 3**. DNA methylation patterns. The figure describes on the left column a normal condition, on the right the altered one. (a) CpG islands when are located at promoters of genes are normally unmethylated, allowing transcription. When they are methylated cause transcriptional inactivation. (b) Same mechanism characterizes CpG shore. (c) Methylation along the gene body in a normal scenario is closely related to transcriptional activation, elongation efficiency and prevention of spurious initiation of transcription. It is positively associated to gene expression. In altered scenario the loss of methylated sites along the gene body could promote origin of transcription at incorrect points. (d) Methylation at repetitive sequences allows to prevent chromosome instability, translocations and gene disruption. Unmethylated scenario is associated to diseases onset (source Portela and Esteller, 2010).

DNA methylation is mediated by DNMT (DNA methyltransferase) enzymes, which allow to transfer a methyl group from S-adenosyl methionine (SAM) to the 5C of DNA cytosine. The members of the DNMT family, which effectively possess the methyltransferase activity, are classified in "*de novo* DNMTs" (DNMT3A and DNMT3B) and "maintenance

DNMTs" (DNMT1). During the embryonic development DNMT3A and DNMT3B are responsible of the methylation pattern setting. DNMT1, the maintenance enzyme, is able to preserve the methylation pattern already existing, because of its affinity for emi-methylated DNA sequences. It has the role of catalyze methylation on a DNA strand associated to a methylated CpG dinucleotides during semi-conservative DNA replication. Indeed, it is the most abundant DNMT in the cell, and is expressed mostly during S phase of the cell cycle.

The 5C of the cytosine could also be modified in 5- hydroxymethylcytosine (5hmC), the oxidative product of 5-metylcytosine, catalyzed by TET (ten-eleven translocation) family enzymes. This epigenetic mark plays a role in embryonic stem cells and blood cells differentiation, in neuronal development, through a mechanism of DNA demethylation able to regulate gene expression (Shock et al., 2011; Pastor et al.,2013).

Altered methylation pattern were highlighted in some pathogenic processes. Neoplastic conditions are generally characterized by gene-specific hypermethylation with a global hypomethylation scenario. While the global hypomethylation leads to chromosome instability, aberrant translocations and mutations, the hypermethylation that occur in CpG islands associated to gene promoters, cause transcriptional inactivation. When the gene is involved in the cell cycle control, DNA repair, Tp53 signaling, apoptosis, then the carcinogenesis is triggered (Portela and Esteller, 2010).

Aberrant DNA hypermethylation has been reported in the promoter regions silencing tumor-suppressor genes such as CDKN2A (cyclin-dependent kinase inhibitor 2A), CDKN2B (cyclin-dependent kinase inhibitor 2B), TP73 (tumor protein p73), MLH1 (mutL homolog 1), APC (adenomatosis polyposis coli), BRCA1 (breast cancer 1), MGMT (O-6-methylguanineDNA methyltransferase), VHL (von Hippel-Lindau tumor-suppressor), GSTP1(glutathione S-transferase pi 1), CDH1 (cadherin 1) and DAPK1 (death-associated protein kinase 1) (Sharma et al., 2010). For this reason, hypermethylated promoters are considered cancer biomarkers, with great diagnostic and prognostic potential (Li et al., 2009). Otherwise, the global hypomethylation could also be caused by dysregulation in DNMTs expression. Furthermore, epigenetic alterations are involved in neurodevelopmental, neurodegenerative and autoimmune diseases (Javierre et al., 2008; Urdinguio et al., 2009).

### 1.5.2 The role of DNA methylation in environmental epigenetics

DNA methylation is the most widely epigenetic mark observed in the environmental epigenetic studies. The exposure to external stressors has the potential to modify gene expression and adult disease susceptibility in different ways through changes in the methylation patterns. These epigenetic changes can be inherited in somatic cells during mitosis, providing a potential mechanism by which the environmental impact on the epigenome can affect gene expression in long-term.

In addition, considering that the methylation pattern is also inherited in germinal cells division, the hypothesis of a transgenerational epigenetic inheritance represents a growing research field, especially referred to environmental exposure inducing epigenetic changes (Jirtle and Skinner, 2007; Feil and Fraga, 2012; Ladd Acosta and Fallin, 2015). Evidence from animal studies indicates that prenatal and post-natal environmental factors exposure (during the life-stages when a series of demethylation and methylation programming occur), can result in altered epigenetic programming and subsequent change in the risk of developing diseases. Likewise, mice germlines show that environmental influences could be linked to disease phenotypes in adulthood through the inherited epigenome modifications (Morgan et al., 1999; Lane et al., 2003; Anway et al., 2005; Anway and Skinner, 2006; Pembrey et al., 2006).

A series of studies on human cohorts were conducted to investigate the relationship between exposure to heavy metals, organic compounds and changes in DNA methylation patterns. Some evidence highlighted by HB studies are reported in Table 2, adapted from Migliore 2018 (Chapter 18).

**Table 2**. Human Biomonitoring using epigenetic biomarkers (adapted from Migliore, 2018 – Chapter 18).

| Exposure | Sample size | Biological matrix | Epigenetic effect reported | Bibliography |
|---|---|---|---|---|
| Benzene | 213 adults | Whole Peripherical Blood | DNA hypomethylation in LINE-1 and AluI | Bollati et al., 2007 |
| PAH - urban smog (Polycyclic Aromatic Hydrocarbons) | 56 children | Leukocytes from Cord Blood | DNA hypermethylation in CpG island associated with ACSL3 gene | Perera et al., 2009 |
| POPs (Persistent Organic Pollutants) | 70 adults | Whole Peripherical Blood | Global DNA hypomethylation | Rusiecki et al., 2008 |
| Particulate Matters | 718 adults | Leukocytes from whole peripherical blood | Global DNA hypomethylation | Baccarelli et al., 2009 |
| Arsenic by drinking water | 16 women | Leukocytes from whole peripherical blood | DNA methylation changes for breast cancer and diabetes correlated genes | Bailey et al., 2013 |
| Endocrine Disruptor | 196 pregnant women | Placenta | DNA hypomethylation in DMR (Differentially Methylated Region) IGF2 and H19 | La Rocca et al., 2014 |

Arsenic exposure from drinking water was associated with pathways involved with breast cancer and diabetes onset by Bailey et al. in 2013. Also, Ameer et al. in 2017 associated arsenic exposure from drinking water with decreased gene expression and DNA hypermethylation in peripheral blood. The pathway analysis performed in this study suggests the involvement of genes related to cell death and cancer.

Benzene low-dose exposure was related, in HB on workers cohort as well in 2007 by Bollati et al., to a significant reduction in LINE-1 and AluI methylation, the repeated sequences representative of global DNA methylation. Results obtained were consistent with global methylation observed in malignant cells and particularly in acute myelogenous leukemia (AML). In addition, they verified hypermethylation in P15 promoter (which normally shows low or not methylation), and hypomethylation in MAGE-1 gene (with high methylation in normal tissues). Even these two observations were consistent with leukemia condition. Likewise, POPs and Particulate Matters from urban smog cause global DNA hypomethylation (Rusiecki et al., 2008; Baccarelli et al., 2009).

A systematic review published in 2015 by Ruiz-Hernandez et al., reported a synthetic overview of the studies evaluating the association of some environmental chemicals and DNA methylation. The Figure 4, adapted from the review, describes possible mechanism of action elucidating these interactions.
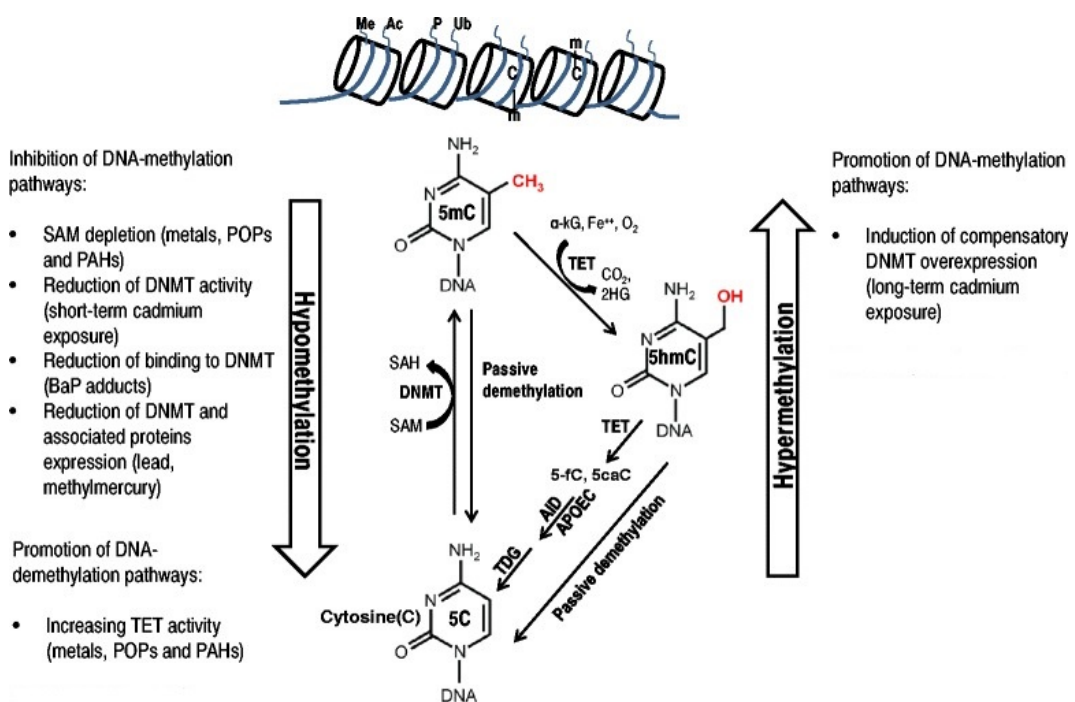
**Figure 4**. Possible mechanisms of action for environmental chemicals on DNA methylation. Metals, POPs and PAH tend to increase reactive oxygen species (ROS) formation. This alters methionine synthesis pathways, leading to a reduced synthesis of S-adenosylmethionine (SAM), substrate for DNMT enzymes. SAM depletion potentially inhibits DNA methylation, resulting in global DNA hypomethylation. Oxidative stress is proposed to stimulate the alpha-ketoglutarate ($\alpha$-KG) production, which activated TET enzyme that, in presence of iron and oxygen, triggers demethylation pathway.

Short-term cadmium, PAH, lead and mercury exposure direct reduce DNMT concentration and activity. Conversely, long-term cadmium exposure induced compensatory DNMT overexpression, which increase global DNA methylation (adapted from Ruiz-Hernandez et al, 2015).

**1.6 Environmental Context Overview: the Campania Region case study**

Scientific literature documents some biomonitoring and epidemiological analyses about national areas of particular interest. Seveso in the Northern of Italy and Taranto in the South are two examples (Hay, 1980; Brambilla et al., 1998; Signorini et al., 2000; Warner et al., 2013; Vimercati et al., 2016; Buononato et al., 2016; Ingelido et al., 2017; Lovreglio et al., 2018). Among them, one of the most famous italian environmental contexts, considered in the last decades as a polluted land, is Campania Region. For several years, some areas of Campania have been scenario of irregular waste disposal and illegal dumping, defining a potential danger for spreading of persistent pollutants in the environment. In addition, due to a series of uncontrolled burning waste events, some of these areas gained the name of "Land of Fires" (LOF) (Legambiente Reports, 2013 and 2015). Over the years, public opinion has focused its attention on potential environmental damage arising from the LOF and its supposed correlations with adverse pathological outcomes, generating alarm. Probably because of an intense and inaccurate media coverage, and probably because of a complete unawareness of people on the true situation, the attention was brought on the Campania products, triggering a widespread distrust for what came from the territory, with a huge damage for agri-food sector. This emergency prompted special intervention by the Italian government, which set up an inter-ministerial task force (Law 6/2014) charged with guaranteeing the safety of agricultural production.

Many substances, chemical products and derivatives of burning and waste disposal are known to be harmful (especially those featured in the IARC ranking, in which substances are classified as carcinogenic on the basis of solid scientific evidences - https://monographs.iarc.fr/agents-classified-by-the-iarc/). Consequently, those associated with practices and events responsible of the LOF phenomenon, have been characterized from epidemiological and analytical point of view in various institutional and research contexts. This has made Campania one of the most thoroughly investigated Regions in Italy regarding the environmental pollution.

Several studies have been carried out (Mazza et al., 2018); some have focused on the measurement of exposure in humans or animals (Basile et al., 2009; Rivezzi et al., 2013; De Felip et al., 2014; Esposito et al., 2014; De Roma et al., 2017), others on the epidemiological evaluation of exposure and health effects (Senior and Mazza, 2004; Fazzo et al., 2011; Pirastu et al., 2013; Comba et al., 2014; Triassi et al., 2015). One series of monitoring activities on environmental and biological matrices showed levels of pollutants consistent with those expected for burning waste, as dioxins, dioxin-like and polychlorinatedbiphenyls compounds (Rivezzi et al., 2013). Likewise, compounds originating from urban areas and from rural and industrial activities have been detected with specific distribution in the provinces (Pacini et al., 2013; Ducci et al., 2017; Thiombane et al., 2018). By contrast, several studies have revealed levels of pollutants in

Campania comparable to or lower than the values registered in other European countries or in the industrialized areas of Northern Italy (Ulaszewska et al., 2011; De Felip et al., 2014). In 2014, Esposito et al. did not detect significantly higher levels of organic compounds (dioxins, dioxin-like, polychlorinated biphenyls congeners) in blood serum from people living in urban Naples and in "triangle of death" area, than those recorded in other European countries and Italian Regions. This was probably due to the lower presence of industrial plants and other recognized sources. In 2013, Pacini et al. reported that the main bodies of water in several areas of the Campania Region were in poor condition, owing to their relatively severe sediment contamination. Nevertheless, in the fish samples analyzed, they did not find high levels of the compounds investigated.

Concerning epidemiological evaluation, data from scientific literature actually reveal a decreased trend for oncological mortality from 1988 to 2009 in Campania, at the regional level, and, to a lesser extent, in the Provinces of Naples and Caserta (where LOF municipalities are concentrated), although in these same areas it was registered a significant deviation, from regional and national trend, with mortality excesses for some neoplastic and chronic conditions (Crispo et al., 2013). Furthermore, in 2014-2015, the SENTIERI report documented cancer incidence and mortality rates in towns in the LOF areas that were comparable to those recorded in areas with a history of industrial pollution in Northern and Southern Italy.

With regard to disease epidemiology, in 2014 Pirastu et al. reported higher rates of all-cause mortality and mortality due to neoplastic and chronic diseases in both sexes in two Campania areas, "Litorale Domizio-Flegreo and Agro-Aversano" and "Area Litorale Vesuviano", in comparison with the regional population; these data were later confirmed by Zona et al. in a 2019 update of the fifth SENTIERI report. However, only some of the medical conditions investigated can be correlated '*a priori*' with environmental causes. While the above authors hypothesize a correlation between disease onset and mortality rates with unauthorized waste disposal or occupational exposure, they also strongly recommend improving biomonitoring, epidemiologic evaluation and primary health interventions.

**1.7 Integrated Approach for Human Biomonitoring**

Diverse scientific publications on Campania Region case study and HB have stoked an epidemiological controversy (Cantoni, 2016). It is well known that the genesis of neoplastic disease is complex and multifactorial, therefore its investigation requires the integration of different evaluations on determining factors. In fact, the epidemiological evidence needs to take in account some other putative determinants of disease, including lifestyle, lack of a culture of prevention, social deprivation, and even the governance of the Regional Health System. Various epidemiological, clinical, geographical models have shown statistically significant correlations between several kinds of chronic or neoplastic diseases and exposure to diverse chemicals. However, much of the evidence available have been limited by the lack of fundamental information, such as environmental characterization in areas of analysis or the failure to investigate biomarkers or to adopt a molecular approach (Barba et al., 2011). None of the above-mentioned epidemiological evaluations have considered all the features of the phenomenon in its entirety, nor was able to analyze a homogeneous data-set: integration of data on the territory (soils, waters and air), food, animals, humans, or full-blown clinical outcomes (including confounding factors). Moreover, none of them have assessed the complex system of variables, environmental matrices and toxicokinetic which contribute to exposure and the putative risk for citizens' health. The real critical point in this type of analysis is the complexity of the environmental system, which includes biotic and abiotic compartments that coexist in a delicate balance.

Hence, to support a complete understanding of the phenomena, the entire system needs to be integrated into a single multilevel analysis, in order to define causal relationships throughout the network of factors involved.

For example, in HB studies, a fundamental contribute should be provided by the monitoring of the environmental contexts, especially by pollutants data in matrices (soil, water and air), in order to acquire a synthetic indicator of the contamination levels in the system analyzed. Additionally, in order to assess the contribution of diet and lifestyle, HB participants should be subjected to a validated questionnaire which collect these evidence (e.g. EPIC – European Prospective Investigation into Cancer and Nutrition studies); they also should undergo a complete medical examination to assess clinical parameters and anamnestic data. Finally, the sample size and the choice of matrices of analysis should fit with the primary endpoints of the study, with the possibility of integrating exposure, effects and susceptibility biomarkers (Pierri et al., 2020).

Concerning LOF and Campania Region case study, since data collected in different HB have so far been unable to demonstrate a causal link between human exposure to pollutants from dumps or other sources and increased mortality due to chronic and degenerative diseases in some municipalities, this implicates an enhanced and compelling requirement of a methodological breakthrough.

As mentioned above, some assessments have not considered a set of specific variables covering environmental features, context observations and confounding aspects (lifestyle and dietary habits, social and economic deprivation, lack of a culture of prevention).

This underscores the pressing need of applying a new biomonitoring model, a new multidisciplinary and systemic approach with an analytical design capable of depicting the real state of contamination, and then to look in depth for the molecular mechanisms and the potential phenotypic consequences, with a view of assessment the exposure risk for residents and susceptible individuals.

## 2. AIMS OF THE STUDY

The purpose of the dissertation is to introduce and describe an innovative HB protocol, carried out in the complex environmental context of the Campania Region, including the "Land of Fire" area. The dissertation is especially focused on innovations in HB study design and procedures and on biomarkers of exposure and effect considered in the health risk assessment for people residing in the regional territory.

The biomonitoring model is based on a whole systemic analytical evaluation of the environmental context. The paradigm of the protocol considers three elements: identification of pollution sources, of pollutants migration ways and of effects on target organisms, and pursues its aims enrolling healthy human cohorts, in order to identify potential risks of exposure to pollutants and to potentially predict correlated clinical outcomes.

The following chapters will describe:

1) Innovative study design and procedures, with environmental background characterization.

2) Exposure and effect biomarkers aimed to have a direct quantification of the environmental impact on the general population.

3) Exploring the environmental epigenetic marks, as DNA methylation, through an "omics" approach.

4) Data integration from several levels of analysis: monitoring of environmental matrices, human biomonitoring, toxicogenomic analysis and epidemiological evaluation, in order to obtain a framework of the regional condition, giving a spatial and geographical reference to pollutants, their sources and their migration ways. This could make up for the lack of methodological sharing and data merging among complementary scientific fields.

The long-term objective of the study, over the current dissertation, is to create an integrated, dynamic map of environmental contamination in the Campania Region, in order to support the implementation of public health interventions and provide a reference model for the evaluation of exposure risk assessment.

Given its holistic nature, the model aspires to be translated in different contexts and for various applications, and to become a useful tool for comparative and multi-criteria analysis elsewhere.

# 3. MATERIALS AND METHODS

## 3.1 Study Design

SPES "Exposure Study on Susceptible People" is an epidemiological observational cohort study promoted by the Istituto Zooprofilattico Sperimentale del Mezzogiorno (IZSM) of Portici (Naples) in collaboration with the National Tumor Institute IRCCS "G. Pascale" of Naples. The protocol of the study was approved by the ethics committee of the IRCCS "G. Pascale" through Commissioner Deliberation n.590 of 03/08/2016.

The study design consists of two parts:

1. A context overview, in order to individuate source of contamination (variables) and the migration pathways of pollutants, and to define, by means of a multi-criteria analysis, geographical areas of interest as "clusters", which are then ranked according to a computed environmental "Pressure Index".
2. A transversal biomonitoring of people living in these clusters, in order to estimate "exposure" by quantifying pollutants in biological samples and investigating its biomolecular effects through in-depth "omics" analysis.

### 3.1.1 Environmental characterization of the Campania Region

Data from the scientific literature, the ARPAC (environmental regional agencies) database, Potential Hazard Maps (Minolfi et al., 2016), local monitoring projects on the Land of Fire and on the Campania Region, and preliminary results from the Campania Trasparente project ([www.campaniatrasparente.it](www.campaniatrasparente.it)) (Fig.5) allowed us to have an overview of the territorial context and potentially contaminated areas, by mapping the spatial distribution of chemical compounds and geo-localizing the putative sources of pollution.
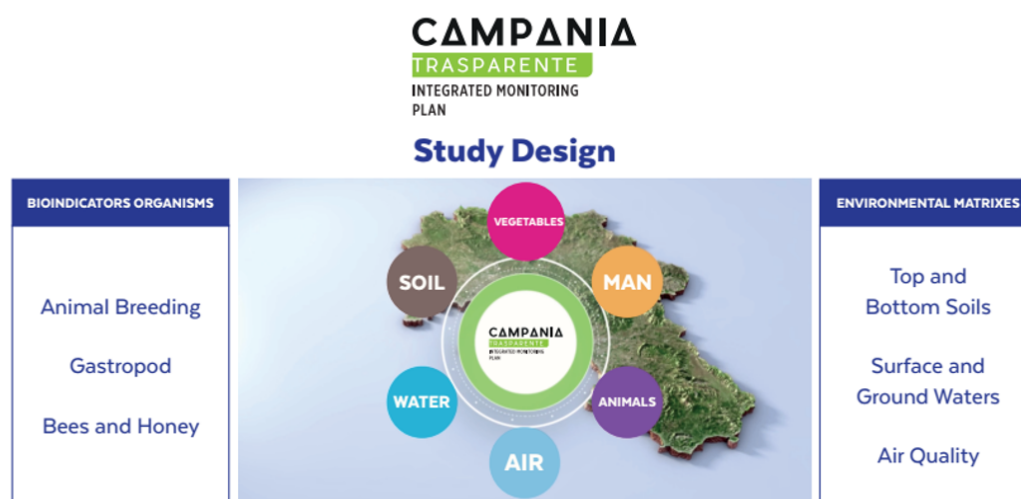
**Figure 5.** Campania Trasparente Environmental Monitoring. The environmental background of SPES biomonitoring. The search for pollutants in bioindicator organisms and environmental matrices yields a guideline for identifying impact areas and Risk Indexes in the municipalities observed.

### 3.1.2 Pressure Index computing for cluster generation and impact areas definition

We adopted an innovative approach in order to construct a "Comparative Risk Assessment Model" based on a multi-criteria analysis and combinatorial calculation of the percentage weight assigned to variables that contribute to determining the exposure.

The paradigm, shown in Figure 6, defines the evaluation chart model, from the selection of sources responsible for contamination, through the identification of migration pathways of pollutants, up to the final targets of exposure.

This approach provides a better understanding of the environmental impact determined by the sum of variables, the concerted action which generates the exposure, or rather creates part of the "exposome" (Wild, 2005 and 2012; Dennis et al., 2017), which could condition human health and putative targets.
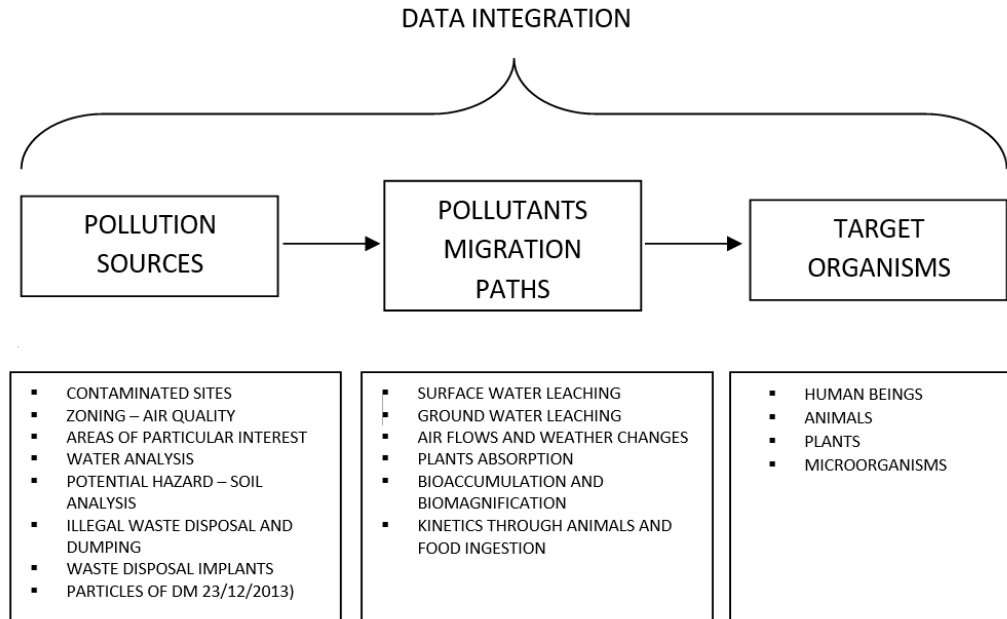
**Figure 6.** Paradigm of the method, from the selection of variables (sources of pollution) for AHP model, through identification of migration pathways of pollutants, to final target organisms. Migration pathways of pollutants represent the junction between the origin of pollutants and the final targets.

The variables shown in Table 3 were selected in such a way as to include all the factors involved in the pollution process, i.e. sources of potential contamination, analytical data on environmental matrices (soil, water, air), migration pathways from contamination sources to potential targets, and the targets themselves. Each variable was inserted into a rank system which attributes a score to each single factor, depending on the weight of the factor in generating the exposure; this operation was done by means of the multi-criteria decision support technique Analytic Hierarchy Process (AHP) (Saaty, 2008).

**Table 3**. Variables selected for Multi-criteria Analysis by the Analytical Hierarchy Process (AHP).

| | VARIABLES – SOURCES OF POLLUTION | Environmental Parameters for IRc computation |
|---|---|---|
| 1 | Contaminated sites | Sites according to Legislative Decree 152/2006; sites of classes 3, 4 and 5 according to Decrees n.56 of 09/03/2015 and n.191 of 08/19/2015 related to the "Land of Fires". |
| 2 | Zoning | Use of land for agriculture, industry and urban purposes; housing density and evaluation of air quality. |
| 3 | Areas of particular interest | Sites of national and local interest present in the regional territory; illegal and pending landfills and potentially contaminated sites surveyed since at least one value above the CSCs was found. |
| 4 | State of groundwater and surface water | Water Analysis data from Campania Trasparente and ARPAC monitoring. |
| 5 | Potential Hazard | Soil analysis data from Minolfi et al., 2016, Campania Trasparente and ARPAC monitoring. |
| 6 | Illegal waste disposal and dumping | Data monitoring by SMA Campania (https://www.smacampania.info/progetto-terra-dei-fuochi/). |
| 7 | Waste disposal plants | Composting plants, waste-to-energy plants, STIR plants, controlled landfills |
| 8 | Particles covered "Land of Fire" Decree | DM 23/12/2013 - TdF 2a, 2b and 5, 4 and 3 of class A particles. |

The AHP enables several alternatives to be compared with regard to a plurality of criteria, and yields an overall evaluation of each variable, which then can be ordered, for instance, according to a preference axis. Thus, all variables were geo-referenced and ordered in a geographic database (using Geographic Information System - GIS) and their percentage weight distributions were defined by means of AHP (Fig. 7).
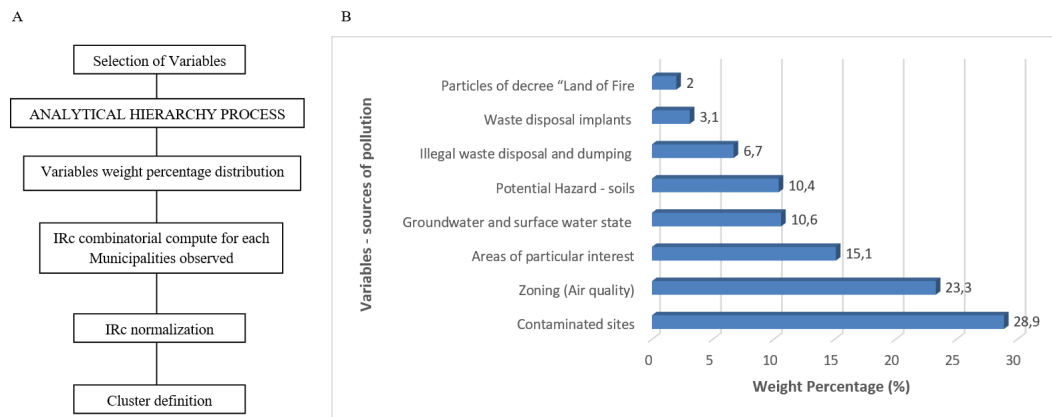
**Figure 7.** (A) Flowchart for the definition of "Municipal Risk Index" (IRc) and cluster generation. (B) Percentage distribution of weights of variables resulting from Analytical Hierarchy Process. For each source of pollution, the AHP model defines the importance of criteria by means of pairwise comparison. Comparisons create a calculation matrix from which the generation of a priority vector gives the final percentage distribution of the weight of each variable.

This enabled us to compute a "Municipal Risk Index" (IRc): our "Pressure Index", given by the sum of the weights of the variables characterizing the territory of the municipality in analysis. This value ensures diversification of the pressures exerted by each source and reproduces different levels of risk; to sum up, this index provides a synthetic representation of the level of pressure exerted by the contamination sources on the municipal territory. The IRcs have been subsequently normalized (by a relativization method with variation range), and divided into homogeneous classes using the Natural Breaks Algorhitm (Dent 1999; Slocum 1999): 21 clusters comprising several municipalities whose score of risk index is included in a given range (Table 4). A completely different approach from the epidemiological computed indexes of Martuzzi et al., 2009 and Musmeci et al., 2010.

**Table 4**. Computed Municipal Index Risk IRc: range and clustering.

| N. CLUSTER | CLUSTER | IRc | Normalized IRc |
|---|---|---|---|
| 1 | HIGH_1 | 833-1015 | 16-19 |
| 2 | HIGH_2 | 1051.7-1216.10 | 21-23 |
| 3 | HIGH_3 | 1240,8 - 1375,9 | 24-26 |
| 4 | HIGH_4 | 1408.4 - 1514 | 27-29 |
| 5 | HIGH_5 | 1563 - 1687.5 | 30- 32 |
| 6 | HIGH_6 | 1692.3 - 1876.2 | 33 - 36 |
| 7 | HIGH_7 | 1916.4 - 2049.7 | 37-40 |
| 8 | HIGH_8 | 2101.3-2286.7 | 41-44 |
| 9 | HIGH_9 | 2429.6 - 2650.1 | 47-51 |
| 10 | HIGH_10 | 2729.6 - 3005.7 | 53-58 |
| 11 | HIGH_11 | 3251.3 - 3347.8 | 63-65 |
| 12 | HIGH_12 | 4125.9 - 5108.1 | 81-100 |
| 13 | MEDIUM_1 | 791.7 - 1200.8 | 15-23 |
| 14 | MEDIUM_2 | 1544.2 - 1749.4 | 30-34 |
| 15 | MEDIUM_3 | 1847.5 - 3373.1 | 36 -66 |
| 16 | LOW_1 | 265.6 - 499 | 4 - 9 |
| 17 | LOW_2 | 525.4 - 815.6 | 9 - 15 |
| 18 | LOW_3 | 812 - 1087.3 | 15 - 21 |
| 19 | SABATO | 1100.6 - 2077.9 | 21 - 40 |
| 20 | IRNO_1 | 687 - 1201.3 | 13 - 23 |
| 21 | IRNO_2 | 1711.1 | 33 |

The spatial distribution of environmental pressures, as yielded by the variables and by the computed IRc, provided an exhaustive representation of the territory, characterized mainly by three increasing levels of impact: high impact, medium impact and low impact (Fig. 8A). The high-impact area (Fig. 8B) included most of the territory of the Naples and Caserta provinces; this area is densely populated, has poor-quality groundwater and exceeds "contamination threshold concentrations" (CSCs) in soil samples. Numerous contaminated or potentially contaminated sites are located in this area, according to Legislative Decree 152/2006, denoting a high level of environmental pressure. The medium-impact area (Fig. 8C) included municipalities in the Naples, Salerno and Avellino provinces, which have poor-quality groundwater and exceed CSCs in soils. In addition, some municipalities show groundwater concentrations of organic halogenated compounds far above CSC values. The "Irno Valley" and "Sabato Valley", which are situated in this area, contribute to this contamination through the presence of large industrial facilities and the orographic conformation of the sites. The low-impact area (Fig. 8D), covering the province of Salerno, the coastline (Cilento) and inland area (Sele-Tanagro), is characterized by a good state of groundwater, a low presence of CSCs and a low anthropization rate.

On the basis of the migration pathways of pollutants, an intervention area was identified for each level.
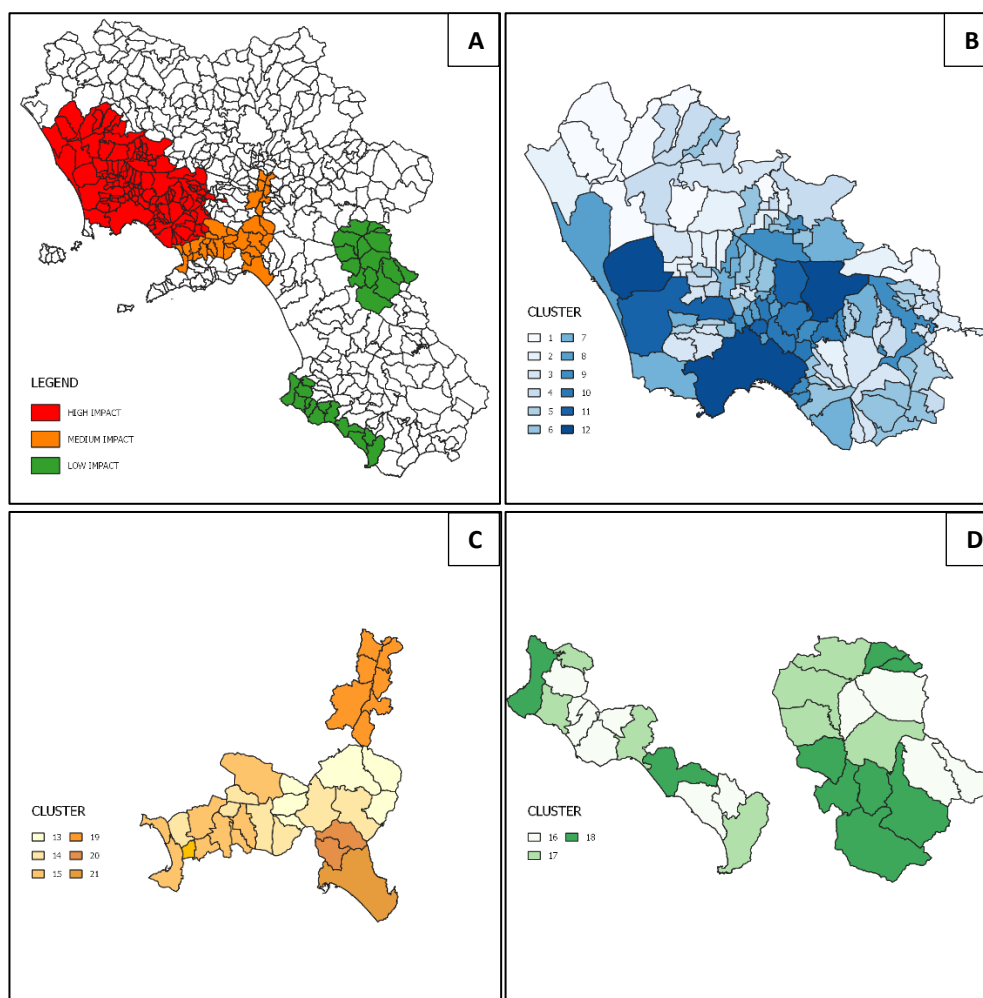
**Figure 8**. Clusters and Impact Areas. (A) Map of Campania Region showing areas of impact and clustering: (B) high impact, (C) medium impact, (D) low impact.

Based on the Comparative Risk Assessment Model and on the clustering of the municipalities in areas linked to different levels of human exposure, a target population in the HB protocol was identified in a ratio of 4:2:1 in high-, medium- and low-impact areas, respectively: 12 clusters were selected in the high-impact area, 6 in the medium-impact area and 3 in the low-impact area.

### 3.1.3 Study Population– SPES "Exposure Study on Susceptible People"

In order to integrate epidemiological observations, biomonitoring and toxicogenomic approaches, the SPES study design envisioned the recruitment of healthy residents in the Campania Region, the aim being to further investigate the state of exposure and its putative pathological consequences.

The scientific protocol involved the evaluation of a set of biomarkers able to quantify exposure and to detect individual genetic and molecular responses to the potential damage suffered.

In detail, exposure biomarkers comprised the presence of organic and inorganic compounds; effect biomarkers included sentinels of morphological, structural and functional alteration of cell systems; individual biomarkers of genetic susceptibility involved subjective features of predisposition or resistance to disease (Fig. 9).
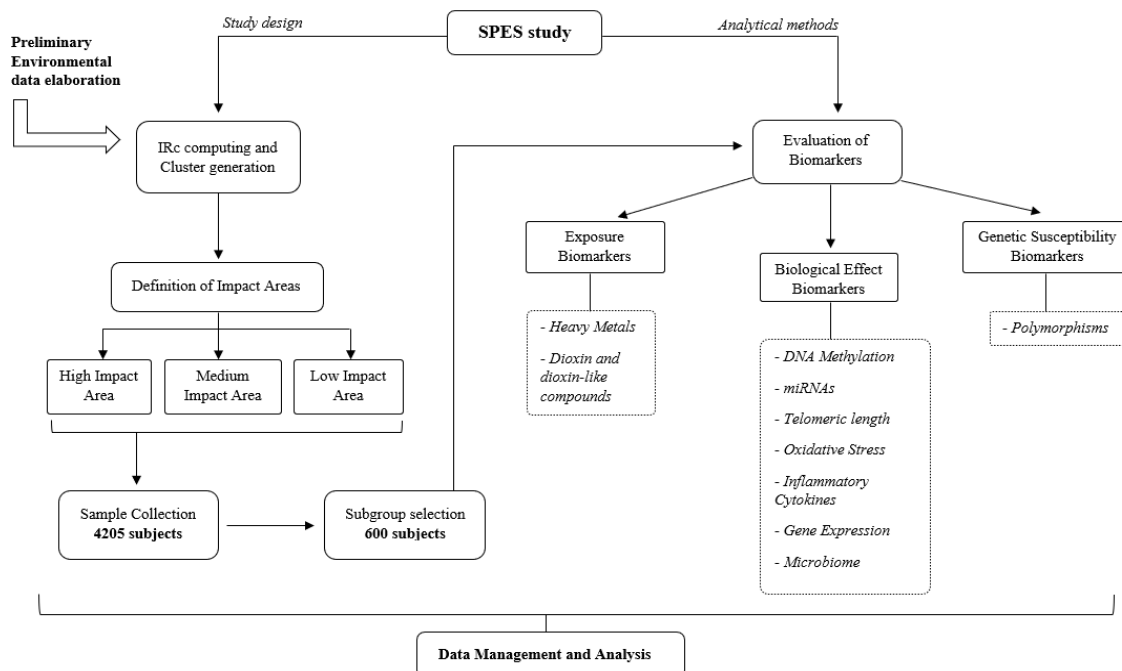


**Figure 9.** SPES Study Design. Selection of target population and analytical methods.

The study design considers an integrated set of assessments on healthy subjects living in several regional areas with different environmental "Pressure Indexes", computed by analyzing environmental data and literature references, as explained in previous paragraph. A total of 4205 subjects were enrolled; the sample size was fixed in such a way as to guarantee optimal power on statistical tests (with a significance level of $\alpha= 0.06$, the power is 90%); subjects were selected from the various impact areas in a ratio of 4:2:1, in cohorts of 200 people for each cluster.

The inclusion criteria were:

- o good health (no severe or neoplastic conditions),
- o age range 20-50,
- o both sexes,
- o no viral infectious (HIV, HCV, HBV),
- o residence in the municipalities under study.

Among the inclusion criteria, it is important to underline that the age-range chosen (20-50 years) allows a better correlation between lifestyle and exposure to work-related risks; indeed, in younger or older age-groups, this correlation may be underestimated or overestimated, respectively. In this way, we tried to reduce the contribution of some confounding factors. In addition, the choice of the age-range guarantees greater adherence

to the project and a higher probability of being able to conduct long-term follow-up. Furthermore, in addition to having a negative history of HIV, HCV, HBV (to ensure the safety of operators and avoid bias) and providing informed consent, the participants must have lived for at least 5 years in these municipalities.


### 3.1.4 People cohorts enrollment and sample collection

The viability of the study was ensured through precise planning of the operating procedures; this guaranteed the ethical and scientific validity of the methods and actions in each phase and the rapid achievement of the final endpoints.

A Contract Research Organization (CRO) supervised all procedures during protocol performing.

The operational steps of the study were divided into five main areas of action:

(i)  volunteers recruiting;
(ii) enrollment and medical examination;
(iii) samples collection;
(iv) experimental phase and reporting of results;
(v)  data management and elaboration.

The selection procedures were implemented through random extrapolation of the population sample by means of an algorithm (Linear Congruential Generator - LCG) that randomly selects potential participants from municipal registries on the basis of the criteria indicated. The alignment of this random sample with the provincial phonebooks permitted to obtain the telephone contacts that the dedicated call center can make use of.

The activity of the call center was accompanied in parallel by the activity of recruitment units throughout the territory; these geo-located the potential participants in the municipalities of interest, identified stakeholders and promoted scientific communication. Indeed, scientific and health communication was one of the secondary endpoints of the study and was aimed at promoting awareness and transferring knowledge of health and prevention issues through specific campaigns tailored on the basis of social deprivation indexes.

On recruitment, participants signed a document in which they consented to the confidential treatment of their personal information, in accordance with current legislation on privacy (EU 679/2016). Eligible subjects, who were willing to participate, were registered in a database (www.openspes.campaniatrasparente.it), in which all the associated information, documents and reports are stored and managed. With the submission of an informed consent the volunteers become enrolled and, in order to ensure the protection of privacy, everyone is associate to an univocal ID code, as well as applied data encryption and policies to restrict access to information. All interactions with the system take place via secure channels using internationally recognized security protocols (for instance https).

Synergic teams of recruiters, doctors and nurses completed the entire recruitment, enrollment, medical examination and sample collection steps in the different impact areas in the same time period (november 2016 – august 2017).

Participants first underwent medical examination, completed a questionnaire on lifestyle and eating habits (EPIC questionnaire – European Prospective Investigation into Cancer and Nutrition -  validated by WHO – World Health Organization; Riboli, 1992; Margetts et al., 1997), and filled in an anamnestic (professional, familial, clinical and pathological) case report form (CRF).

Finally, samples of blood (about 100 ml in several aliquots) urine and stool were collected. These biological samples constituted the biological matrices in which biomarkers of exposure, effect and genetic susceptibility were measured, in order to further investigate any causal link between environmental contamination and health.

Every sample collected was identified by a unique barcode, associate with the ID of the subject.

Samples were transported with secondary and thirdly boxes and GPS- Data Logger Device, in order to guarantee transport temperature control and traceability.

Aliquots not used for immediate analysis have been stored in the IZSM Biobank dedicated to the study ([www.biobancaizsm.it](www.biobancaizsm.it)).

### 3.1.5 Subgroup selection and analytical methods

In addition to routine biochemical-clinical investigations and virologic investigations (HBV, HCV, HIV, HEV), which define an entrance profile of the individual, the analytical step has provided the search in the biological samples of exposure, effect and susceptible biomarkers.

Regarding biomarkers of exposure, we have considered Potentially Toxic Metals (MPT), Dioxins, Furans and PCBs linked to exposure to different sources of pollution, able to persist in the environment and in living organisms for a long time (WHO 2015). They were detected by chemical and biological methods: Inductively Coupled Plasma - Mass Spectrometry methods (ICP-MS) for MPT;  High Resolution - Gas Chromatography (HR-GC) (adapted from US EPA Methods 1613 e 1668) and CALUX Assay (Warner et al. 2005; Sakthivel et al. 2016) for organic pollutants, dioxins, furans, dioxin-like, PCBs, PAH and other metabolites.

About Effect biomarkers, SPES protocol have considered: evaluation of the telomeric length (De Felice et al. 2012; Borghini et al. 2016), of the inflammatory cytokines (Gruzieva et al., 2017), of the oxidative stress (Rossner et al. 2013; Brucker et al. 2013), total plasmatic protein, as well as insights on epigenomic mechanisms such as the DNA methylation (by pyrosequencing and microarray) (Plusquin et al. 2017; Demanelis et al. 2019), circulating miRNA (La Rocca et al. 2016; Vrijens et al. 2016), panel of gene

expression profile (Ameer et al. 2017; Espin-Perez et al. 2018), microbiome (Claus et al. 2016) and metabolomic panel (Saberi et al. 2013; Li et al. 2019b).

Finally, biomarkers of genetic susceptibility. Details are shown in table 5.

**Table 5**. Scheme of Exposure, Effects and Susceptible Biomarkers.

| BIOMARKER | BIOLOGICAL MATRIX | N. SAMPLES TESTED |
|---|---|---|
| ➢ EXPOSURE BIOMARKERS: | | |
| - Heavy Metals (20) | Serum | 4205 |
| - Organic - Dioxins, Furans, dioxin -like PCBs, non-dioxin-like PCBs | serum | 600 |
| - Bioassay CALUX for determination of organic pollutants | plasma | 600 |
| - Trace Elements | serum | 600 |
| - Speciation Cr VI (hexavalent chromium) | erythrocytes | 600 |
| - Speciation As (Arsenic) | urine | 180 |
| ➢ EFFECT BIOMARKERS: | | |
| - Telomeric length | DNA from whole blood | 600 |
| - Inflammatory cytokines (Luminex assay pane l) | plasma | 600 |
| - Redox status | erythrocytes e mRNA from whole blood | 600 |
| - miRNA (panel of 18 miRNA) | miRNA from whole blood | 600 |
| - DNA Methylation (Pyrosequencing) | DNA from whole blood | 600 |
| - DNA Methylation (Array Infinium Methylation 850k Illumina) | DNA from whole blood | 600 |
| - Plasmatic markers (AOPP, MDA, homocystein, thiol, total protein) | plasma | 600 |
| - Copy Number Variation mitochondrial DNA | DNA from whole blood | 600 |
| - Gene Expression – Nanostring Panel | RNA from whole blood | 600 |
| - Microbiome | stool | 600 |
| - Metabolome | serum | 600 |
| ➢ GENETIC SUSCEPTIBILITY BIOMARKERS: | | |
| - Polymorphisms (hOGG1, XRCC3, XRCC1, CYPA1) | DNA from whole blood | 600 |

In order to preliminary test all the biomarkers and identify the set of specific and trustable ones (especially in view of optimizing funding resources), a subgroup of 600 subjects has been selected between the 4205 enrolled.

The subgroup represents a specific part of SPES population, eligible for the following inclusion criteria:

- No smoking;
- No alcohol abuse;
- No use of drugs for chronic diseases (in order to avoid iatrogenic alterations);

and balance criteria:

- Ratio of 2:2:1 between high, medium and low impact areas, in such way as to maximizing the difference between clusters;
- Stratified by body mass index (no obesity of II, III and IV classes);

and, finally, with Professional Identification.

Details in Table 6.

**Table 6**. Subgroup stratification per gender, age, clusters and impact areas.

| | | | male | | | female | | | total per | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Clusters | Age range 20-29 | Age range 30-39 | Age range 40-49 | Age range 20-29 | Age range 30-39 | Age range 40-49 | Cluster | Impact Areas |
| HIGH | | 9 | 10 | 10 | 10 | 10 | 10 | 10 | 60 | |
| | | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 60 | 240 |
| | | 11 | 10 | 10 | 10 | 10 | 10 | 10 | 60 | |
| | | 12 | 10 | 10 | 10 | 10 | 10 | 10 | 60 | |
| MEDIUM | | 13 | 10 | 10 | 10 | 10 | 10 | 10 | 60 | |
| | | 14 | 10 | 10 | 10 | 10 | 10 | 10 | 60 | 240 |
| | | 19 | 10 | 10 | 10 | 10 | 10 | 10 | 60 | |
| | | 20+21 | 10 | 10 | 10 | 10 | 10 | 10 | 60 | |
| LOW | | 16 | 10 | 10 | 10 | 10 | 10 | 10 | 60 | |
| | | 17 | 10 | 10 | 10 | 10 | 10 | 10 | 60 | 120 |
| | | | 100 | 100 | 100 | 100 | 100 | 100 | | |
| | | | | male total | 300 | | female total | 300 | | |
| | | | | | | | | TOTAL | | 600 |

### 3.1.6 Sample processing and Biobanking

Biological specimens collected were blood, urine and stool.

Blood was collected in tubes vacutainer as follow:

- n. 8 vacutainers for serum (BD REF 366468 SST II Advance Tube);

- n. 6 vacutainers for plasma and whole blood (BD REF 367864 $K_2$EDTA Tube);

- n. 2 vacutainers for plasma (BD REF 368886 LH Lithium Heparin);

- n. 2 Tempus Blood RNA (REF 4342792);

for a total amount of about 100 ml.

Fresh samples were allocated to biochemical and virological analysis, within 3 hours from venous sampling (1 vacutainer for serum and 2 vacutainers with $K_2EDTA$ for each subject). The remaining part was processed as follow:

- Serum was separated by centrifugation at +4°C, 10 minutes at 2000 g. Aliquots obtained were stored at -80°C in cryovials until analysis.
- Plasma from $K_2EDTA$ and Lithium Heparin vacutainers was obtained after centrifugation at +4°C, 10 minutes at 2500 g. Then separated and stored at -80°C in cryovials until analysis.
- Whole blood aliquots ($K_2EDTA$ vacutainers) were inverted several times and then transferred to cryovials for storage at -80°C until analysis.
- Tempus Blood RNA were vortexed and then stored at -80°C as manufacturer's instructions.
- Urine, collected in sterile urine container, was aliquoted in cryovials and stored at -20°C until analysis.
- Stool, collected in sterile container, was directly stored at -80°C until DNA extraction for microbiome analysis.

The biobank dedicated to the study ([www.biobancaizsm.it](www.biobancaizsm.it)) has a system of remote monitoring of temperature. Each aliquot is registered on the platform by a sample code and position coordinates in the fridge.


## 3.2 Biomarkers of Exposure

### 3.2.1 Heavy Metals

The determinations were performed by IZSM's Department of Chemistry.

All study participants samples (4205) were analyzed for 19 metals (arsenic [As], beryllium [Be], cadmium [Cd], cobalt [Co], chromium [Cr], copper [Cu], iron [Fe56 and Fe57], mercury [Hg], lithium [Li], manganese [Mn], molybdenum [Mo], nickel [Ni], lead [Pb], antimony [Sb], selenium [Se], strontium [Sr], thallium [Tl], vanadium [V], and zinc [Zn].

The measurement was carried out in serum by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) NexION 350 (PerkinElmer, Waltham, USA) equipped with a concentric nebulizer (Meinhard Associates, Golden, USA), a cyclonic spray chamber and a quartz torch with a quartz injector tube (2 mm internal diameter). Argon gas (99.9999%) and Helium 6.0 were supplied from Rivoira (Italy).

For each analyte, accuracy and precision were assessed by internal quality controls and using certified reference materials.

Calibration standard solutions and internal standards were prepared by successive dilution of a high purity multi-element Standard Solution at 10 mg/L, obtained from Perkin Elmer (Norwalk, CT). Internal standard solution containing yttrium (Y), rhodium (Rh), and

lutetium (Lu) (each at 1000 mg/L) were acquired from Perkin Elmer. Superpure grade nitric acid 68% (v/v) was obtained from VWR International (Belgium).

Serum was thawed at room temperature and mixed by gentle rotation. Then, 500 μl of sample were diluted 1:10 (v/v) with nitric acid 67-69 %.

Trace element concentrations were determined in serum by ICP-MS and the signal intensities were compared to a standard calibration curve for each trace element.

The following isotopes were selected: 75As, 111Cd, 9Be, 59Co, 52Cr, 63Cu, 57Fe, 202Hg, 7Li, 55Mn, 98Mo, 60Ni, 208Pb, 121Sb, 78Se, 88Sr, 205Tl, 51V and 66Zn.

The correlation coefficient ($R^2$) of calibration curves for all the trace elements was always greater than 0.99, showing a good linear relationship throughout the selected ranges of concentration. Each sample was analyzed in duplicate and the mean concentration was used in all statistical analyses.

### 3.2.2 Dioxins, Furans and PCBs congeners by High Resolution Gas Chromatography – Mass Spectrometry

The determinations were performed by IZSM's Department of Chemistry in partnership with University of Liegi (Belgium). The analysis was carried out on the 600 subgroup samples. Analytical method used was adapted from Brasseur et al. (2014).

This protocol allowed the determination of congeners belonging to classes of compounds as Dioxins PCDD (polychlorinated dibenzo-p-dioxin), Furans PCDF (polychlorinated dibenzofuran), DL-PCB (Dioxin like - polychlorinated biphenyl) and NDL-PCB (Non-Dioxin like - polychlorinated biphenyl) were analyzed.

For the purpose of the present dissertation will be considered only the following variables:

- Sum of PCDD/PCDF pg WHO-TEQ/g lipids.
- Sum of DL-PCB pg WHO-TEQ/g lipids.
- Sum of PCDD/PCDF + DL-PCB pg WHO-TEQ/g lipids.
- Sum of NDL-PCB ng/g lipids.

A volume between 10 to 20 ml of serum for each sample was processed. After the accelerated solvent extraction using a mixture of hexane-acetone, and a clean-up step, the serum was analysed by High Resolution Gas Chromatograph – High Resolution Mass Spectrometer (DFS Magnetic Sector HRGC-HRMS system, Thermo Fisher Scientific) operating at a resolution of at least 10000 at a 10% valley.

Each batch of analysis was monitored for Quality Assurance and Quality Control by testing control samples.

Given the lipophilic nature of the congeners, data obtained from biochemical analysis (through enzymatic method) were considered for the computing of fats percentage: the total lipid (TL) content was estimated using triglycerides (TG), total cholesterol (TC), non-esterified (free) cholesterol (FC) and phospholipids (PL). For each serum sample, the TL content, expressed in mg $dL^{-1}$, was calculated by the following equation:

$$TL = 1.677 \times (TC\text{-}FC) + TG + PL$$

where 1.677 is the ratio of the mean molecular weight of esterified cholesterol to unesterified cholesterol (Bernert et al., 2007; Brasseur et al., 2014).

For comparing the estimate of the lipid content in human serum obtained using alternative formulas, the TL contents calculated with the above-mentioned equation was compared with the results calculated using the formula described by Phillips et al. (1989):

$$TL = (2.27 \times TC) + TG + 62.3 \text{ mg dL}^{-1}$$

Two equations provided comparable values for TL.

Analytical results were expressed not only as measured value, but also according to Toxic Equivalent (TEQ) concentrations, by means Toxic Equivalent Factors (TEF) (Van den Berg et al., 2006). Congeners and their sums were also expressed used the approach of Lower, Medium and Upper Bound, that means to replace 0.00 (zero), ½ LOQ (Limit of Quantification) or LOQ, if the detected value was below the LOQ itself (De Felip et al., 2008; Miniero et al., 2017).

### 3.2.3 Chemical Activated LUciferase gene eXpression (CALUX) Bioassay

These determinations were performed in partnership with Bio-Detection System (BDS) Laboratory (The Netherlands). The analysis was carried out on the 600 subgroup samples. Chemical Activated LUciferase gene eXpression (CALUX) is a ligand-dependent nuclear receptor- bioassay used in the detection of classes of chemicals in food, environmental and biological matrices. It could be considered as a screening method because it estimates the total effect that ligands have on the receptor observed. Engineered cell lines are stably transfected with a luciferase reporter gene. If activated by ligands presence, DNA responsive elements stimulate transcription of the inserted luciferase gene and produce the light-generating enzyme which can be measured by Luciferin addition (Fig.10).
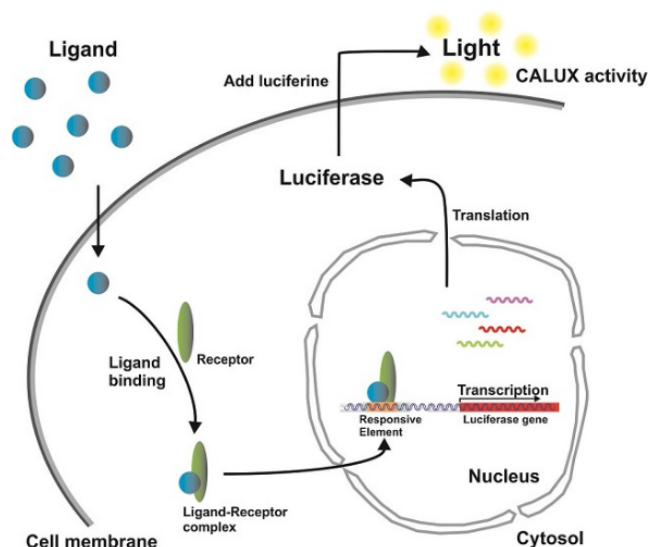
**Figure 10.** CALUX bioassay mechanism. The presence of the ligand by binding to the nuclear receptor allows its dimerization and transfer to the nucleus, where an intracellular signal transduction cascade activates regulatory regions and responsive elements of the DNA which, associated with the luciferase reporter gene, permit gene expression and luminescence emission as a response to enzymatic activity in the presence of luciferin.

SPES protocol have considered the DR CALUX® (Garrison et al., 1996), PAH- CALUX® (Pieterse et al, 2013) and ERα CALUX® (Sonneveld et al., 2005). They are all based on stably transfected cell lines: DR and PAH CALUX are based on stably transfected rat hepatoma H4IIe cell lines, while the ERα CALUX assay is based on a stably transfected human osteoblastic osteosarcoma U2-OS cell line (American Type Culture Collection). Cells that are exposed to compounds of interest not only express proteins that are under normal circumstances associated to the receptors, but also luciferase. By addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor binding, which is benchmarked against the relevant reference compounds, i.e., 2,3,7,8-TCDD (2,3,7,8-tetrachlorodibenzodioxin) for DR CALUX; B(a)P (benzo(a)pyrene) for PAH CALUX and 17-β-estradiol for ER CALUX.

The results are expressed as bioassay equivalents (BEQ) per gram of lipid (DR, PAH) or per ml of plasma (ERα).

In detail, the human plasma (5 ml obtained from Lithium Heparin Vacutainers) was extracted with hexane/ethyl ether solvent, subsequently purified (clean-up) on a silica column; the extraction product is resuspended in DMSO (dimethyl sulfoxide).

The ERα CALUX cells, U2-OS cells, were routinely sub-cultured every 3–4 days in growth medium consisting of DMEM (Gibco) supplemented with 7.5% dextran-coated charcoal stripped fetal calf serum (DCC-FCS), 1×nonessential amino acids (Gibco) and 10 U/ml penicillin and 10 μg/ml streptomycin. DR and PAH-CALUX cells were routinely sub-

cultured every 3–4 days in growth medium consisting of αMEM (Gibco) supplemented with 10% fetal calf serum. All cell types were always maintained at 37 °C and 5% $CO_2$. CALUX® cells were seeded in 96 wells plates in assay medium and fetal calf serum according to conditions indicated in Table 7. Exposure of the cells started when > 95% confluency was reached (DR, PAH) or at 10000 cells per well (ERα).

The cell cultures are then incubated with the DMSO eluted extract for 24 hours. Incubation is in triplicate for each biological sample and takes place in 96-well plates. A solution containing luciferin is added to estimate the luciferase activity by means of a luminometer (Berthold Centro XS3). Each plate contains a calibration curve with reference standards relating to the class of compounds investigated: full dose-response curve of the reference compound, 2,3,7,8-TCDD for DR CALUX, B(a)P for PAH CALUX and 17-β-estradiol for the ERα CALUX.

**Table 7.** CALUX cell type, culture and exposure information.

| Assay | **ERα CALUX** | **DR CALUX** | **PAH CALUX** |
|---|---|---|---|
| Cell type | U2OS | H4IIe | H4IIe |
| Species | Human | Rat | Rat |
| %DMSO exposure | 0,1%, or 1%* | 0,8% | 0,8% |
| %$CO_2$ incubation | 5% | 5% | 5% |
| Exposure time | 24 hrs | 24 hrs | 4 hrs |
| Confluence/cells | 10000 cells/well | >95% confluence | >95% confluence |
| Medium used | DMEM/F12 | αMEM | αMEM |
| % FCS | 7,5% DCC-stripped** | 10% | 10% |
| Additions to medium | Non-essential amino acids | | |

* All samples were first tested at 0,1% DMSO; when low values were observed (below Limit of quantification) these samples were re-analyzed using 1% DMSO

** FCS was treated with dextran-coated charcoal (DCC) to strip the endogenously present hormones

Analysis results of plasma extracts expressed as relative light units (RLUs) are interpolated in the calibration curves of each respective bioassay for quantitative determination of the induction potential using the statistical software package GraphPad Prism V5.03. All analysis results are expressed as amount of reference compound equivalents per ml processed sample (BEQ/ml plasma), or per gram of fat (BEQ/g fat).

Fats percentage follows gravimetrically measurement and, in parallel, the same formulas of Dioxins/Furans/PCBs in previous paragraph.

### 3.3 Biomarkers of Effect and Susceptibility

### 3.3.1 DNA Methylation Array

The determinations were performed in partnership with Lab. Med. Mol. Ge., University of Salerno. The analysis was carried out on the 600 subgroup samples.

Genomic DNA was extracted from whole blood aliquots (obtained from $K_2$EDTA vacutainers) stored at -80°C in IZSM Biobank.

The nucleic acid extraction protocol using the QIAsymphony Biorobot (QIAGEN), Custom B_400 protocol, was carried out according to DSP DNA MIDI QIASymphony Kit (REF 937255). A volume of 400 µl of whole blood was the input material. Extracted DNA was finally eluted in 100 µl of nuclease-free $H_2O$ (Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water).

Genomic DNA was checked for integrity by means of Genomic DNA protocol (REF 5067-5365/5366) on Agilent TapeStation System 4200. Concentration and purity were assessed by spectrophotometer Nanodrop ND-1000: A260/A280 and A260/A230 ratio. Accurate concentration measurement was performed by means of Qubit 2.0 Fluorometer, according to Qubit dsDNA BR – Broad Range kit (REF Q32850).

All samples showed A260/A280 ratio around 1.8, and A260/A230 ratio around 2.00, confirming the good quality of DNA. Tape Station DINs (DNA Integrity Number) were for all samples in acceptable range for integrity 6.00 to 10.00.

The bisulfite conversion step was performed with EZ DNA Methylation Zymo kit (ZYD5001) with 500 ng of genomic DNA as input material, according to manufacturer's instructions.

DNA methylation arrays were performed according to Illumina Infinium HD Arrays procedure. Particularly, Infinium Methylation EPIC 850K (Infinium HD Assay Methylation Protocol Guide Material #20002138 Document #15019519v01), which allows the identification and quantitative analysis of the methylation state of approximately 850000 CpG dinucleotides distributed throughout the entire human genome, was used.

The Infinium HD Assay for Methylation combines bisulfite conversion of genomic DNA and whole-genome amplification (WGA) with direct, array-based capture and scoring of the CpG loci. During the chemical conversion induced by bisulfite, the unmethylated cytosines are deaminated to uracil while the methylated ones are protected and remain so. The enzymatic reaction that occurs on the surface of the array determines the incorporation of complementary fluorescent bases which, thanks to different fluorophores, allows to discriminate one case from another for each dinucleotide analyzed.

This method considers two chemistry technologies, Infinium I and Infinium II. The Infinium I probe design has two probes per site (one for methylated, one for unmethylated) and the 3' end of the probes is positioned directly across from the CpG site (Fig.11A). The Infinium II probe has one probe per site (for the methylated state) and the 3' end of the probes is positioned immediately adjacent to the site (Fig.11B).

**Figure 11.** Broader Coverage using Illumina Infinium I and II Assay Design. (A) The Infinium I assay employs 2 probes per CpG locus: 1 "unmethylated" and 1 "methylated" query probe. The 3'terminus of each probe is designed to match either the protected cytosine (methylated design) or the thymine base resulting from bisulfite conversion and whole-genome amplification (unmethylated design). (B) The Infinium II assay design requires only one probe per locus. The 3' terminus of the probe complements the base directly upstream of the query site. A single base extension results in the addition of a labeled G or A base, complementary to either the 'methylated' C or 'unmethylated' T (source www.emea.illumina.com).

This approach allows to evaluate 95% of the cytosines present in the best functionally characterized regions along the whole human genome and, in particular, > 99% of the human gene regulatory sites (RefSeq), including promoters, 5 'UTR, gene body (exons and introns) and 3 'UTR, with excellent coverage also of the enhancer regions (Moran et al, 2016).

### 3.3.2 DNA Methylation Array data analysis

Signal intensity was measured with the Illumina iScan system. DMAP files (containing the spatial maps of the probes along each bead-chip), were downloaded following the "Decode File Client" instructions, the windows-based software application supplied for this purpose. The input data from the Illumina iScan were analyzed using the Illumina GenomeStudio® Methylation Module v1.9 software, in order to generate Beta Values, the measure of the level of methylation at a locus. Beta values represent the estimate methylation level of the

CpG locus as ratio of intensities between methylated and unmethylated alleles; it is computed by means the following expression: β = M/M+U+100, where M indicates the methylated locus, M+U the sum of methylated and unmethylated loci and 100 a mathematical constant. Beta values are intended to allow sample to sample comparisons for each individual CpG and can be interrogated and compared across samples for powerful large-scale studies.

Genome Studio Software allows to check and validate array sessions through a series of controls: sample independent controls, for quality workflow steps, and sample dependent controls, that evaluate performance across samples. Among them for example, the bisulfite conversion rate check.

First step analysis considers the background subtraction, in order to avoid any non-specific signal, and normalization. Then a filter was applied: only the probes that showed a detection p-value <0.05 (98% for each sample) were selected, considering for the subsequent steps only the CpGs with a high detection quality.

Together with Genome Studio Software, provided by Illumina, thanks to the partnership with The Laboratory of Statistics and computational tools for Bioinformatics (BioinfoLab) – Istituto per le applicazioni del calcolo "Mauro Picone", National Research Council of Naples (CNR), data analysis were also performed by means R software (4.0.3 version) and Bioconductor, with ChAMP package (https://www.bioconductor.org/packages/release/bioc/html/ChAMP.html) as follows:

- Probes normalization;
- Correction for Cell Heterogeneity (step considered when sample matrix is blood);
- Singular Value Decomposition (SVD) for the batch effect correction;
- Batch correction on SVD indication;
- Differential Methylated Probes identification (DMP);
- Differential Methylated Region identification (DMR);
- Gene set enrichment for DMP and DMR.

In addition, both Genome Studio and R Bioconductor-ChAMP display valuable information such as chromosomal coordinates, percent GC, location in CpG Island, and β values useful for perform an in-depth analysis.

### 3.4 Statistical Analysis

Standard descriptive statistics were used for characterizing the study sample. Numerical variables were described using mean ± standard deviation (std. dev.) or median (25th - 75th percentile) in case of distribution showing a consistent asymmetry. Absolute frequencies and percentages were used to describe categorical factors. Several median regression models were estimated to explore the association between serum exposure biomarkers in the assessed population and impact areas, as well as clusters.

In those models where the impact area was considered as the geographical reference unit, the low impact area was always used as reference category. When analyzing geographical difference at cluster's level, the reference cluster was the one with the median concentration.

Considering Calux bioassay data analysis, statistical significance was set at a level p-value<0,05 and paired datasets were analyzed by the Mann Whitney test, while differences between multiple groups were analyzed using one-way Anova test.

For methylation data analysis, in order to highlight a potential correlation between methylation levels and exposure biomarkers, data analysis proceeded through a robust linear multiple regression model, by M-value transformation from methylation beta values. All the analyses were performed using the statistical platform R.

### 3.5 Data management

The study protocol has been supported by web-based software (openspes.campaniatrasparente.it), developed by IZSM, and custom-made for the SPES study. The software interface works in the Linux Apache POSTGRESsql (LAPP) environment to ensure maximum interoperability and scalability. The programming language operated is PHP interfaced with Apache server. The management and permanent storage of data has been carried out using POSTGRESql equipped with PostGis extension in order to ensure maximum interoperability with GIS systems. The graphical interface is based on the twitter Bootstrap framework, so to optimize the web platform for displaying on several kinds of devices of different degrees of resolution. All users are able to access the system using a web browser; they therefore, using a TC/ IP network and communicating via the HTTPs protocol to send data and make requests to the Apache web server, without installing additional software. All restriction and cryptographic policies have been applied to guarantee the protection and privacy of data. The openspes system also communicates with the GIS system to collect environmental data from "Campania Trasparente" and literature references (www.geojizz.it) in order to integrate data into a single software.

## 4. RESULTS

### 4.1 Environmental background

A preliminary analysis on the environmental background was performed to compute the environmental pressure index for each municipality.

Data from scientific literature and from "Campania Trasparente" monitoring (www.campaniatrasparente.it; Allocca et al., 2016; Qu et al., 2019;; Sellerino et al., 2019; Nasta et al., 2020) were combined in the "Comparative Risk Assessment Model" (see Paragraph 3.1.2), in order to obtain a synthetic representation of the level of pressure exerted by the pollutant sources, on the municipal territory. This synthetic index is represented by the IRc (see Table 4). IRc values allow to cluster municipalities. Figure 12 shows a graphical representation of the IRcs, according to the geographical distribution.
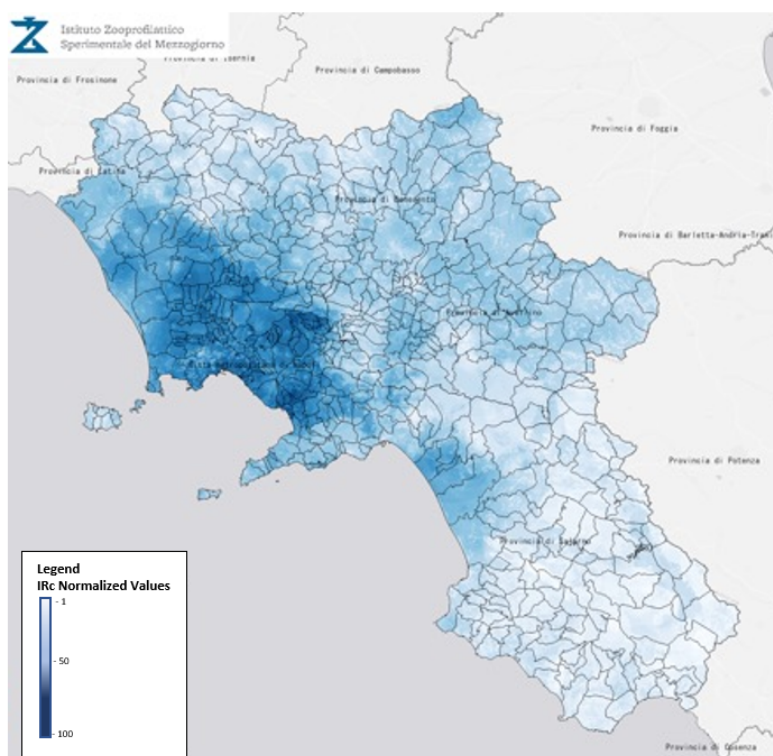


**Figure 12.** Campania region and geographical distribution of IRcs. The spatial distribution of the environmental pressures provides an exhaustive representation of the territory, mainly characterized by three increasing levels of impact: high impact, medium impact and low impact.

The evidence of critical issues on the regional territory could be divided, from an environmental point of view, in different macro-areas:

1. Volturno and Regi Lagni areas (Caserta province), where pollutants values above normative referments (Legislative Decree 152/2006; Annex 5, Part IV) are Lead (Pb) and Zinc (Zn) in soil matrix, both attributable to vehicular traffic. Spreading of Volatil Organic Compounds (VOC) in surface waters is attributable to industrial

waste disposal, especially for Villa Literno and Marcianise municipalities. Finally, nitrates peaks are attributable to agricultural and urban contributes.

2. Flegrea-Neapolitan area (Naples and north of Naples), where high levels of toxic metals are recorded both in urban and in agricultural areas. Lead (Pb) and Zinc (Zn) in soils are attributable to vehicular traffic, while polycyclic aromatic hydrocarbons in waters are probably linked to intentional or accidental waste disposal.

3. Sarno-Vesuvio area (south of Naples), where surface pollution for coast line regards heavy metals and organochlorine pesticides, while the most critical issue is registered for Sarno river area and for Terzigno municipality, this last with ground water contamination attributable to landfill leachate (Cava Sari and Cava Ranieri). Finally, the wide presence of copper (Cu) is linked to the indiscriminate use of pesticides in viticulture.

4. Irno and Sabato Valley, where the presence of industrial plants gives an important contribute to the spreading of heavy metals and polycyclic aromatic hydrocarbons.

According to the environmental monitoring and to the IRcs computing, three areas with increasing levels of impact were identified: high-, medium- and low-impact (Paragraph 3.1.2, Fig. 8).

As mentioned in Materials and Methods chapter, the target population in the SPES protocol was identified in a ratio of 4:2:1 in high-, medium- and low-impact areas, respectively: 12 clusters were selected in the high-impact area, 6 in the medium-impact area and 3 in the low-impact area. A subgroup of 600 subjects has been selected among the 4205 enrolled, in ratio of 2:2:1 between high-, medium- and low-impact areas for further investigation.

**4.2 People demographic features**

Among the 4205 volunteers enrolled for the purposes of the biomonitoring study, a total of 2404 subjects (57.2% of the study sample) were recruited in the high impact area, 1201 (28.6% of the study sample) were recruited in the medium impact area and 600 (14.3% of the study sample) were recruited in the low impact area. Distribution of enrolled subjects according to age, gender, impact area and cluster is shown in table 8.

Mean age of the population was 34.9±8.8 years, with women representing 53.9% of the study sample. Age and gender distributions were consistent across the three study areas.

Median weight of the entire population was 71 kg [IQR: 61 ; 82] , while median height was 170 cm [IQR: 163 ; 175], with a median body mass index of 24.6 [IQR: 22.3 ; 27.7] kg/m² (table 9). Median weight in the high-, medium- and low-impact area was 71 [IQR: 60 ; 83], 71 [IQR: 61; 82] and 72 [IQR: 62 ; 82] kg, respectively, while median height was 170 [IQR: 163 ; 176], 168 [IQR: 161.2 ; 175] and 170 [IQR: 163 ; 175] cm, respectively (Table 9).

Blood count, blood biochemistry and endocrinological panel results were consistent with those of a healthy population, with adequate function of bone marrow, liver, kidney and of the endocrine system. No clinically relevant differences were reported among the three areas assessed.

**Table 8.** Number of participants according to age. gender. impact area and cluster.

| Study sample | # Cluster | Number of subjects (% with respect to the overall sample) |
|---|---|---|
| Total Population | | 4205 (100.0) |
| **Age** | | |
| 20-29 | | 1394 (33.2) |
| 30-39 | | 1296 (30.8) |
| 40-50 | | 1515 (36) |
| **Gender** | | |
| Male | | 2268 (53.9) |
| Female | | 1937 (46.1) |
| **Area** | | |
| High | | 2404 (57.2) |
| Low | | 600 (14.3) |
| Medium | | 1201 (28.6) |
| **Cluster** | **# Cluster** | |
| HIGH_1 | 1 | 204 (4.9) |
| HIGH_2 | 2 | 199 (4.7) |
| HIGH_3 | 3 | 200 (4.8) |
| HIGH_4 | 4 | 200 (4.8) |
| HIGH_5 | 5 | 200 (4.8) |
| HIGH_6 | 6 | 200 (4.8) |
| HIGH_7 | 7 | 199 (4.7) |
| HIGH_8 | 8 | 201 (4.8) |
| HIGH_9 | 9 | 200 (4.8) |
| HIGH_10 | 10 | 199 (4.7) |
| HIGH_11 | 11 | 200 (4.8) |
| HIGH_12 | 12 | 202 (4.8) |
| MEDIUM_1 | 13 | 179 (4.3) |
| MEDIUM_2 | 14 | 209 (5) |
| MEDIUM_3 | 15 | 212 (5) |
| IRNO_1 | 20 | 188 (4.5) |
| IRNO_2 | 21 | 212 (5) |
| SABATO | 19 | 201 (4.8) |
| LOW_1 | 16 | 200 (4.8) |
| LOW_2 | 17 | 205 (4.9) |
| LOW_3 | 18 | 195 (4.6) |

**Table 9.** Demographic characteristics of the overall sample population, according to the impact area.

| | Overall (n=4205; 100%) | IMPACT AREAS (HIGH vs MEDIUM vs LOW) | | |
|---|---|---|---|---|
| | | High (n=2404; 57.2%) | Medium (n=1201; 28.6%) | Low (n=600; 14.3%) |
| **Age; years** | 34.9+-8.8 (20 to 50) | 34.6+-8.8 (20 to 50) | 35.5+-8.8 (20 to 50) | 35.1+-8.9 (20 to 50) |
| **Gender; female** | 2268 (53.9) | 1306 (54.3) | 639 (53.2) | 323 (53.8) |
| **Deprivation Index*; %** | 26+-8.8 (0 to 92.1) | 25.5+-8.6 (0 to 74.7) | 25.9+-8.9 (5.7 to 92.1) | 28+-9.3 (8.6 to 65.3) |
| Below 1st quartile | 1045 (25.1) | 652 (27.1) | 293 (25.3) | 100 (16.7) |
| Below 2nd quartile | 1037 (24.9) | 610 (25.4) | 293 (25.3) | 134 (22.3) |
| Below 3rd quartile | 1071 (25.7) | 591 (24.6) | 294 (25.4) | 186 (31) |
| Above 3rd quartile | 1008 (24.2) | 550 (22.9) | 278 (24) | 180 (30) |
| **Occupational status; %** | | | | |
| Full time | 1846 (51.4) | 1069 (51.4) | 522 (52.8) | 255 (48.6) |
| Partial | 957 (26.6) | 518 (24.9) | 272 (27.5) | 167 (31.8) |
| Unemployed | 789 (22) | 492 (23.7) | 194 (19.6) | 103 (19.6) |
| **Current Potential Occupational Exposure; %** | 605 (14.4) | 330 (13.7) | 177 (14.7) | 98 (16.3) |
| **Weight; (kg)** | 71 [61; 82] (39 to 166) | 71 [60; 83] (42 to 166) | 72 [62; 82] (39 to 140) | 71 [61; 82] (41 to 135) |
| **Height; (cm)** | 170 [163; 175] (108 to 198) | 170 [163; 176] (108 to 198) | 170 [163; 175] (124 to 196) | 168 [161.2; 175] (108 to 196) |
| **BMI; (kg/m²)** | 24.6 [22.3; 27.7] (15.8 to 63.4) | 24.6 [22.2; 27.5] (15.8 to 63.4) | 24.6 [22.2; 27.8] (16.9 to 54.6) | 25 [22.5; 28.2] (16 to 48.8) |

*Deprivation index was computed taking into account both educational characteristics of the study participants (number of years of education, education degree), housing characteristics (number of rooms, number of occupants) and ownership of basic consumption goods (refrigerator and car). These types of data were collected by means EPIC questionnaire (see paragraph 3.1.4).

**4.3 Exposure Biomarkers**

**4.3.1 Heavy Metals**

Heavy metals results, expressed as serum concentrations in µg/L, were measured on the overall population. They are reported as geometric mean in the general population and in the three impact areas as well as in the 21 clusters (Tables 10a and 10b). Median and inter-quartile ranges are reported in tables 10c, 10d and 10e.

Geometric mean values were generally comparable among the three impact areas, except for mercury levels that were approximately twice in the medium impact areas vs. the high- and low-impact areas. Considering the 21 clusters defined and the 19 metals assessed, the most relevant differences of a single cluster *vs* average serum levels in the overall study sample, were reported in the Sabato valley cluster for cadmium (Cd111), nickel (Ni60) and antimony (Sb121) and for the two Irno valley clusters for mercury (Hg202), which were approximately five times higher than those found in the entire population.

Percentage fold decreases/increases of interesting 6 of 19 heavy metals, for which percentage fold increase with respect to the general population was ≥ 100% in at least one cluster, are reported in Table 11 and graphically represented in Figures 13 a to f.

In Median regression models, where impact area was the geographical analysis unit (Table 12 a/b), using the low impact area as reference, the high impact area was not associated with higher levels of any of the 19 heavy metals assessed, except for thallium (Tl205) whose median levels were significantly but only slightly higher in the high impact *vs* the low impact area (Difference between medians: 0.009; 95% C.I.: 0.006 to 0.011).

Compared to the low impact area, the medium impact one was instead associated with higher levels of mercury (Hg202) (Difference between medians: 0.87; 95% C.I.: 0.62 to 113), lithium (Li7) (Difference between medians: 0.09; 95% C.I.: 0.03 to 0.16), cobalt (Co59) (Difference between medians: 0.13; 95% C.I.: 0.09 to 0.17), manganese (Mn55) (Difference between medians: 0.15; 95% C.I.: 0.06 to 0.23), antimony (Sb121) (Difference between medians: 0.03; 95% C.I.: 0.01 to 0.04), thallium (Tl205) (Difference between medians: 0.004; 95% C.I.: 0.002 to 0.007) and zinc (Zn66) (Difference between medians: 138; 95% C.I.: 100 to 176). Older age was consistently associated with higher levels of arsenic (As75), cobalt (Co59), copper (Cu63) and strontium (Sr88). Male gender was characterized by higher levels of lithium (Li7), iron (Fe), manganese (Mn55), selenium (Se78) and zinc (Zn66) but lower levels of copper (Cu63).When analyzed by cluster (Table 13 a/b/c), it emerged that the associations found for the medium impact area were mainly due to the contribute of the Irno and Sabato Valley's clusters.

Finally, Tables 13d, 13e, 13f, report a correlation matrix showing how correlated metals are to each other, for impact areas. Noteworthy, there is a significant association between Hg and Cd in high impact areas and between Cd and Mn in medium impact areas.

**Table 10a.** Geometric mean of the heavy metals assessed in the serum (µg/L) by cluster and area.

| Cluster | Li7 | Be9 | Cd111 | As75 | Cr52 | Co59 | Cu63 | Fe | Hg202 | Mn55 |
|---|---|---|---|---|---|---|---|---|---|---|
| **High Impact Area** | **1.008** | **0.004** | **0.007** | **0.940** | **0.049** | **0.852** | **981.5** | **2311** | **0.573** | **0.612** |
| HIGH_1 | 1.102 | 0.004 | 0.006 | 1.033 | 0.045 | 0.948 | 1106 | 2363 | 0.631 | 0.831 |
| HIGH_2 | 0.948 | 0.004 | 0.007 | 0.735 | 0.046 | 0.806 | 966.1 | 2215 | 0.587 | 0.518 |
| HIGH_3 | 0.979 | 0.004 | 0.007 | 1.106 | 0.058 | 0.842 | 978.5 | 2153 | 0.686 | 0.562 |
| HIGH_4 | 1.002 | 0.004 | 0.009 | 1.082 | 0.037 | 0.814 | 1007 | 2258 | 0.55 | 0.56 |
| HIGH_5 | 1.151 | 0.003 | 0.007 | 0.744 | 0.036 | 0.966 | 992.4 | 2378 | 0.486 | 0.618 |
| HIGH_6 | 1.046 | 0.004 | 0.008 | 1.236 | 0.063 | 0.856 | 967.9 | 2399 | 0.518 | 0.685 |
| HIGH_7 | 0.809 | 0.005 | 0.007 | 0.457 | 0.048 | 0.786 | 942.9 | 2189 | 0.473 | 0.323 |
| HIGH_8 | 1.021 | 0.005 | 0.008 | 1.17 | 0.051 | 0.854 | 975.9 | 2433 | 0.622 | 0.688 |
| HIGH_9 | 1.104 | 0.004 | 0.007 | 1.096 | 0.044 | 0.905 | 973.7 | 2399 | 0.575 | 0.422 |
| HIGH_10 | 0.98 | 0.003 | 0.008 | 1.09 | 0.078 | 0.888 | 916.6 | 2364 | 0.628 | 0.845 |
| HIGH_11 | 0.96 | 0.003 | 0.008 | 0.911 | 0.063 | 0.777 | 984.2 | 2256 | 0.521 | 0.893 |
| HIGH_12 | 1.031 | 0.006 | 0.009 | 0.967 | 0.039 | 0.799 | 975.7 | 2341 | 0.658 | 0.666 |
| **Medium Impact Area** | **1.093** | **0.005** | **0.011** | **1.084** | **0.065** | **0.812** | **966.3** | **2154** | **1.505** | **0.689** |
| MEDIUM_1 | 0.958 | 0.004 | 0.007 | 0.765 | 0.056 | 0.975 | 1025 | 2592 | 0.648 | 1.12 |
| MEDIUM_2 | 0.949 | 0.004 | 0.008 | 0.779 | 0.063 | 0.855 | 1000 | 2475 | 0.564 | 0.592 |
| MEDIUM_3 | 0.912 | 0.004 | 0.005 | 0.799 | 0.028 | 0.833 | 995 | 2372 | 0.463 | 0.441 |
| IRNO_1 | 1.287 | 0.004 | 0.012 | 1.566 | 0.07 | 0.582 | 868.1 | 1656 | 4.517 | 0.499 |
| IRNO_2 | 1.245 | 0.005 | 0.011 | 1.221 | 0.078 | 0.697 | 881 | 1641 | 4.971 | 0.459 |
| SABATO | 1.292 | 0.007 | 0.053 | 1.794 | 0.151 | 1.021 | 1044 | 2425 | 1.678 | 1.74 |
| **Low Impact Area** | **1.037** | **0.004** | **0.009** | **1.182** | **0.061** | **0.904** | **1034.0** | **2379** | **0.756** | **0.922** |
| LOW_1 | 1.044 | 0.005 | 0.009 | 1.226 | 0.036 | 0.878 | 1006 | 2316 | 0.865 | 0.658 |
| LOW_2 | 1.021 | 0.003 | 0.008 | 1.327 | 0.076 | 0.941 | 1049 | 2487 | 0.899 | 1.058 |
| LOW_3 | 1.047 | 0.003 | 0.011 | 1.007 | 0.086 | 0.894 | 1048 | 2334 | 0.561 | 1.129 |
| **Overall** | **1.036** | **0.004** | **0.009** | **1.011** | **0.055** | **0.847** | **984.4** | **2274** | **0.804** | **0.671** |

**Table 10b.** Geometric mean of the heavy metals assessed in the serum (µg/L) by cluster and area.

| Cluster | Mo98 | Ni60 | Pb208 | Sb121 | Se78 | Sr88 | Tl205 | V51 | Zn66 |
|---|---|---|---|---|---|---|---|---|---|
| **High Impact Area** | **0.405** | **0.457** | **0.038** | **0.023** | **187** | **28.3** | **0.014** | **0.287** | **1342** |
| HIGH_1 | 0.408 | 0.321 | 0.036 | 0.02 | 191 | 29.8 | 0.017 | 0.374 | 1434 |
| HIGH_2 | 0.308 | 0.315 | 0.04 | 0.02 | 188 | 27.7 | 0.015 | 0.299 | 1311 |
| HIGH_3 | 0.466 | 0.526 | 0.036 | 0.02 | 189 | 28.5 | 0.013 | 0.344 | 1388 |
| HIGH_4 | 0.379 | 0.471 | 0.043 | 0.03 | 183 | 27.7 | 0.016 | 0.275 | 1270 |
| HIGH_5 | 0.275 | 0.586 | 0.032 | 0.02 | 207 | 30.9 | 0.014 | 0.27 | 1502 |
| HIGH_6 | 0.611 | 0.31 | 0.048 | 0.03 | 186 | 28.4 | 0.012 | 0.25 | 1287 |
| HIGH_7 | 0.445 | 0.306 | 0.03 | 0.02 | 165 | 26.1 | 0.011 | 0.213 | 1246 |
| HIGH_8 | 0.429 | 0.648 | 0.048 | 0.03 | 188 | 28.8 | 0.014 | 0.309 | 1367 |
| HIGH_9 | 0.53 | 0.829 | 0.037 | 0.04 | 192 | 28.5 | 0.015 | 0.234 | 1390 |
| HIGH_10 | 0.429 | 0.412 | 0.039 | 0.03 | 184 | 27.5 | 0.015 | 0.37 | 1287 |
| HIGH_11 | 0.399 | 0.36 | 0.037 | 0.03 | 190 | 28.5 | 0.019 | 0.343 | 1321 |
| HIGH_12 | 0.299 | 0.887 | 0.035 | 0.02 | 183 | 27.4 | 0.016 | 0.225 | 1314 |
| **Medium Impact Area** | **0.378** | **0.515** | **0.041** | **0.057** | **193** | **29.1** | **0.014** | **0.246** | **1507** |
| MEDIUM_1 | 0.391 | 0.309 | 0.044 | 0.04 | 193 | 28.7 | 0.018 | 0.32 | 1460 |
| MEDIUM_2 | 0.374 | 0.381 | 0.04 | 0.02 | 190 | 28.0 | 0.013 | 0.361 | 1385 |
| MEDIUM_3 | 0.423 | 0.724 | 0.031 | 0.02 | 199 | 29.2 | 0.007 | 0.245 | 1472 |
| IRNO_1 | 0.276 | 0.23 | 0.043 | 0.09 | 184 | 27.4 | 0.015 | 0.171 | 1580 |
| IRNO_2 | 0.229 | 0.226 | 0.034 | 0.08 | 181 | 27.3 | 0.009 | 0.176 | 1697 |
| SABATO | 0.752 | 3.198 | 0.06 | 0.23 | 209 | 34.3 | 0.033 | 0.26 | 1451 |
| **Low Impact Area** | **0.598** | **0.404** | **0.047** | **0.038** | **196** | **29.0** | **0.011** | **0.321** | **1331** |
| LOW_1 | 0.464 | 0.33 | 0.034 | 0.03 | 177 | 29.0 | 0.009 | 0.34 | 1294 |
| LOW_2 | 0.663 | 0.632 | 0.043 | 0.05 | 212 | 29.6 | 0.015 | 0.29 | 1377 |
| LOW_3 | 0.698 | 0.406 | 0.07 | 0.03 | 201 | 28.4 | 0.01 | 0.336 | 1321 |
| **Overall** | **0.42** | **0.472** | **0.04** | **0.03** | **190** | **28.6** | **0.014** | **0.279** | **1386** |

**Table 10c**. Median (IQR) of the heavy metals assessed in the serum (µg/L) by cluster and area. Data are reported as median [25th ; 75th percentile].

| Cluster | Li7 | Be9 | Cd111 | As75 | Cr52 | Co59 |
|---|---|---|---|---|---|---|
| **High Impact Area** | **1.01 (0.727 ; 1.444)** | **0.002 (0.002 ; 0.012)** | **0.005 (0.001 ; 0.059)** | **1.446 (0.615 ; 3.237)** | **0.196 (0.002 ; 0.832)** | **0.909 (0.688 ; 1.162)** |
| HIGH_1 | 1.048 (0.75 ; 1.427) | 0.002 (0.002 ; 0.018) | 0.001 (0.001 ; 0.057) | 1.565 (0.673 ; 3.182) | 0.168 (0.002 ; 0.727) | 0.965 (0.744 ; 1.232) |
| HIGH_2 | 0.958 (0.655 ; 1.353) | 0.002 (0.002 ; 0.011) | 0.002 (0.001 ; 0.07) | 1.432 (0.483 ; 3.198) | 0.149 (0.002 ; 0.727) | 0.857 (0.67 ; 1.135) |
| HIGH_3 | 0.919 (0.692 ; 1.397) | 0.002 (0.002 ; 0.012) | 0.001 (0.001 ; 0.062) | 1.478 (0.724 ; 2.962) | 0.239 (0.002 ; 0.862) | 0.914 (0.662 ; 1.167) |
| HIGH_4 | 1.02 (0.733 ; 1.385) | 0.002 (0.002 ; 0.002) | 0.005 (0.001 ; 0.07) | 1.749 (0.692 ; 3.405) | 0.08 (0.002 ; 0.784) | 0.94 (0.706 ; 1.18) |
| HIGH_5 | 1.136 (0.789 ; 1.625) | 0.002 (0.002 ; 0.002) | 0.001 (0.001 ; 0.042) | 1.328 (0.589 ; 3.178) | 0.088 (0.002 ; 0.633) | 1.01 (0.785 ; 1.268) |
| HIGH_6 | 1.044 (0.722 ; 1.538) | 0.002 (0.002 ; 0.015) | 0.001 (0.001 ; 0.073) | 1.711 (0.723 ; 3.233) | 0.314 (0.002 ; 1.045) | 0.914 (0.66 ; 1.207) |
| HIGH_7 | 0.961 (0.684 ; 1.429) | 0.002 (0.002 ; 0.021) | 0.003 (0.001 ; 0.067) | 1.151 (0.278 ; 2.904) | 0.167 (0.002 ; 1.122) | 0.796 (0.6 ; 1.054) |
| HIGH_8 | 1.02 (0.736 ; 1.426) | 0.002 (0.002 ; 0.028) | 0.001 (0.001 ; 0.069) | 1.575 (0.724 ; 4.03) | 0.189 (0.002 ; 0.882) | 0.877 (0.665 ; 1.141) |
| HIGH_9 | 1.087 (0.803 ; 1.573) | 0.002 (0.002 ; 0.009) | 0.009 (0.001 ; 0.029) | 1.47 (0.552 ; 3.477) | 0.165 (0.002 ; 0.751) | 0.925 (0.717 ; 1.192) |
| HIGH_10 | 0.972 (0.732 ; 1.358) | 0.002 (0.002 ; 0.005) | 0.008 (0.001 ; 0.053) | 1.591 (0.684 ; 3.23) | 0.348 (0.002 ; 0.891) | 0.966 (0.778 ; 1.18) |
| HIGH_11 | 0.942 (0.654 ; 1.338) | 0.002 (0.002 ; 0.006) | 0.007 (0.001 ; 0.033) | 1.174 (0.577 ; 2.947) | 0.253 (0.002 ; 0.777) | 0.838 (0.689 ; 1.023) |
| HIGH_12 | 1.035 (0.751 ; 1.413) | 0.002 (0.002 ; 0.031) | 0.006 (0.001 ; 0.102) | 1.437 (0.701 ; 3.469) | 0.077 (0.002 ; 0.9) | 0.89 (0.623 ; 1.129) |
| **Medium Impact Area** | **1.099 (0.805 ; 1.581)** | **0.002 (0.002 ; 0.022)** | **0.017 (0.001 ; 0.082)** | **1.69 (0.631 ; 3.89)** | **0.288 (0.002 ; 0.906)** | **1.039 (0.739 ; 1.352)** |
| MEDIUM_1 | 0.929 (0.717 ; 1.296) | 0.002 (0.002 ; 0.025) | 0.005 (0.001 ; 0.04) | 1.091 (0.528 ; 2.696) | 0.227 (0.002 ; 0.645) | 0.996 (0.802 ; 1.242) |
| MEDIUM_2 | 0.973 (0.7 ; 1.325) | 0.002 (0.002 ; 0.007) | 0.007 (0.001 ; 0.069) | 1.313 (0.421 ; 3.702) | 0.294 (0.002 ; 0.908) | 0.965 (0.716 ; 1.25) |
| MEDIUM_3 | 0.973 (0.76 ; 1.312) | 0.002 (0.002 ; 0.008) | 0.001 (0.001 ; 0.031) | 1.051 (0.44 ; 2.912) | 0.002 (0.002 ; 0.822) | 0.895 (0.657 ; 1.19) |
| IRNO_1 | 1.244 (0.921 ; 1.938) | 0.002 (0.002 ; 0.014) | 0.019 (0.001 ; 0.055) | 2.843 (0.872 ; 5.978) | 0.291 (0.002 ; 0.805) | 1.189 (0.772 ; 1.508) |
| IRNO_2 | 1.247 (0.854 ; 1.882) | 0.002 (0.002 ; 0.032) | 0.019 (0.001 ; 0.058) | 2.235 (0.926 ; 4.872) | 0.345 (0.002 ; 0.81) | 1.232 (0.839 ; 1.599) |
| SABATO | 1.197 (0.958 ; 1.643) | 0.002 (0.002 ; 0.059) | 0.095 (0.038 ; 0.196) | 2.319 (1.242 ; 3.69) | 0.697 (0.002 ; 1.26) | 1.042 (0.812 ; 1.319) |
| **Low Impact Area** | **0.99 (0.697 ; 1.537)** | **0.002 (0.002 ; 0.006)** | **0.013 (0.001 ; 0.041)** | **1.45 (0.62 ; 3.168)** | **0.277 (0.002 ; 0.804)** | **0.923 (0.742 ; 1.15)** |
| LOW_1 | 1.017 (0.686 ; 1.427) | 0.002 (0.002 ; 0.031) | 0.013 (0.001 ; 0.059) | 1.64 (0.666 ; 3.774) | 0.062 (0.002 ; 0.703) | 0.906 (0.74 ; 1.162) |
| LOW_2 | 0.956 (0.675 ; 1.561) | 0.002 (0.002 ; 0.002) | 0.012 (0.001 ; 0.029) | 1.413 (0.685 ; 3.632) | 0.331 (0.002 ; 0.94) | 0.935 (0.776 ; 1.155) |
| LOW_3 | 0.995 (0.709 ; 1.704) | 0.002 (0.002 ; 0.002) | 0.017 (0.001 ; 0.044) | 1.422 (0.503 ; 2.547) | 0.347 (0.002 ; 0.982) | 0.925 (0.696 ; 1.101) |
| **Overall** | **1.026 (0.736 ; 1.504)** | **0.002 (0.002 ; 0.012)** | **0.009 (0.001 ; 0.062)** | **1.513 (0.624 ; 3.462)** | **0.242 (0.002 ; 0.854)** | **0.941 (0.709 ; 1.213)** |

**Table 10d**. Median (IQR) of the heavy metals assessed in the serum (µg/L) by cluster and area. Data are reported as median [25th ; 75th percentile].

| Cluster | Cu63 | Fe | Hg202 | Mn55 | Mo98 | Ni60 | Pb208 |
|---|---|---|---|---|---|---|---|
| **High Impact Area** | **985.9 (832.5 ; 1159.9)** | **2389.2 (1794.8 ; 3127.9)** | **0.693 (0.119 ; 1.881)** | **1.031 (0.543 ; 1.506)** | **0.756 (0.305 ; 1.207)** | **1.121 (0.021 ; 2.537)** | **0.018 (0.018 ; 0.044)** |
| HIGH_1 | 1093.4 (935.8 ; 1310.8) | 2382.7 (1833.3 ; 3216.4) | 0.783 (0.119 ; 1.796) | 1.18 (0.797 ; 1.538) | 0.754 (0.322 ; 1.209) | 0.907 (0.021 ; 1.956) | 0.018 (0.018 ; 0.02) |
| HIGH_2 | 999.7 (814.5 ; 1152.4) | 2334.6 (1643 ; 3073.4) | 0.792 (0.119 ; 1.798) | 0.934 (0.504 ; 1.346) | 0.638 (0.128 ; 1.128) | 0.765 (0.021 ; 2.092) | 0.018 (0.018 ; 0.088) |
| HIGH_3 | 989.8 (814.2 ; 1165.7) | 2232.8 (1674.4 ; 2867.3) | 0.929 (0.119 ; 2.351) | 1.015 (0.473 ; 1.449) | 0.76 (0.389 ; 1.202) | 1.407 (0.021 ; 2.262) | 0.018 (0.018 ; 0.036) |
| HIGH_4 | 1009.4 (897.2 ; 1159.4) | 2327.9 (1770 ; 3069.1) | 0.684 (0.119 ; 1.86) | 0.981 (0.493 ; 1.47) | 0.744 (0.29 ; 1.084) | 1.027 (0.021 ; 2.503) | 0.018 (0.018 ; 0.073) |
| HIGH_5 | 980.6 (819.2 ; 1170.3) | 2516.6 (1832.3 ; 3161.3) | 0.449 (0.119 ; 1.901) | 0.966 (0.506 ; 1.416) | 0.628 (0.057 ; 1.151) | 1.526 (0.021 ; 3.475) | 0.018 (0.018 ; 0.018) |
| HIGH_6 | 982 (826.4 ; 1165.9) | 2434.7 (1814.8 ; 3321.6) | 0.528 (0.119 ; 1.741) | 1.148 (0.625 ; 1.575) | 0.945 (0.438 ; 1.28) | 0.819 (0.021 ; 2.808) | 0.018 (0.018 ; 0.142) |
| HIGH_7 | 957.6 (815.3 ; 1162.2) | 2239.3 (1670.3 ; 3101.3) | 0.316 (0.119 ; 1.765) | 0.781 (0.156 ; 1.317) | 0.774 (0.364 ; 1.223) | 0.641 (0.021 ; 2.011) | 0.018 (0.018 ; 0.018) |
| HIGH_8 | 985.4 (847.2 ; 1151.4) | 2472.2 (1924 ; 3111.9) | 0.728 (0.142 ; 1.996) | 1.019 (0.595 ; 1.52) | 0.855 (0.428 ; 1.172) | 1.151 (0.021 ; 3.112) | 0.018 (0.018 ; 0.098) |
| HIGH_9 | 963.2 (851.2 ; 1127.2) | 2427.3 (1800 ; 3250.7) | 0.697 (0.141 ; 1.534) | 0.86 (0.383 ; 1.504) | 0.833 (0.387 ; 1.21) | 1.753 (0.556 ; 3.056) | 0.018 (0.018 ; 0.026) |
| HIGH_10 | 921.4 (795.5 ; 1065.5) | 2437.4 (1918.2 ; 3123.2) | 0.854 (0.119 ; 1.762) | 1.135 (0.714 ; 1.611) | 0.81 (0.296 ; 1.23) | 0.78 (0.021 ; 2.266) | 0.018 (0.018 ; 0.048) |
| HIGH_11 | 993.7 (832.5 ; 1155.9) | 2420.2 (1899.8 ; 3022.3) | 0.654 (0.119 ; 1.583) | 1.158 (0.737 ; 1.527) | 0.742 (0.322 ; 1.168) | 0.774 (0.021 ; 1.96) | 0.018 (0.018 ; 0.033) |
| HIGH_12 | 967.7 (836.5 ; 1120) | 2449.7 (1851.1 ; 3261.1) | 0.863 (0.146 ; 2.086) | 1.081 (0.535 ; 1.595) | 0.669 (0.193 ; 1.072) | 1.578 (0.692 ; 2.703) | 0.018 (0.018 ; 0.021) |
| **Medium Impact Area** | **982 (842.7 ; 1148.3)** | **2442.9 (1787 ; 3176.5)** | **1.937 (0.564 ; 4.284)** | **1.309 (0.729 ; 1.78)** | **0.794 (0.27 ; 1.215)** | **1.054 (0.021 ; 3.042)** | **0.018 (0.018 ; 0.06)** |
| MEDIUM_1 | 1017.8 (848.6 ; 1169.6) | 2597 (2016.9 ; 3408) | 0.826 (0.119 ; 2.029) | 1.353 (0.847 ; 1.884) | 0.789 (0.317 ; 1.209) | 0.416 (0.021 ; 2.401) | 0.018 (0.018 ; 0.041) |
| MEDIUM_2 | 1002.4 (854.6 ; 1200.9) | 2534.3 (1820.7 ; 3306.8) | 0.568 (0.119 ; 1.792) | 1.086 (0.563 ; 1.556) | 0.792 (0.176 ; 1.355) | 1.068 (0.021 ; 2.47) | 0.018 (0.018 ; 0.047) |
| MEDIUM_3 | 981.5 (848.3 ; 1172.5) | 2441.6 (1876.3 ; 3110) | 0.385 (0.119 ; 1.65) | 0.952 (0.438 ; 1.368) | 0.809 (0.244 ; 1.27) | 1.845 (0.021 ; 4.269) | 0.018 (0.018 ; 0.018) |
| IRNO_1 | 903.8 (812.2 ; 1077.3) | 2233.2 (1424.1 ; 3170.7) | 5.14 (2.874 ; 8.812) | 1.4 (0.614 ; 1.735) | 0.728 (0.06 ; 1.112) | 0.383 (0.021 ; 1.478) | 0.018 (0.018 ; 0.064) |
| IRNO_2 | 925.8 (755.3 ; 1083.4) | 2326.2 (1286 ; 2998) | 5.046 (2.667 ; 10.542) | 1.246 (0.643 ; 1.654) | 0.757 (0.01 ; 1.165) | 0.415 (0.021 ; 1.169) | 0.018 (0.018 ; 0.021) |
| SABATO | 1040.3 (900.9 ; 1176.4) | 2468.8 (1999.9 ; 3090.4) | 1.812 (1.056 ; 2.972) | 1.785 (1.421 ; 2.248) | 0.895 (0.602 ; 1.168) | 3.43 (1.873 ; 11.147) | 0.018 (0.018 ; 0.281) |
| **Low Impact Area** | **1008.6 (870.8 ; 1210.5)** | **2456 (1847.6 ; 3177.3)** | **0.877 (0.189 ; 2.15)** | **1.157 (0.749 ; 1.622)** | **0.902 (0.541 ; 1.33)** | **0.858 (0.021 ; 3.423)** | **0.018 (0.018 ; 0.144)** |
| LOW_1 | 983.4 (839 ; 1202.3) | 2381.9 (1810.9 ; 3061.3) | 1.059 (0.253 ; 2.635) | 0.956 (0.585 ; 1.456) | 0.859 (0.405 ; 1.306) | 0.653 (0.021 ; 3.29) | 0.018 (0.018 ; 0.018) |
| LOW_2 | 1034.5 (865.9 ; 1224.6) | 2561.7 (1904.3 ; 3301.1) | 1.006 (0.333 ; 2.521) | 1.187 (0.813 ; 1.594) | 0.942 (0.557 ; 1.283) | 1.449 (0.021 ; 3.807) | 0.018 (0.018 ; 0.085) |
| LOW_3 | 1024.3 (899 ; 1191.3) | 2378.1 (1840.2 ; 3139.5) | 0.734 (0.119 ; 1.398) | 1.303 (0.832 ; 1.791) | 0.908 (0.624 ; 1.402) | 0.81 (0.021 ; 3.243) | 0.018 (0.018 ; 0.354) |
| **Overall** | **988.1 (841 ; 1163.6)** | **2413.9 (1799.7 ; 3155.7)** | **1.004 (0.119 ; 2.619)** | **1.129 (0.609 ; 1.618)** | **0.795 (0.326 ; 1.224)** | **1.057 (0.021 ; 2.752)** | **0.018 (0.018 ; 0.057)** |

**Table 10e**. Median (IQR) of the heavy metals assessed in the serum (µg/L) by cluster and area. Data are reported as median [25th ; 75th percentile].

| Cluster | Pb208 | Sb121 | Se78 | Sr88 | Tl205 | V51 | Zn66 |
|---|---|---|---|---|---|---|---|
| **High Impact Area** | **0.018 (0.018 ; 0.044)** | **0.063 (0.002 ; 0.168)** | **194.3 (159 ; 228.1)** | **28.3 (22.3 ; 35.3)** | **0.025 (0.001 ; 0.054)** | **0.418 (0.216 ; 0.693)** | **1387 (1178.3 ; 1618.6)** |
| HIGH_1 | 0.018 (0.018 ; 0.02) | 0.029 (0.002 ; 0.18) | 198.3 (162.9 ; 233) | 29.2 (24.3 ; 35.9) | 0.031 (0.001 ; 0.06) | 0.477 (0.292 ; 0.787) | 1462.2 (1262.6 ; 1679.6) |
| HIGH_2 | 0.018 (0.018 ; 0.088) | 0.002 (0.002 ; 0.144) | 194.1 (158.5 ; 230.2) | 27.7 (22.2 ; 35.2) | 0.03 (0.001 ; 0.065) | 0.501 (0.232 ; 0.876) | 1363.6 (1123.5 ; 1614.8) |
| HIGH_3 | 0.018 (0.018 ; 0.036) | 0.029 (0.002 ; 0.141) | 195.8 (157.4 ; 238.1) | 27.6 (20.8 ; 37.2) | 0.022 (0.001 ; 0.045) | 0.45 (0.251 ; 0.744) | 1404.6 (1159.7 ; 1638.7) |
| HIGH_4 | 0.018 (0.018 ; 0.073) | 0.103 (0.002 ; 0.182) | 187 (159 ; 219.3) | 28.2 (21.7 ; 35.9) | 0.029 (0.001 ; 0.067) | 0.451 (0.188 ; 0.774) | 1371 (1121.1 ; 1617.6) |
| HIGH_5 | 0.018 (0.018 ; 0.018) | 0.002 (0.002 ; 0.15) | 207.2 (175 ; 243.2) | 29.8 (25.4 ; 35.9) | 0.022 (0.002 ; 0.046) | 0.424 (0.223 ; 0.639) | 1529.2 (1308.9 ; 1709.9) |
| HIGH_6 | 0.018 (0.018 ; 0.142) | 0.071 (0.002 ; 0.155) | 195.9 (157.7 ; 228.1) | 28.5 (21.8 ; 35.3) | 0.022 (0.001 ; 0.049) | 0.387 (0.163 ; 0.678) | 1380.3 (1114.9 ; 1602.1) |
| HIGH_7 | 0.018 (0.018 ; 0.018) | 0.002 (0.002 ; 0.132) | 175.8 (135.3 ; 206.6) | 25.8 (20.3 ; 33.8) | 0.017 (0.001 ; 0.058) | 0.395 (0.184 ; 0.609) | 1304 (1040.8 ; 1568.9) |
| HIGH_8 | 0.018 (0.018 ; 0.098) | 0.078 (0.002 ; 0.182) | 194.7 (154.7 ; 218.9) | 27.9 (23 ; 36.5) | 0.027 (0.001 ; 0.054) | 0.446 (0.218 ; 0.781) | 1408.9 (1173.9 ; 1608.5) |
| HIGH_9 | 0.018 (0.018 ; 0.026) | 0.106 (0.002 ; 0.187) | 195.1 (164.2 ; 228.8) | 28.5 (22.9 ; 34.6) | 0.023 (0.005 ; 0.042) | 0.361 (0.184 ; 0.558) | 1392.7 (1218.2 ; 1676.3) |
| HIGH_10 | 0.018 (0.018 ; 0.048) | 0.103 (0.002 ; 0.185) | 187.4 (155.5 ; 218.1) | 27.9 (22.4 ; 33.6) | 0.023 (0.004 ; 0.048) | 0.409 (0.266 ; 0.631) | 1317.1 (1103.8 ; 1545.1) |
| HIGH_11 | 0.018 (0.018 ; 0.033) | 0.102 (0.002 ; 0.174) | 197.7 (168.4 ; 234.5) | 28.9 (22.2 ; 35.4) | 0.028 (0.008 ; 0.057) | 0.433 (0.299 ; 0.645) | 1369.8 (1195.3 ; 1578.4) |
| **HIGH_12** | **0.018 (0.018 ; 0.021)** | **0.03 (0.002 ; 0.156)** | **195.2 (157.2 ; 225.9)** | **27.9 (22 ; 33.6)** | **0.026 (0.001 ; 0.061)** | **0.345 (0.14 ; 0.613)** | **1348.2 (1150.6 ; 1533.3)** |
| **Medium Impact Area** | **0.018 (0.018 ; 0.06)** | **0.144 (0.002 ; 0.23)** | **196 (163.4 ; 233.9)** | **28.4 (23.5 ; 36.2)** | **0.021 (0.002 ; 0.043)** | **0.446 (0.193 ; 0.731)** | **1539.5 (1320.8 ; 1753.8)** |
| MEDIUM_1 | 0.018 (0.018 ; 0.041) | 0.143 (0.002 ; 0.228) | 195.9 (163.9 ; 233.2) | 27.8 (23.6 ; 34.2) | 0.026 (0.012 ; 0.045) | 0.462 (0.257 ; 0.641) | 1493.9 (1322.9 ; 1703.3) |
| MEDIUM_2 | 0.018 (0.018 ; 0.047) | 0.074 (0.002 ; 0.167) | 202.7 (161.2 ; 231.6) | 27.4 (22 ; 35.4) | 0.02 (0.001 ; 0.046) | 0.534 (0.27 ; 0.812) | 1428.5 (1224.5 ; 1642.5) |
| MEDIUM_3 | 0.018 (0.018 ; 0.018) | 0.03 (0.002 ; 0.151) | 197.7 (165.8 ; 238.2) | 28.6 (24.7 ; 35) | 0.008 (0.001 ; 0.028) | 0.363 (0.185 ; 0.646) | 1457.7 (1265.5 ; 1688.4) |
| IRNO_1 | 0.018 (0.018 ; 0.064) | 0.138 (0.084 ; 0.215) | 184 (155 ; 221.8) | 28.2 (22.6 ; 34.3) | 0.022 (0.013 ; 0.042) | 0.404 (0.009 ; 0.671) | 1651.7 (1467.6 ; 1899.3) |
| IRNO_2 | 0.018 (0.018 ; 0.021) | 0.156 (0.07 ; 0.216) | 183.5 (156.3 ; 227.6) | 27 (21.8 ; 33.6) | 0.017 (0.001 ; 0.03) | 0.469 (0.009 ; 0.694) | 1708.7 (1522.4 ; 1973.6) |
| SABATO | 0.018 (0.018 ; 0.281) | 0.246 (0.183 ; 0.304) | 206.6 (184 ; 243.8) | 32.8 (27.1 ; 41.3) | 0.038 (0.016 ; 0.085) | 0.533 (0.186 ; 0.836) | 1456.2 (1288.9 ; 1627.7) |
| **Low Impact Area** | **0.018 (0.018 ; 0.144)** | **0.118 (0.002 ; 0.192)** | **204 (169.3 ; 240.8)** | **28.1 (22.5 ; 36.2)** | **0.016 (0.003 ; 0.031)** | **0.428 (0.245 ; 0.681)** | **1379.3 (1170.8 ; 1607)** |
| LOW_1 | 0.018 (0.018 ; 0.018) | 0.094 (0.002 ; 0.176) | 188 (152 ; 222.4) | 28.3 (22.3 ; 35.7) | 0.013 (0.001 ; 0.031) | 0.439 (0.248 ; 0.739) | 1353.7 (1161.4 ; 1596.6) |
| LOW_2 | 0.018 (0.018 ; 0.085) | 0.154 (0.002 ; 0.209) | 216.2 (181.3 ; 250.8) | 28.1 (22.5 ; 37.5) | 0.02 (0.009 ; 0.033) | 0.416 (0.229 ; 0.584) | 1401.6 (1172.1 ; 1598.7) |
| LOW_3 | 0.018 (0.018 ; 0.354) | 0.105 (0.002 ; 0.196) | 207.6 (172.5 ; 236.2) | 27.4 (22.6 ; 34.8) | 0.015 (0.001 ; 0.027) | 0.439 (0.258 ; 0.75) | 1387.7 (1187.6 ; 1622.2) |
| **Overall** | **0.018 (0.018 ; 0.057)** | **0.103 (0.002 ; 0.194)** | **196.1 (161.9 ; 232.1)** | **28.3 (22.7 ; 35.6)** | **0.022 (0.001 ; 0.046)** | **0.425 (0.215 ; 0.703)** | **1431.4 (1216.8 ; 1661.8)** |

**Table 11.** Percentage fold-increase/decrease of the geometric mean of six of the heavy metals assessed in the population indwelling in each cluster/area vs the entire population by cluster and area. Green and red colors express the fold-decrease and increase, respectively.

| Cluster | Cd111 | Hg202 | Mn55 | Ni60 | Sb121 | Tl205 |
|---|---|---|---|---|---|---|
| **High Impact Area** | -19.1 | -28.7 | -8.8 | -3.2 | -28.7 | 2.8 |
| HIGH_1 | -29.4 | -21.6 | 23.9 | -32 | -36.0 | 24.3 |
| HIGH_2 | -15.9 | -27.0 | -22.7 | -33.3 | -50.0 | 8.6 |
| HIGH_3 | -24.3 | -14.7 | -16.2 | 11.4 | -37.5 | -4.9 |
| HIGH_4 | -0.4 | -31.6 | -16.5 | -0.2 | -14.7 | 18.3 |
| HIGH_5 | -21.7 | -39.5 | -7.8 | 24.2 | -48.5 | -0.2 |
| HIGH_6 | -10.6 | -35.6 | 2.1 | -34.3 | -16.3 | -15.1 |
| HIGH_7 | -14.4 | -41.1 | -51.8 | -35.2 | -50.1 | -22.2 |
| HIGH_8 | -9.9 | -22.6 | 2.5 | 37.3 | -14.7 | 3.8 |
| HIGH_9 | -14.7 | -28.5 | -37.0 | 75.8 | 23.0 | 8.1 |
| HIGH_10 | -6.8 | -21.9 | 26.0 | -12.7 | -3.5 | 8.0 |
| HIGH_11 | -13.1 | -35.2 | 33.1 | -23.6 | -9.6 | 38.7 |
| HIGH_12 | 5.5 | -18.2 | -0.7 | 88.1 | -38.2 | 16.6 |
| **Medium Impact Area** | 4.0 | -5.9 | 37.5 | -14.4 | 17.7 | -19.2 |
| MEDIUM_1 | -22.4 | -19.3 | 66.9 | -34.5 | 22.6 | 34.9 |
| MEDIUM_2 | -3.8 | -29.8 | -11.7 | -19.3 | -24.3 | -7.5 |
| MEDIUM_3 | -45.4 | -42.4 | -34.2 | 53.4 | -32.8 | -51.5 |
| IRNO_1 | 40.7 | 461.9 | -25.7 | -51.2 | 180.9 | 12.4 |
| IRNO_2 | 30.9 | 518.4 | -31.5 | -52 | 146.0 | -33.8 |
| SABATO | 517.5 | 108.7 | 159.5 | 577.6 | 626.6 | 142.2 |
| **Low Impact Area** | 27.1 | 87.2 | 2.7 | 9.1 | 76.6 | 2.8 |
| LOW_1 | 9.3 | 7.6 | -1.9 | -30 | 3.8 | -34.4 |
| LOW_2 | -6.2 | 11.8 | 57.8 | 33.9 | 48.3 | 8.6 |
| LOW_3 | 22.2 | -30.3 | 68.3 | -13.9 | 6.4 | -27.9 |
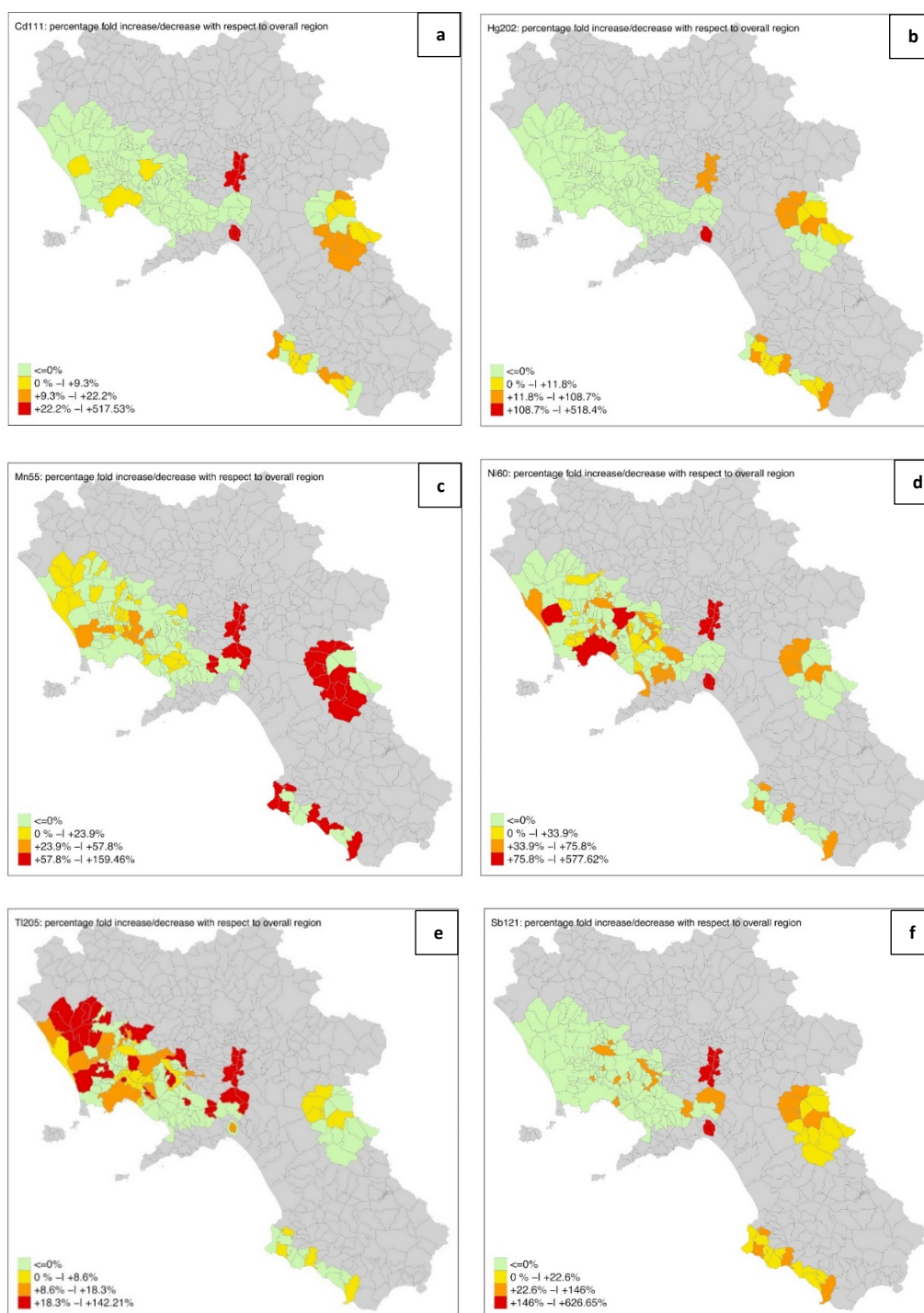
**Figure 13.** Campania Region representations with percentage fold decreases/increases of heavy metals for which percentage fold increase with respect to the general population was ≥ 100% in at least one cluster: (a) Cadmium – Cd111. (b) Mercury – Hg202. (c) Manganese – Mn55. (d) Nichel – Ni60. (e) Antimony – Sb121. (f) Thallium – Tl205.

**Table 12a.** Median regression models where low impact area is the geographical analysis unit. Likewise, age and gender are considered for the median regression model. Bold results indicate differences with p<0.05. Results are expressed as difference between adjusted medians.

| | Se78 | Sr88 | Tl205 | V51 | Zn66 | Co59 | Cr52 |
|---|---|---|---|---|---|---|---|
| **Area; Low impact ref.** | | | | | | | |
| Medium impact | **-8.7 (-15 to -2.4) p=0.007** | -0.05 (-1.22 to 1.11) p=0.929 | **0.004 (0.002 to 0.007) p=0.002** | 0.02 (-0.03 to 0.07) p=0.393 | **138.1 (100.1 to 176) p<0.001** | **0.13 (0.09 to 0.17) p<0.001** | 0.05 (-0.05 to 0.15) p=0.332 |
| High Impact | **-11.7 (-17.8 to -5.6) p<0.001** | -0.21 (-1.28 to 0.87) p=0.707 | **0.009 (0.006 to 0.011) p<0.001** | -0.01 (-0.05 to 0.03) p=0.586 | -5.9 (-43.3 to 31.5) p=0.758 | -0.01 (-0.04 to 0.03) p=0.677 | -0.06 (-0.16 to 0.03) p=0.203 |
| **Gender; Male** | **21.2 (15.8 to 26.5) p<0.001** | -0.26 (-1.12 to 0.61) p=0.557 | -0.002 (-0.005 to 0.001) p=0.153 | 0 (-0.04 to 0.04) p=0.906 | **132.8 (99.3 to 166.2) p<0.001** | 0.01 (-0.03 to 0.04) p=0.721 | 0.03 (-0.03 to 0.1) p=0.321 |
| **Age; 20-30 ref** | | | | | | | |
| 30-40 | -0.1 (-5.2 to 5) p=0.974 | **1.23 (0.37 to 2.09) p=0.005** | 0.001 (-0.002 to 0.004) p=0.553 | -0.01 (-0.04 to 0.02) p=0.428 | -18.8 (-52.8 to 15.2) p=0.279 | **0.04 (0.01 to 0.08) p=0.021** | 0.05 (-0.02 to 0.12) p=0.181 |
| 40-50 | 1.1 (-4.3 to 6.5) p=0.7 | **1.78 (0.92 to 2.64) p=0.00** | 0.002 (-0.001 to 0.004) p=0.126 | -0.02 (-0.06 to 0.01) p=0.192 | -0.6 (-32.3 to 31.2) p=0.973 | **0.07 (0.03 to 0.1) p=0.00** | 0.03 (-0.04 to 0.09) p=0.409 |
| | Hg202 | Li7 | Cd111 | As75 | Cr52 | Ni60 | Sb121 |
| **Area; Low impact ref.** | | | | | | | |
| Medium impact | **0.87 (0.62 to 1.13) p<0.001** | **0.09 (0.03 to 0.16) p=0.003** | 0.003 (-0.003 to 0.009) p=0.296 | 0.14 (-0.1 to 0.37) p=0.251 | 0.05 (-0.05 to 0.15) p=0.332 | 0.12 (-0.31 to 0.55) p=0.584 | **0.03 (0.01 to 0.04) p=0.005** |
| High Impact | **-0.3 (-0.45 to -0.15) p<0.001** | 0.01 (-0.05 to 0.07) p=0.694 | **-0.007 (-0.011 to -0.003) p=0.001** | -0.06 (-0.26 to 0.15) p=0.588 | -0.06 (-0.16 to 0.03) p=0.203 | 0.21 (-0.2 to 0.62) p=0.316 | **-0.06 (-0.08 to -0.04) p<0.001** |
| **Gender; Male** | 0.16 (0 to 0.32) p=0.053 | **0.12 (0.08 to 0.17) p<0.001** | -0.004 (-0.007 to 0) p=0.058 | 0.17 (-0.04 to 0.39) p=0.109 | 0.02 (-0.03 to 0.1) p=0.321 | -0.02 (-0.31 to 0.28) p=0.915 | 0 (-0.02 to 0.02) p=0.779 |
| **Age; 20-30 ref** | | | | | | | |
| 30-40 | 0.15 (-0.02 to 0.33) p=0.087 | -0.03 (-0.08 to 0.02) p=0.25 | 0 (-0.002 to 0.002) p=1 | **0.25 (0.04 to 0.45) p=0.017** | 0.05 (-0.02 to 0.12) p=0.181 | -0.02 (-0.31 to 0.28) p=0.905 | 0.01 (-0.01 to 0.03) p=0.186 |
| 40-50 | 0.15 (-0.03 to 0.33) p=0.107 | 0.03 (-0.04 to 0.07) p=0.589 | (-0.001 to 0.005) p=0.161 | **0.26 (0.05 to 0.46) p=0.015** | 0.03 (-0.04 to 0.09) p=0.409 | 0.02 (-0.21 to 0.26) p=0.86 | 0.01 (-0.01 to 0.03) p=0.272 |

**Table 12b.** Median regression models where low impact area is the geographical analysis unit. Likewise, age and gender are considered for the median regression model. Bold results indicate differences with p<0.05. Results are expressed as difference between adjusted medians.

| | Cu63 | Fe | Mn55 | Mo98 |
|---|---|---|---|---|
| **Area; Low impact ref.** | | | | |
| Medium impact | **-43.8 (-67.4 to -20.2) p<0.001** | 6 (-104 to 117) p=0.91 | **0.15 (0.06 to 0.23) p=0.001** | **-0.09 (-0.16 to -0.02) p=0.016** |
| High Impact | **-42.2 (-63.8 to -20.7) p<0.001** | -94 (-200 to 13) p=0.085 | **-0.12 (-0.2 to -0.04) p=0.004** | **-0.14 (-0.2 to -0.08) p<0.001** |
| **Gender; Male** | **-159 (-183.5 to -135.1) p<0.001** | **544 (440 to 649) p<0.001** | **0.11 (0.02 to 0.19) p=0.013** | 0 (-0.06 to 0.06) p=0.991 |
| **Age; 20-30 ref** | | | | |
| 30-40 | **52.4 (33 to 71.7) p=0.00** | 34 (-77 to 144) p=0.553 | 0.01 (-0.06 to 0.08) p=0.77 | 0.05 (-0.01 to 0.11) p=0.134 |
| 40-50 | **80 (59.8 to 100.2) p=0.00** | -93 (-198 to 11) p=0.079 | 0.02 (-0.03 to 0.1) p=0.344 | 0.03 (-0.02 to 0.11) p=0.169 |

**Table 13a.** Median regression models for the heavy metals assessed in the population, using cluster as geographical reference unit. Data are reported as adjusted difference between medians with the corresponding 95% CIs. Bold results indicate differences with p<0.005. It is considered as reference value the cluster with the lower geometric mean for each metal.

| Cluster | Hg202 | Li7 | Cd111 | As75 | Cr52 | Co59 |
|---|---|---|---|---|---|---|
| HIGH_1 | ref | 0 (-0.16 to 0.16) | -0.004 (-0.012 to 0.003) | 0.12 (-0.4 to 0.64) | -0.07 (-0.27 to 0.13) | 0.04 (-0.06 to 0.13) |
| HIGH_2 | -0.02 (-0.45 to 0.4) | -0.1 (-0.25 to 0.05) | -0.003 (-0.012 to 0.005) | -0.01 (-0.55 to 0.54) | -0.08 (-0.29 to 0.12) | -0.07 (-0.15 to 0.01) |
| HIGH_3 | 0.14 (-0.42 to 0.7) | -0.1 (-0.24 to 0.05) | -0.004 (-0.011 to 0.004) | 0.22 (-0.31 to 0.75) | -0.01 (-0.21 to 0.18) | -0.01 (-0.1 to 0.09) |
| HIGH_4 | 0.02 (-0.46 to 0.5) | -0.03 (-0.18 to 0.12) | -0.002 (-0.013 to 0.009) | 0.39 (-0.19 to 0.96) | -0.14 (-0.33 to 0.05) | 0.01 (-0.07 to 0.09) |
| HIGH_5 | -0.33 (-0.69 to 0.03) | 0.11 (-0.04 to 0.26) | -0.003 (-0.01 to 0.005) | -0.11 (-0.59 to 0.36) | -0.09 (-0.28 to 0.1) | 0.08 (-0.02 to 0.18) |
| HIGH_6 | -0.22 (-0.62 to 0.18) | 0.06 (-0.1 to 0.22) | -0.003 (-0.011 to 0.005) | 0.26 (-0.3 to 0.82) | 0.06 (-0.14 to 0.26) | 0.01 (-0.1 to 0.11) |
| HIGH_7 | -0.28 (-0.7 to 0.14) | -0.07 (-0.23 to 0.08) | -0.002 (-0.011 to 0.007) | -0.26 (-0.73 to 0.22) | -0.08 (-0.29 to 0.13) | **-0.13 (-0.23 to -0.04)** |
| HIGH_8 | 0.03 (-0.37 to 0.43) | 0 (-0.13 to 0.13) | -0.004 (-0.012 to 0.005) | 0.23 (-0.31 to 0.77) | -0.02 (-0.23 to 0.19) | -0.05 (-0.13 to 0.04) |
| HIGH_9 | -0.1 (-0.48 to 0.29) | 0.07 (-0.08 to 0.22) | 0.001 (-0.008 to 0.009) | ref | -0.08 (-0.27 to 0.1) | ref |
| HIGH_10 | 0.05 (-0.35 to 0.44) | -0.02 (-0.16 to 0.11) | 0.001 (-0.009 to 0.01) | 0.15 (-0.37 to 0.66) | 0.16 (-0.06 to 0.37) | 0.05 (-0.04 to 0.14) |
| HIGH_11 | -0.15 (-0.54 to 0.25) | -0.08 (-0.22 to 0.06) | 0 (-0.008 to 0.008) | -0.07 (-0.54 to 0.41) | 0.01 (-0.21 to 0.23) | **-0.11 (-0.18 to -0.03)** |
| HIGH_12 | 0.09 (-0.33 to 0.51) | -0.02 (-0.15 to 0.12) | ref | -0.1 (-0.59 to 0.39) | -0.12 (-0.31 to 0.07) | -0.05 (-0.15 to 0.04) |
| MEDIUM_1 | 0.16 (-0.28 to 0.61) | -0.06 (-0.2 to 0.09) | -0.002 (-0.01 to 0.007) | -0.33 (-0.79 to 0.14) | ref | 0.07 (-0.02 to 0.17) |
| MEDIUM_2 | -0.07 (-0.58 to 0.44) | -0.03 (-0.16 to 0.1) | -0.002 (-0.011 to 0.008) | -0.14 (-0.65 to 0.36) | 0.09 (-0.12 to 0.29) | 0.03 (-0.06 to 0.13) |
| MEDIUM_3 | -0.3 (-0.66 to 0.06) | -0.02 (-0.15 to 0.1) | -0.005 (-0.012 to 0.001) | -0.4 (-0.88 to 0.07) | -0.16 (-0.32 to 0.01) | -0.03 (-0.14 to 0.07) |
| IRNO_1 | **4.17 (3.28 to 5.05)** | **0.26 (0.1 to 0.42)** | **0.012 (0.003 to 0.021)** | **1.47 (0.68 to 2.26)** | 0.08 (-0.14 to 0.3) | **0.29 (0.16 to 0.42)** |
| IRNO_2 | **4.17 (3.22 to 5.13)** | **0.22 (0.05 to 0.38)** | **0.013 (0.002 to 0.023)** | **0.8 (0.17 to 1.42)** | 0.08 (-0.11 to 0.27) | **0.33 (0.22 to 0.45)** |
| SABATO | **1.03 (0.54 to 1.51)** | **0.17 (0.03 to 0.32)** | **0.092 (0.065 to 0.119)** | **0.82 (0.26 to 1.37)** | **0.45 (0.18 to 0.72)** | 0.09 (-0.03 to 0.2) |
| LOW_1 | 0.34 (-0.06 to 0.74) | ref | 0.004 (-0.005 to 0.012) | 0.31 (-0.26 to 0.88) | -0.12 (-0.3 to 0.06) | -0.02 (-0.11 to 0.06) |
| LOW_2 | 0.34 (-0.13 to 0.8) | -0.05 (-0.18 to 0.08) | 0.003 (-0.005 to 0.012) | 0.22 (-0.34 to 0.78) | 0.09 (-0.12 to 0.3) | 0 (-0.08 to 0.08) |
| LOW_3 | -0.03 (-0.4 to 0.34) | -0.05 (-0.19 to 0.09) | 0.009 (0 to 0.018) | -0.02 (-0.5 to 0.45) | 0.12 (-0.08 to 0.32) | 0 (-0.09 to 0.08) |

**Table 13b.** Median regression models for the heavy metals assessed in the population, using cluster as geographical reference unit. Data are reported as adjusted difference between medians with the corresponding 95% CIs. Bold results indicate differences with p<0.005. It is considered as reference value the cluster with the lower geometric mean for each metal.

| Cluster | Cu63 | Fe | Mn55 | Mo98 | Ni60 | Sb121 |
|---|---|---|---|---|---|---|
| HIGH_1 | **79.5 (13.1 to 145.9)** | -4.6 (-287 to 277.8) | 0.1 (-0.07 to 0.26) | -0.02 (-0.17 to 0.12) | ref | **-0.05 (-0.1 to -0.01)** |
| HIGH_2 | -36.2 (-97.2 to 24.9) | -58.9 (-343.4 to 225.5) | **-0.18 (-0.36 to 0)** | -0.13 (-0.3 to 0.04) | -0.37 (-1.13 to 0.38) | **-0.07 (-0.1 to -0.05)** |
| HIGH_3 | -46 (-105.3 to 13.4) | -175.1 (-426.5 to 76.3) | -0.08 (-0.24 to 0.08) | 0 (-0.15 to 0.16) | 0.39 (-0.38 to 1.15) | **-0.06 (-0.1 to -0.02)** |
| HIGH_4 | -2.8 (-57.2 to 51.6) | -99.9 (-351.6 to 151.9) | -0.08 (-0.25 to 0.08) | -0.04 (-0.21 to 0.12) | 0.22 (-0.54 to 0.99) | 0.01 (-0.03 to 0.05) |
| HIGH_5 | -18.5 (-75 to 38) | -1.4 (-300.6 to 297.7) | -0.12 (-0.3 to 0.05) | -0.17 (-0.37 to 0.02) | 0.58 (-0.21 to 1.36) | **-0.08 (-0.1 to -0.05)** |
| HIGH_6 | -0.5 (-59.9 to 59) | 67.3 (-210 to 344.5) | 0.08 (-0.1 to 0.26) | 0.16 (-0.02 to 0.33) | -0.38 (-1.03 to 0.27) | -0.02 (-0.07 to 0.02) |
| HIGH_7 | -38.8 (-94.1 to 16.5) | -121.7 (-413.8 to 170.5) | **-0.29 (-0.46 to -0.12)** | -0.02 (-0.19 to 0.15) | 0.04 (-0.74 to 0.83) | **-0.07 (-0.1 to -0.04)** |
| HIGH_8 | ref | 53.7 (-221.8 to 329.3) | -0.07 (-0.23 to 0.09) | 0.07 (-0.08 to 0.23) | 0.17 (-0.54 to 0.89) | -0.01 (-0.06 to 0.04) |
| HIGH_9 | -38.6 (-89.4 to 12.3) | ref | -0.19 (-0.39 to 0.01) | 0.03 (-0.13 to 0.19) | 0.8 (0.07 to 1.52) | 0.01 (-0.02 to 0.04) |
| HIGH_10 | **-62.5 (-115.7 to -9.4)** | -76.7 (-313.8 to 160.5) | 0.05 (-0.09 to 0.19) | 0.03 (-0.16 to 0.21) | -0.2 (-0.95 to 0.55) | 0.01 (-0.03 to 0.04) |
| HIGH_11 | -33.6 (-96.3 to 29.1) | -29.3 (-287.7 to 229.1) | 0.08 (-0.07 to 0.22) | -0.06 (-0.22 to 0.11) | -0.17 (-0.85 to 0.51) | 0.01 (-0.04 to 0.05) |
| HIGH_12 | -9.5 (-67.5 to 48.4) | -47.4 (-300 to 205.2) | 0 (-0.18 to 0.17) | -0.1 (-0.24 to 0.04) | 0.54 (-0.18 to 1.25) | -0.06 (-0.09 to -0.02) |
| MEDIUM_1 | -10.3 (-70 to 49.3) | 201.5 (-76.4 to 479.4) | **0.26 (0.07 to 0.45)** | ref | -0.35 (-1.09 to 0.4) | 0.05 (0.01 to 0.08) |
| MEDIUM_2 | 15.8 (-43 to 74.7) | 161.6 (-79.9 to 403.2) | ref | 0.01 (-0.19 to 0.22) | 0 (-0.72 to 0.72) | -0.01 (-0.06 to 0.03) |
| MEDIUM_3 | -36.5 (-89.7 to 16.7) | 56.9 (-199.4 to 313.2) | -0.13 (-0.32 to 0.07) | 0.04 (-0.14 to 0.21) | 0.95 (-0.05 to 1.95) | **-0.06 (-0.1 to -0.03)** |
| IRNO_1 | **-72.5 (-122 to -23)** | 11.6 (-294.5 to 317.7) | **0.34 (0.17 to 0.51)** | -0.01 (-0.19 to 0.18) | -0.53 (-1.12 to 0.06) | **0.05 (0.03 to 0.08)** |
| IRNO_2 | **-58.3 (-109.5 to -7.1)** | -33.3 (-296.1 to 229.5) | **0.24 (0.08 to 0.41)** | 0.02 (-0.15 to 0.18) | -0.51 (-1.09 to 0.07) | **0.07 (0.04 to 0.09)** |
| SABATO | 23.5 (-34.1 to 81) | 17.3 (-206.9 to 241.6) | **0.69 (0.54 to 0.85)** | 0.1 (-0.06 to 0.25) | **2.26 (1.58 to 2.95)** | **0.15 (0.12 to 0.18)** |
| LOW_1 | -7.4 (-64.9 to 50.1) | 31.1 (-196.7 to 259) | -0.12 (-0.27 to 0.03) | 0.07 (-0.09 to 0.23) | -0.33 (-1.19 to 0.54) | ref |
| LOW_2 | 37 (-13.7 to 87.6) | 132.8 (-111.6 to 377.2) | 0.09 (-0.07 to 0.25) | 0.14 (0 to 0.28) | 0.6 (-0.54 to 1.73) | **0.06 (0.03 to 0.08)** |
| LOW_3 | 40.5 (-16.1 to 97.1) | -3.2 (-251.9 to 245.5) | **0.21 (0.04 to 0.38)** | **0.14 (0.01 to 0.28)** | -0.13 (-0.94 to 0.67) | 0.01 (-0.02 to 0.05) |

**Table 13c.** Median regression models for the heavy metals assessed in the population, using cluster as geographical reference unit. Data are reported as adjusted difference between medians with the corresponding 95% CIs. Bold results indicate differences with p<0.005. It is considered as reference value the cluster with the lower geometric mean for each metal.

| Cluster | Se78 | Sr88 | Tl205 | V51 | Zn66 |
|---|---|---|---|---|---|
| HIGH_1 | 4.7 (-11.4 to 20.8) | 0.45 (-1.59 to 2.49) | 0.008 (-0.001 to 0.017) | 0.023 (-0.068 to 0.115) | 58.8 (-37.5 to 155.2) |
| HIGH_2 | 2 (-14.2 to 18.1) | -1.17 (-3.68 to 1.33) | 0.007 (-0.004 to 0.017) | 0.04 (-0.06 to 0.139) | -37.4 (-129.2 to 54.3) |
| HIGH_3 | ref | -1.32 (-4.17 to 1.52) | 0 (-0.01 to 0.011) | -0.016 (-0.106 to 0.073) | -14.3 (-106.8 to 78.1) |
| HIGH_4 | -3.8 (-20.4 to 12.8) | -0.78 (-3.3 to 1.74) | 0.007 (-0.005 to 0.018) | -0.002 (-0.105 to 0.101) | -53.6 (-151.8 to 44.6) |
| HIGH_5 | 13.7 (-1.8 to 29.2) | 1.22 (-0.94 to 3.38) | 0 (-0.01 to 0.01) | -0.028 (-0.118 to 0.062) | **103.7 (16.6 to 190.8)** |
| HIGH_6 | -0.4 (-15.5 to 14.8) | 0.03 (-2.4 to 2.45) | ref | -0.063 (-0.144 to 0.018) | -23.8 (-107.4 to 59.8) |
| HIGH_7 | **-19.3 (-37 to -1.7)** | -2.42 (-4.87 to 0.03) | -0.004 (-0.017 to 0.008) | -0.056 (-0.131 to 0.02) | -80.6 (-162.9 to 1.7) |
| HIGH_8 | -3.3 (-17.8 to 11.2) | -0.05 (-2.06 to 1.97) | 0.005 (-0.004 to 0.015) | -0.003 (-0.109 to 0.103) | -18.1 (-110.6 to 74.4) |
| HIGH_9 | 0.4 (-15.7 to 16.6) | -0.14 (-2.2 to 1.92) | -0.001 (-0.009 to 0.008) | **-0.089 (-0.166 to -0.011)** | -22.9 (-114 to 68.1) |
| HIGH_10 | -4.6 (-20.6 to 11.5) | -1.09 (-3.32 to 1.15) | 0.001 (-0.008 to 0.01) | -0.04 (-0.116 to 0.036) | **-97.6 (-179.8 to -15.5)** |
| HIGH_11 | 4.1 (-12.5 to 20.8) | 0.91 (-1.52 to 3.33) | 0.007 (-0.001 to 0.016) | -0.023 (-0.099 to 0.054) | -26.3 (-112.7 to 60.1) |
| HIGH_12 | -2.4 (-18.5 to 13.6) | -0.55 (-2.73 to 1.64) | 0.004 (-0.005 to 0.013) | **-0.112 (-0.203 to -0.021)** | -54.1 (-144.7 to 36.5) |
| MEDIUM_1 | 1.3 (-14.5 to 17.2) | -0.51 (-2.88 to 1.86) | 0.002 (-0.006 to 0.011) | 0.011 (-0.076 to 0.099) | 74.1 (-15 to 163.2) |
| MEDIUM_2 | 8.2 (-6.6 to 22.9) | -1.08 (-3.14 to 0.99) | -0.001 (-0.009 to 0.008) | 0.084 (-0.02 to 0.188) | -6.2 (-99.4 to 87) |
| MEDIUM_3 | 4.5 (-10.4 to 19.5) | 0 (-1.94 to 1.93) | **-0.013 (-0.021 to -0.005)** | **-0.096 (-0.178 to -0.014)** | 48.2 (-38.8 to 135.2) |
| IRNO_1 | -13.7 (-31.6 to 4.2) | -0.56 (-2.63 to 1.52) | -0.001 (-0.009 to 0.008) | -0.038 (-0.134 to 0.059) | **235.8 (136.4 to 335.3)** |
| IRNO_2 | -11.9 (-27.2 to 3.4) | -1.72 (-3.79 to 0.35) | -0.004 (-0.012 to 0.004) | 0.029 (-0.061 to 0.118) | **287.5 (202.1 to 373)** |
| SABATO | 14.7 (-1.9 to 31.2) | 4.79 (2.25 to 7.33) | **0.016 (0.006 to 0.026)** | -0.019 (-0.153 to 0.115) | 51.2 (-33.7 to 136.2) |
| LOW_1 | -6.5 (-21.9 to 8.9) | 0.11 (-2.35 to 2.56) | -0.008 (-0.016 to 0) | ref | -42.3 (-125.7 to 41.2) |
| LOW_2 | **23.4 (8 to 38.8)** | ref | -0.002 (-0.01 to 0.006) | -0.054 (-0.138 to 0.029) | ref |
| LOW_3 | 14.4 (-1.4 to 30.1) | -0.31 (-2.71 to 2.1) | -0.008 (-0.016 to 0.001) | -0.019 (-0.107 to 0.07) | -9.4 (-100.7 to 81.9) |

**Table 13d.** Metals correlation matrix for low impact areas and clusters. Data are reported as Spearman Correlation Coefficient (p-value). Red color expresses the significant correlations.

| Low | Li7 | Be9 | Cd111 | As75 | Cr52 | Co59 | Cu63 | Fe | Hg202 | Mn55 | Mo98 | Ni60 | Pb208 | Sb121 | Se78 | Sr88 | Tl205 | V51 | Zn66 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Li7 | 1 | 0.107 (0.009) | 0.178 (<0.001) | 0.139 (0.001) | 0.061 (0.135) | 0.236 (<0.001) | 0.104 (0.011) | 0.152 (<0.001) | 0.23 (<0.001) | 0.213 (<0.001) | 0.207 (<0.001) | 0.141 (0.028) | 0.033 (0.418) | 0.174 (<0.001) | 0.201 (<0.001) | **0.526 (<0.001)** | 0.113 (0.006) | 0.173 (<0.001) | 0.251 (<0.001) |
| Be9 | | 1 | 0.092 (0.025) | 0.014 (0.727) | 0.08 (0.052) | 0.086 (0.036) | 0.093 (0.024) | 0.03 (0.465) | 0.202 (<0.001) | 0.011 (0.78) | 0.143 (<0.001) | 0.087 (0.175) | -0.135 (0.001) | 0.018 (0.654) | 0.042 (0.306) | 0.053 (0.197) | 0.074 (0.07) | 0.088 (0.031) | 0.094 (0.024) |
| Cd111 | | | 1 | 0.096 (0.019) | 0.252 (<0.001) | 0.091 (0.027) | 0.099 (0.016) | 0.068 (0.099) | 0.242 (<0.001) | 0.265 (<0.001) | **0.312 (<0.001)** | 0.173 (0.007) | 0.218 (<0.001) | 0.206 (<0.001) | 0.056 (0.171) | 0.078 (0.056) | 0.241 (<0.001) | 0.214 (<0.001) | 0.105 (0.013) |
| As75 | | | | 1 | 0.045 (0.277) | 0.142 (0.001) | 0.089 (0.03) | 0.096 (0.019) | 0.202 (<0.001) | 0.005 (0.911) | -0.06 (0.143) | 0.149 (0.02) | -0.055 (0.179) | 0.032 (0.432) | 0.152 (<0.001) | 0.116 (0.004) | 0.155 (<0.001) | 0.004 (0.927) | 0.123 (0.003) |
| Cr52 | | | | | 1 | 0.034 (0.411) | -0.036 (0.385) | 0.051 (0.215) | 0.215 (<0.001) | **0.332 (<0.001)** | 0.096 (0.02) | 0.283 (<0.001) | 0.192 (<0.001) | 0.226 (<0.001) | 0.033 (0.425) | -0.047 (0.248) | 0.178 (<0.001) | 0.067 (0.111) | |
| Co59 | | | | | | 1 | 0.269 (<0.001) | 0.212 (<0.001) | 0.101 (0.036) | 0.227 (<0.001) | 0.112 (0.006) | 0.111 (0.083) | 0.062 (0.13) | 0.157 (<0.001) | **0.32 (<0.001)** | 0.25 (<0.001) | 0.079 (0.054) | 0.116 (0.004) | 0.261 (<0.001) |
| Cu63 | | | | | | | 1 | 0.164 (<0.001) | 0.055 (0.256) | 0.206 (<0.001) | 0.17 (<0.001) | 0.025 (0.693) | 0.026 (0.521) | 0.18 (<0.001) | **0.415 (<0.001)** | 0.269 (<0.001) | 0.064 (0.121) | 0.116 (0.004) | 0.293 (<0.001) |
| Fe | | | | | | | | 1 | 0.05 (0.299) | **0.322 (<0.001)** | 0.186 (<0.001) | 0.035 (0.586) | 0.054 (0.185) | 0.172 (<0.001) | **0.448 (<0.001)** | 0.235 (<0.001) | 0.073 (0.074) | 0.187 (<0.001) | **0.424 (<0.001)** |
| Hg202 | | | | | | | | | 1 | 0.068 (0.154) | 0.197 (<0.001) | 0.032 (0.509) | 0.271 (<0.001) | -0.018 (0.713) | 0.119 (0.013) | 0.207 (<0.001) | 0.157 (0.001) | 0.17 (<0.001) | |
| Mn55 | | | | | | | | | | 1 | 0.298 (<0.001) | 0.178 (0.005) | 0.276 (<0.001) | **0.301 (<0.001)** | 0.258 (<0.001) | 0.131 (0.001) | 0.142 (<0.001) | 0.171 (<0.001) | **0.3 (<0.001)** |
| Mo98 | | | | | | | | | | | 1 | **0.309 (<0.001)** | 0.151 (<0.001) | **0.331 (<0.001)** | 0.219 (<0.001) | 0.158 (<0.001) | 0.072 (0.078) | 0.087 (0.034) | 0.217 (<0.001) |
| Ni60 | | | | | | | | | | | | 1 | 0.202 (0.001) | **0.391 (<0.001)** | -0.105 (0.1) | -0.072 (0.265) | 0.11 (0.087) | -0.027 (0.671) | -0.031 (0.646) |
| Pb208 | | | | | | | | | | | | | 1 | 0.205 (<0.001) | 0.029 (0.472) | -0.025 (0.541) | 0.114 (0.005) | 0.058 (0.158) | 0.046 (0.268) |
| Sb121 | | | | | | | | | | | | | | 1 | 0.254 (<0.001) | 0.134 (<0.001) | 0.218 (<0.001) | -0.01 (0.815) | 0.186 (<0.001) |
| Se78 | | | | | | | | | | | | | | | 1 | **0.389 (<0.001)** | 0.098 (0.017) | 0.13 (0.001) | **0.542 (<0.001)** |
| Sr88 | | | | | | | | | | | | | | | | 1 | 0.05 (0.222) | 0.13 (0.001) | **0.308 (<0.001)** |
| Tl205 | | | | | | | | | | | | | | | | | 1 | 0.061 (0.136) | 0.109 (0.018) |
| V51 | | | | | | | | | | | | | | | | | | 1 | 0.279 (<0.001) |
| Zn66 | | | | | | | | | | | | | | | | | | | 1 |

**Table 13e.** Metals correlation matrix for medium impact areas and clusters. Data are reported as Spearman Correlation Coefficient (p-value). Red color expresses the significant correlations.

| Medium | Li7 | Be9 | Cd111 | As75 | Cr52 | Co59 | Cu63 | Fe | Hg202 | Mn55 | Mo98 | Ni60 | Pb208 | Sb121 | Se78 | Sr88 | Tl205 | V51 | Zn66 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Li7 | 1 | 0.171 (<0.001) | 0.212 (<0.001) | 0.055 (0.06) | 0.152 (<0.001) | 0.04 (0.167) | 0.097 (0.001) | 0.154 (<0.001) | 0.145 (<0.001) | 0.061 (0.035) | 0.048 (0.154) | 0.128 (<0.001) | 0.145 (<0.001) | 0.138 (<0.001) | **0.377 (<0.001)** | 0.104 (<0.001) | 0.029 (0.31) | 0.154 (<0.001) | |
| Be9 | | 1 | 0.045 (0.118) | 0.12 (<0.001) | -0.05 (0.084) | -0.05 (0.085) | -0.014 (0.633) | -0.049 (0.091) | 0.074 (0.016) | -0.027 (0.357) | -0.021 (0.459) | -0.072 (0.034) | 0.084 (0.004) | 0.032 (0.268) | -0.007 (0.82) | -0.06 (0.039) | 0.042 (0.147) | -0.071 (0.014) | 0.042 (0.151) |
| Cd111 | | | 1 | 0.084 (0.003) | 0.211 (<0.001) | 0.153 (<0.001) | 0.098 (0.001) | 0.048 (0.1) | 0.171 (<0.001) | **0.322 (<0.001)** | 0.26 (<0.001) | 0.26 (<0.001) | 0.164 (<0.001) | 0.299 (<0.001) | -0.012 (0.669) | 0.14 (<0.001) | 0.221 (<0.001) | 0.201 (<0.001) | -0.037 (0.208) |
| As75 | | | | 1 | 0.032 (0.275) | -0.016 (0.572) | 0.004 (0.883) | 0.003 (0.908) | 0.276 (<0.001) | 0.029 (0.322) | -0.131 (<0.001) | -0.002 (0.953) | 0.052 (0.075) | 0.023 (0.429) | 0.1 (0.001) | 0.127 (<0.001) | 0.077 (0.008) | -0.002 (0.942) | 0.138 (<0.001) |
| Cr52 | | | | | 1 | 0.171 (<0.001) | 0.037 (0.205) | 0.112 (<0.001) | 0.142 (<0.001) | **0.316 (<0.001)** | 0.252 (<0.001) | 0.279 (<0.001) | 0.015 (0.616) | 0.286 (<0.001) | -0.102 (<0.001) | 0.028 (0.327) | 0.074 (0.01) | 0.166 (<0.001) | -0.046 (0.12) |
| Co59 | | | | | | 1 | 0.235 (<0.001) | 0.264 (<0.001) | 0.165 (<0.001) | **0.354 (<0.001)** | 0.124 (<0.001) | 0.204 (<0.001) | 0.068 (0.019) | 0.235 (<0.001) | 0.026 (0.362) | 0.203 (<0.001) | 0.044 (0.128) | 0.233 (<0.001) | 0.217 (<0.001) |
| Cu63 | | | | | | | 1 | 0.066 (0.022) | -0.069 (0.024) | 0.2 (<0.001) | 0.224 (<0.001) | 0.178 (<0.001) | 0.082 (0.005) | 0.089 (0.002) | 0.275 (<0.001) | 0.235 (<0.001) | 0.075 (0.01) | 0.19 (<0.001) | 0.157 (<0.001) |
| Fe | | | | | | | | 1 | -0.046 (0.134) | **0.392 (<0.001)** | 0.148 (<0.001) | 0.116 (<0.001) | 0.178 (<0.001) | 0.186 (<0.001) | 0.195 (<0.001) | 0.147 (<0.001) | 0.072 (0.013) | 0.182 (<0.001) | 0.167 (<0.001) |
| Hg202 | | | | | | | | | 1 | 0.118 (<0.001) | -0.01 (0.749) | -0.08 (0.02) | 0.029 (0.347) | 0.234 (<0.001) | -0.188 (<0.001) | 0.032 (0.292) | 0.165 (<0.001) | 0.01 (0.737) | 0.205 (<0.001) |
| Mn55 | | | | | | | | | | 1 | **0.379 (<0.001)** | **0.347 (<0.001)** | 0.292 (<0.001) | **0.451 (<0.001)** | 0.022 (0.447) | 0.204 (<0.001) | 0.2 (<0.001) | 0.244 (<0.001) | 0.079 (0.007) |
| Mo98 | | | | | | | | | | | 1 | **0.307 (<0.001)** | 0.197 (<0.001) | **0.307 (<0.001)** | 0.033 (0.255) | 0.091 (0.002) | 0.092 (0.001) | 0.164 (<0.001) | -0.051 (0.082) |
| Ni60 | | | | | | | | | | | | 1 | 0.121 (<0.001) | **0.306 (<0.001)** | -0.012 (0.715) | 0.179 (<0.001) | 0.188 (<0.001) | 0.181 (<0.001) | -0.13 (<0.001) |
| Pb208 | | | | | | | | | | | | | 1 | 0.168 (<0.001) | 0.005 (0.87) | 0.029 (0.318) | 0.078 (0.007) | 0.053 (0.065) | -0.029 (0.319) |
| Sb121 | | | | | | | | | | | | | | 1 | -0.017 (0.562) | 0.242 (<0.001) | 0.105 (<0.001) | 0.049 (0.093) | |
| Se78 | | | | | | | | | | | | | | | 1 | 0.181 (<0.001) | -0.043 (0.135) | -0.065 (0.024) | **0.358 (<0.001)** |
| Sr88 | | | | | | | | | | | | | | | | 1 | 0.153 (<0.001) | 0.116 (<0.001) | 0.068 (0.021) |
| Tl205 | | | | | | | | | | | | | | | | | 1 | 0.098 (0.001) | -0.074 (0.012) |
| V51 | | | | | | | | | | | | | | | | | | 1 | -0.036 (0.215) |
| Zn66 | | | | | | | | | | | | | | | | | | | 1 |

**Table 13f.** Metals correlation matrix for high impact areas and clusters. Data are reported as Spearman Correlation Coefficient (p-value). Red color expresses the significant correlations.

| High | Li7 | Be9 | Cd111 | As75 | Cr52 | Co59 | Cu63 | Fe | Hg202 | Mn55 | Mo98 | Ni60 | Pb208 | Sb121 | Se78 | Sr88 | Tl205 | V51 | Zn66 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Li7 | 1 | 0.073 (<0.001) | 0.139 (<0.001) | 0.144 (<0.001) | 0.077 (<0.001) | 0.198 (<0.001) | 0.147 (<0.001) | 0.179 (<0.001) | 0.102 (<0.001) | 0.206 (<0.001) | 0.116 (<0.001) | 0.114 (<0.001) | 0.066 (0.001) | 0.058 (0.005) | 0.196 (<0.001) | 0.386 (<0.001) | 0.091 (<0.001) | 0.16 (<0.001) | 0.21 (<0.001) |
| Be9 | | 1 | 0.142 (<0.001) | 0.087 (<0.001) | 0.07 (0.001) | 0.076 (<0.001) | 0.031 (0.133) | -0.022 (0.286) | 0.14 (<0.001) | 0.046 (0.026) | 0.049 (0.016) | 0.037 (0.2) | 0.001 (0.951) | 0.046 (0.024) | -0.087 (<0.001) | -0.02 (0.336) | 0.085 (<0.001) | 0.106 (<0.001) | 0.033 (0.12) |
| Cd111 | | | 1 | 0.152 (<0.001) | 0.254 (<0.001) | 0.114 (<0.001) | 0.117 (<0.001) | -0.018 (0.379) | 0.344 (<0.001) | 0.294 (<0.001) | 0.269 (<0.001) | 0.273 (<0.001) | 0.259 (<0.001) | 0.266 (<0.001) | -0.034 (0.1) | 0.023 (0.251) | 0.23 (<0.001) | 0.131 (<0.001) | 0.06 (0.004) |
| As75 | | | | 1 | 0.085 (<0.001) | 0.079 (<0.001) | 0.064 (0.002) | 0.087 (<0.001) | 0.163 (<0.001) | 0.111 (<0.001) | 0.055 (0.007) | -0.005 (0.852) | 0.034 (0.099) | 0.021 (0.31) | 0.077 (<0.001) | 0.113 (<0.001) | 0.103 (<0.001) | 0.097 (<0.001) | 0.162 (<0.001) |
| Cr52 | | | | | 1 | 0.056 (0.006) | 0.054 (0.008) | -0.029 (0.158) | 0.215 (<0.001) | 0.331 (<0.001) | 0.185 (<0.001) | 0.22 (<0.001) | 0.192 (<0.001) | 0.33 (<0.001) | -0.062 (0.003) | -0.039 (0.054) | 0.074 (<0.001) | 0.144 (<0.001) | -0.044 (0.035) |
| Co59 | | | | | | 1 | 0.288 (<0.001) | 0.182 (<0.001) | 0.094 (<0.001) | 0.207 (<0.001) | 0.117 (<0.001) | 0.113 (<0.001) | 0.021 (0.303) | 0.065 (0.001) | 0.258 (<0.001) | 0.217 (<0.001) | 0.036 (0.077) | 0.167 (<0.001) | 0.31 (<0.001) |
| Cu63 | | | | | | | 1 | 0.126 (<0.001) | 0.102 (<0.001) | 0.227 (<0.001) | 0.211 (<0.001) | 0.129 (<0.001) | 0.106 (<0.001) | 0.106 (<0.001) | 0.334 (<0.001) | 0.315 (<0.001) | 0.109 (<0.001) | 0.176 (<0.001) | 0.306 (<0.001) |
| Fe | | | | | | | | 1 | -0.003 (0.907) | 0.243 (<0.001) | 0.093 (<0.001) | 0.001 (0.986) | 0.034 (0.1) | 0.093 (<0.001) | 0.378 (<0.001) | 0.2 (<0.001) | 0.015 (0.475) | 0.087 (<0.001) | 0.394 (<0.001) |
| Hg202 | | | | | | | | | 1 | 0.195 (<0.001) | 0.231 (<0.001) | 0.34 (<0.001) | 0.153 (<0.001) | 0.286 (<0.001) | -0.089 (<0.001) | 0.059 (0.01) | 0.281 (<0.001) | 0.135 (<0.001) | 0.097 (<0.001) |
| Mn55 | | | | | | | | | | 1 | 0.31 (<0.001) | 0.281 (<0.001) | 0.264 (<0.001) | 0.337 (<0.001) | 0.16 (<0.001) | 0.197 (<0.001) | 0.13 (<0.001) | 0.193 (<0.001) | 0.224 (<0.001) |
| Mo98 | | | | | | | | | | | 1 | 0.209 (<0.001) | 0.202 (<0.001) | 0.398 (<0.001) | 0.097 (<0.001) | 0.121 (<0.001) | 0.088 (<0.001) | 0.063 (0.002) | 0.12 (<0.001) |
| Ni60 | | | | | | | | | | | | 1 | 0.198 (<0.001) | 0.24 (<0.001) | -0.017 (0.548) | 0.013 (0.658) | 0.173 (<0.001) | 0.007 (0.805) | 0.138 (<0.001) |
| Pb208 | | | | | | | | | | | | | 1 | 0.249 (<0.001) | 0.025 (0.224) | 0.049 (0.016) | 0.073 (<0.001) | 0.102 (<0.001) | 0.016 (0.443) |
| Sb121 | | | | | | | | | | | | | | 1 | 0.01 (0.629) | 0.054 (0.008) | 0.215 (<0.001) | 0.046 (0.024) | 0.057 (0.006) |
| Se78 | | | | | | | | | | | | | | | 1 | 0.353 (<0.001) | 0.054 (0.008) | 0.03 (0.139) | 0.501 (<0.001) |
| Sr88 | | | | | | | | | | | | | | | | 1 | 0.078 (<0.001) | 0.141 (<0.001) | 0.336 (<0.001) |
| Tl205 | | | | | | | | | | | | | | | | | 1 | 0.032 (0.123) | 0.129 (<0.001) |
| V51 | | | | | | | | | | | | | | | | | | 1 | 0.136 (<0.001) |
| Zn66 | | | | | | | | | | | | | | | | | | | 1 |

**4.3.2 Dioxins, Furans and PCBs congeners by High Resolution Gas Chromatography – Mass Spectrometry**

Results, expressed as serum concentrations, were measured on the subgroup of 600 subjects. All congeners were expressed as estimated value on volume of serum and on gram of fats. It was also considered the TEQ (Toxicity Equivalence) value that is the product of the concentration of the compound and its corresponding TEF (Toxic Equivalency Factor) on gram of fats (Van den Berg et al., 1998; Van den Berg et al., 2006).

To avoid any bias for the substitution of measured values under the Limit of Quantification (LOQ), the "Lower Bound" (LB) approach was applied for statistical analysis. This means that each value registered under the LOQ was substituted with 0.00 (zero).

The figures below show the median of the sum of classes of organic compounds among the three impact areas and among the clusters included in the subgroup.

In order to estimate a cumulative effect on the people potentially exposed, we firstly analyzed the sum of PCDD/PCDF + DL-PCB (pg WHO-TEQ/g lipids) (Fig. 14).
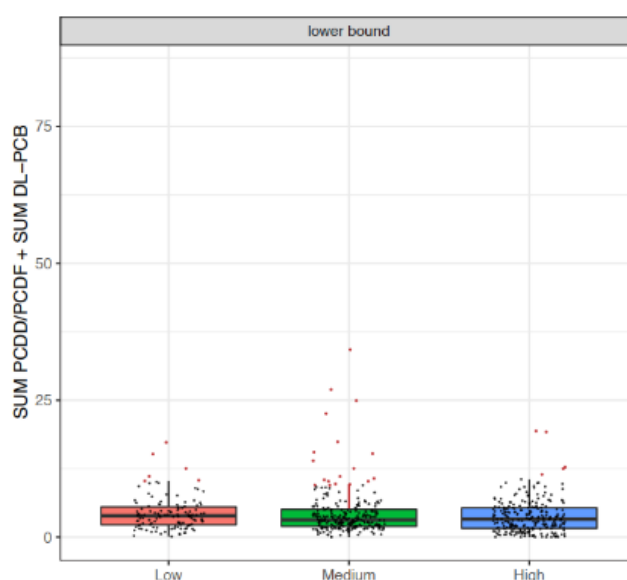


**Figure 14.** Sum of PCDD/PCDF + sum of DL-PCB expressed as pg WHO-TEQ/g lipids among the three different impact areas: low, medium and high impact area.

No significative evidence was shown among the three impact areas, considering the median values. If the same parameter is observed among clusters (Table 14 and Fig.15), it shows a sensitive increase of median value for Sabato Valley cluster compared to the overall median. Irno 1 and Irno 2 clusters, together with High 9 and High 12, show a slight increase compared to the other clusters.

**Table 14.** Median of Sum of PCDD/PCDF + sum of DL-PCB assessed in the serum (pg WHO-TEQ/g lipids) by cluster and grouped cluster for areas. LB=Lower Bound; IQR=Interquartile Range.

| Cluster | Median [IQR] (Range) LB | CLUSTER GROUPED | Average value |
|---|---|---|---|
| LOW_1 | 3.919 [2.469; 6.011] (from 0.795 to 12.544) | LOW | 3.894 |
| LOW_2 | 3.869 [1.929; 4.694] (from 0.108 to 17.293) | | |
| MEDIUM_1 | 3.016 [1.957; 3.826] (from 0.468 to 9.076) | MEDIUM | 2.989 |
| MEDIUM_2 | 2.963 [2.079; 4.13] (from 0.015 to 34.244) | | |
| HIGH_9 | 3.047 [0.833; 5.079] (from 0.007 to 19.201) | HIGH | 3.265 |
| HIGH_10 | 2.755 [1.342; 6.296] (from 0.013 to 19.386) | | |
| HIGH_11 | 3.207 [2.212; 4.601] (from 0.219 to 9.899) | | |
| HIGH_12 | 4.051 [1.797; 5.685] (from 0.052 to 11.466) | | |
| IRNO_1 | 4.951 [2.371; 8.145] (from 0.355 to 22.572) | IRNO | 4.165 |
| IRNO_2 | 3.38 [1.796; 5.994] (from 0.025 to 15.244) | | |
| SABATO | 4.192 [2.027; 6.5] (from 0.374 to 24.923) | SABATO | 4.195 |
| OVERALL | 3.385 [1.897; 5.317] (from 0.007 to 34.244) | OVERALL | 3.385 |
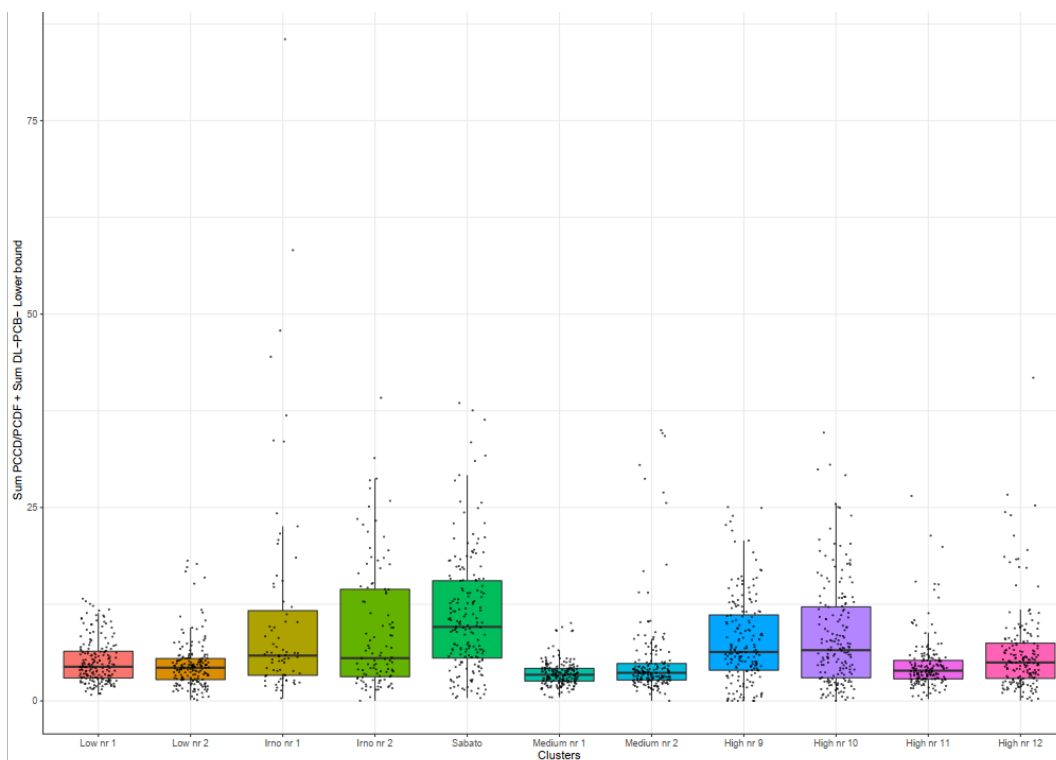


**Figure 15.** Sum of PCDD/PCDF + sum of DL-PCB expressed as pg WHO-TEQ/g lipids among the clusters.

The same scenario is presented when considering the only contribute of dioxins and furans, without DL-PCB addition (Table 15 and Fig. 16).

**Table 15.** Median of Sum of PCDD/PCDF assessed in the serum (pg WHO-TEQ/g lipids) by cluster and grouped cluster for areas. LB=Lower Bound; IQR=Interquartile Range.

| Cluster | Median [IQR] (Range) LB | CLUSTER GROUPED | Average value |
|---------|------------------------|-----------------|---------------|
| LOW_1 | 1.041 [0.762; 1.687] (from 0.074 to 3.16) | LOW | 1.109 |
| LOW_2 | 1.177 [0.649; 1.895] (from 0.1 to 6.988) | | |
| MEDIUM_1 | 1.628 [1.168; 2.413] (from 0.302 to 5.683) | MEDIUM | 1.675 |
| MEDIUM_2 | 1.722 [1.166; 2.664] (from 0 to 26.414) | | |
| HIGH_9 | 1.069 [0.171; 3.126] (from 0 to 17.691) | HIGH | 1.662 |
| HIGH_10 | 1.727 [0.769; 3.925] (from 0 to 18.996) | | |
| HIGH_11 | 1.77 [1.229; 2.811] (from 0.158 to 8.81) | | |
| HIGH_12 | 2.085 [1.012; 3.336] (from 0 to 9.933) | | |
| IRNO_1 | 1.881 [0.832; 3.572] (from 0.036 to 19.84) | IRNO | 1.866 |
| IRNO_2 | 1.851 [0.995; 3.936] (from 0 to 13.505) | | |
| SABATO | 3.127 [0.42; 5.648] (from 0 to 16.347) | SABATO | 3.127 |
| OVERALL | 1.618 [0.825; 2.891] (from 0 to 26.414) | OVERALL | 1.618 |
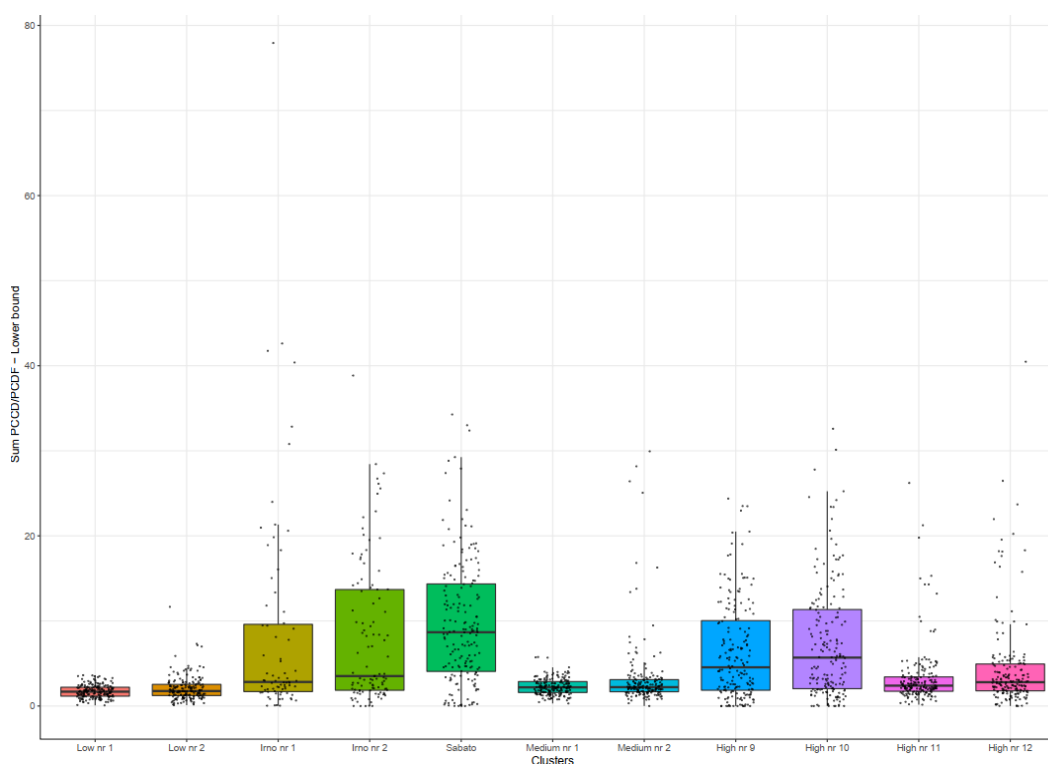


**Figure 16.** Sum of PCDD/PCDF expressed as pg WHO-TEQ/g lipids among the clusters.

Finally, NDL-PCB are shown in Figure 17. Median of Low 1 cluster is higher compared to those of other clusters (Table 16).

**Table 16.** Median of Sum of NDL-PCB assessed in the serum (ng/g lipids) by cluster and grouped cluster for areas. LB=Lower Bound; IQR=Interquartile Range.

| Cluster | Median [IQR] (Range) LB | CLUSTER GROUPED | Average value |
|---|---|---|---|
| LOW_1 | 57.802 [36.375; 108.759] (from 14.226 to 342.419) | LOW | 1.109 |
| LOW_2 | 63.646 [29.554; 102.164] (from 4.942 to 355.678) | | |
| MEDIUM_1 | 38.67 [30.292; 61.9] (from 3.218 to 159.285) | MEDIUM | 1.675 |
| MEDIUM_2 | 40.953 [25.97; 73.585] (from 11.718 to 429.657) | | |
| HIGH_9 | 23.335 [16.732; 43.549] (from 6.29 to 285.887) | HIGH | 1.662 |
| HIGH_10 | 25.065 [17.488; 47.998] (from 7.91 to 438.94) | | |
| HIGH_11 | 44.24 [25.263; 65.679] (from 4.571 to 171.898) | | |
| HIGH_12 | 51.77 [29.158; 101.514] (from 10.2 to 560.58) | | |
| IRNO_1 | 46.12 [26.369; 82.549] (from 17.95 to 1340.27) | IRNO | 1.866 |
| IRNO_2 | 40.983 [22.835; 67.873] (from 11.53 to 635.98) | | |
| SABATO | 47.61 [31.962; 63.532] (from 11.98 to 272.26) | SABATO | 3.127 |
| OVERALL | 42.611 [24.763; 73.566] (from 3.218 to 1340.27) | OVERALL | 1.618 |
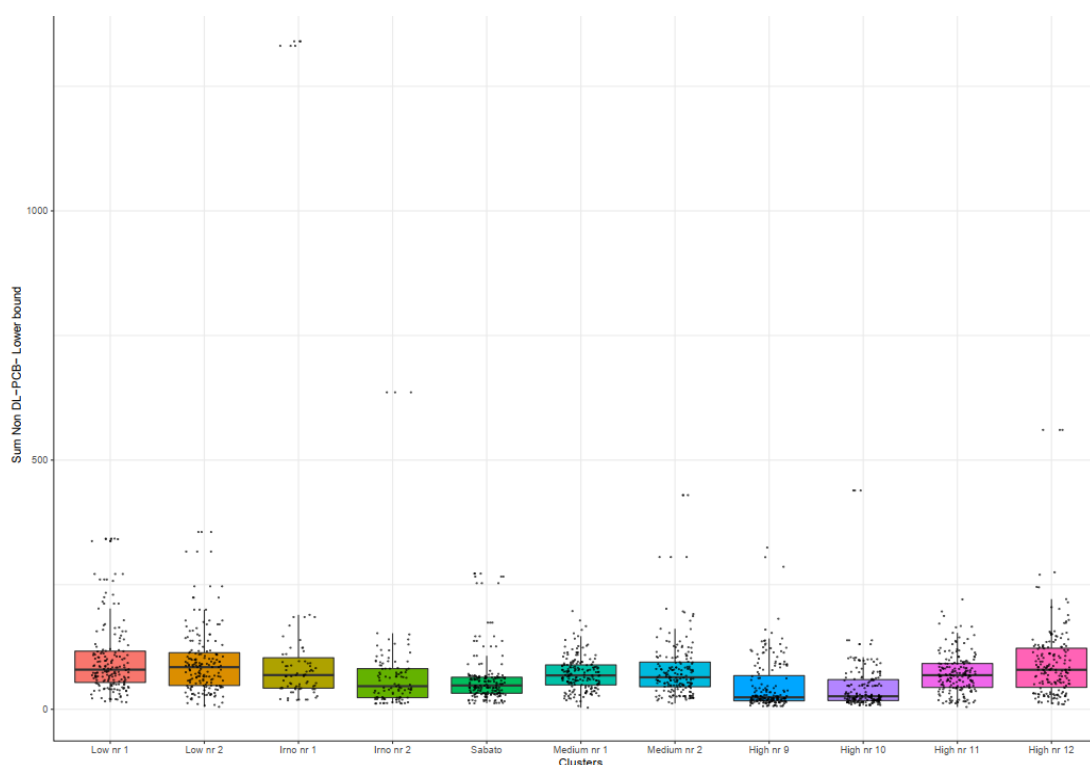


**Figure 17.** Sum of NDL-PCB expressed as ng/g lipids among the clusters.

Table 17 reports the percentage fold-increase/decrease of the median of compounds assessed per cluster and area. Noteworthy: the Irno and Sabato valley percentage increase for the sum of PCDD/F + DL-PCB, as well as in the high impact cluster 12; the NDL-PCB increase evidence for both clusters low_1 and low_2.

Results are also reported in Table 18 and 19 as median regression models using impact areas or clusters as geographical reference unit, respectively. It emerged that older age was consistently associated with the variable sum NDL-PCB and, to a lesser extent, with sum of PCDD/F, sum of PCDD/F+DL-PCB, sum of NDL-PCB.

Medium and high impact areas revealed association with higher levels of sum PCDD/F, and lower levels of DL-PCB and NDL-PCB.

Concerning median regression model data per clusters, all clusters of medium impact area were positively associated with higher levels of sum of PCDD/F, especially considering Irno_2 and Sabato valley. Also High_10, High_11, High_12 showed the same association. Conversely, low impact clusters were associated with higher levels of DL-PCB and NDL-PCB. Finally, clusters High_11 and High_12, Irno_1, Sabato, Medium_1 and Medium_2 were associated with high levels of NDL-PCB.

**Table 17.** Percentage fold-increase/decrease of the median of compounds assessed in the population indwelling in each cluster/area vs. the entire population. Green and red colors express the fold-decrease and increase, respectively.

| Cluster | SUM PCDD/PCDF pg WHO-TEQ/g lipids | SUM PCDD/PCDF + SUM DL-PCB pg WHO-TEQ/g lipids | SUM DL-PCB pg WHO-TEQ/g lipids | SUM NDL-PCB ng/g lipids |
|---|---|---|---|---|
| **High Impact Area** | 7,6 | -2,3 | -24,6 | -19 |
| HIGH_9 | -33,9 | -9,9 | -42,8 | -45,2 |
| HIGH_10 | 6,7 | -18,6 | -59,6 | -41,1 |
| HIGH_11 | 9,3 | -5,2 | -4,5 | 3,8 |
| HIGH_12 | 28,8 | 19,6 | 21,3 | 21,4 |
| **Medium Impact Area** | 16,8 | -6,5 | -12,8 | 1,4 |
| MEDIUM_1 | 0,6 | -10,9 | -2,9 | -9,2 |
| MEDIUM_2 | 6,4 | -12,4 | -15,5 | -3,8 |
| IRNO_1 | 16,2 | 46,2 | 9,9 | 8,2 |
| IRNO_2 | 14,4 | -0,1 | -37,4 | -3,8 |
| SABATO | 93,2 | 23,8 | -12,3 | 11,7 |
| **Low Impact Area** | -33,1 | 15,3 | 93,6 | 41,9 |
| LOW_1 | -35,6 | 15,7 | 113,7 | 35,6 |
| LOW_2 | -27,2 | 14,2 | 68,9 | 49,3 |

**Table 18.** Results of median regression models for the compounds assessed in the population, using impact area as geographical reference unit. Data are reported as adjusted difference between medians with the corresponding 95% CIs. Bold results indicate differences with p<0.05.

| | SUM PCDD/PCDF<br>pg WHO-TEQ/g lipids | SUM PCDD/PCDF + SUM DL-PCB<br>pg WHO-TEQ/g lipids | SUM DL-PCB<br>pg WHO-TEQ/g lipids | SUM NDL-PCB<br>ng/g lipids |
|---|---|---|---|---|
| **Area; Low impact ref.** | | | | |
| Medium impact | **0.68 (0.41 to 1.01); p<0.001** | -0.34 (-0.93 to 0.27); p=0.183 | **-1.12 (-1.56 to -0.8); p<0.001** | **-12.28 (-24.62 to -1.48); p=0.032** |
| High Impact | **0.58 (0.27 to 0.86); p=0.001** | -0.47 (-1.02 to 0.22); p=0.151 | **-1.18 (-1.6 to -0.77); p<0.001** | **-16.94 (-29.62 to -6.5); p=0.005** |
| **Gender; Male** | **0.32 (0.01 to 0.54); p=0.047** | **0.6 (0.1 to 1.04); p=0.014** | 0.08 (-0.17 to 0.46); p=0.636 | **11.73 (3.2 to 18.66); p=0.002** |
| **Age; 20-30 ref** | | | | |
| 30-40 | 0.17 (-0.01 to 0.65); p=0.274 | **0.63 (0.11 to 1.09); p=0.023** | 0.19 (0 to 0.45); p=0.177 | **10.17 (4.68 to 16.29); p<0.001** |
| 40-50 | **1.06 (0.69 to 1.5); p<0.001** | **2.04 (1.53 to 2.67); p<0.001** | **0.9 (0.59 to 1.15); p= p<0.001** | **43.15 (35.17 to 50.58); p<0.001** |

**Table 19.** Results of median regression models for the compounds assessed in the population, using cluster as geographical reference unit. Data are reported as adjusted difference between medians with the corresponding 95% CIs. Bold results indicate differences with p<0.05.

| Cluster | SUM PCDD/PCDF<br>pg WHO-TEQ/g lipids | SUM PCDD/PCDF + SUM DL-PCB<br>pg WHO-TEQ/g lipids | SUM DL-PCB<br>pg WHO-TEQ/g lipids | SUM NDL-PCB<br>ng/g lipids |
|---|---|---|---|---|
| LOW_1 | Reference | 0.96 (-0.42 to 2.13); p=0.14 | **1.53 (0.75 to 2.34); p<0.001** | **32.33 (11.49 to 48.62); p<0.001** |
| LOW_2 | 0.25 (-0.18 to 0.57); p=0.229 | 0.63 (-0.94 to 1.63); p=0.337 | **0.98 (0.32 to 1.78); p=0.012** | **25.54 (12.32 to 47.95); p=0.004** |
| MEDIUM_1 | **0.72 (0.22 to 1.09); p=0.001** | 0.12 (-1.49 to 1); p=0.838 | 0.04 (-0.5 to 0.61); p=0.888 | **10.6 (-0.53 to 18); p=0.024** |
| MEDIUM_2 | **0.88 (0.42 to 1.18); p<0.001** | 0.57 (-0.99 to 1.41); p=0.37 | 0.17 (-0.43 to 0.61); p=0.534 | **18.13 (6.01 to 29.85); p=0.005** |
| HIGH_9 | 0.26 (-0.36 to 1.01); p=0.406 | 0.33 (-1.64 to 1.46); p=0.688 | Reference | Reference |
| HIGH_10 | **0.98 (0.12 to 1.35); p=0.003** | Reference | -0.34 (-0.95 to 0.09); p=0.212 | 4.61 (-9.32 to 11.33); p=0.394 |
| HIGH_11 | **0.70 (0.27 to 1.17); p=0.003** | 0.48 (-1.02 to 1.39); p=0.42 | 0.23 (-0.46 to 0.72); p=0.439 | **16.62 (3.97 to 24.67); p=0.002** |
| HIGH_12 | **0.87 (0.33 to 1.37); p=0.002** | 0.87 (-0.79 to 2.1); p=0.228 | 0.49 (-0.19 to 1.02); p=0.107 | **16.61 (5.2 to 37.91); p=0.038** |
| IRNO_1 | 0.50 (-0.17 to 1.44); p=0.295 | 0.92 (-0.67 to 3.62); p=0.396 | 0.68 (-0.53 to 1.68); p=0.238 | **17.68 (4.83 to 32.12); p=0.008** |
| IRNO_2 | **1.05 (0.07 to 2.02); p=0.034** | 0.59 (-0.96 to 1.87); p=0.419 | -0.07 (-0.67 to 0.56); p=0.833 | 12.09 (-1.03 to 24.5); p=0.079 |
| SABATO | **1.77 (0.37 to 3.13); p=0.016** | 1.41 (-0.22 to 2.52); p=0.062 | 0.09 (-0.56 to 0.59); p=0.753 | **19.71 (6.77 to 29.31); p=0.001** |

### 4.3.3 Chemical Activated LUciferase gene eXpression (CALUX) Bioassay

Results of the Calux gene reporter assay, expressed as plasma concentrations depending on the cell line observed, were estimated on the subgroup of 600 subjects. Considering the DR Calux® assay, with Ahrilic (AhR) receptor activation measurement, 597 samples had sufficient amount of plasma for the determinations.

In Figure 18 a Yardstick representation of the results, lined up from lowest to highest value is given, while in Figure 19 a frequency distribution of these data is presented.

A mean value of 44.89 ± 15.74 pg 2,3,67,8-TCDD BEQ/g fat and a median value of 42.19 pg 2,3,7,8-TCDD BEQ/g fat was observed (Fig. 18 and 19, table 20). 90% of the DR CALUX® values were between 24.8 and 73.8 pg 2,3,7,8-TCDD BEQ/g fat, while the 5% highest values ranged from 73.8 to 150.0 pg 2,3,7,8-TCDD BEQ/g fat. The lowest quantifiable result obtained was 6.0 pg 2,3,7,8-TCDD BEQ/g fat, equal to the LOQ of the DR CALUX® method. Two samples had results below the LOQ.
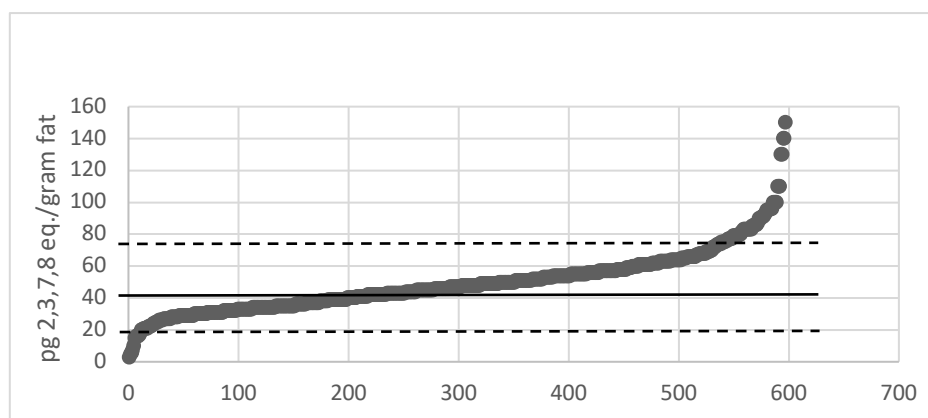


**Figure 18.** Yardstick-based representation of DR CALUX® results in study sample plasma analyzed. DR CALUX® bioassay results are lined up from lowest to highest obtained result expressed in pg 2,3,7,8-TCDD BEQ/g fat. Back line represents the mean value of 44.89 pg 2,3,7,8-TCDD BEQ/g fat. The top line indicates the 95% percentile and the bottom line the 5% percentile of respectively 73.8 and 24.8 pg 2,3,7,8-TCDD BEQ/g fat.
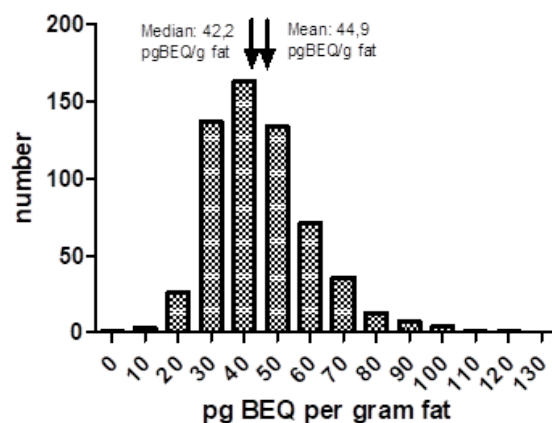
**Figure 19.** Frequency distribution of DR CALUX® results expressed as pg BEQ/g fat.

In Table 20 a summary of results obtained in plasma, measured by DR CALUX®, is given per impact areas and clusters. Medium impact area is divided in two groups, in order to better highlight differences among clusters.

**Table 20.** DR CALUX® results (pgBEQ/g fat) compared to areas and cluster of living. In bold are values significantly higher (p-value: >0.001, one-way Anova), as compared to all other clusters; SEM = standard error of mean.

| Impact Area | Cluster (grouped) | N. subjects | Mean | Median | SEM | Min. | Max. |
|---|---|---|---|---|---|---|---|
| Low | 16-17 | 120 | 41.62 | 39.66 | 1.74 | 12.41 | 99.51 |
| Medium 1 | 13-14 | 118 | 43.31 | 41.65 | 1,58 | 20.76 | 88.41 |
| Medium 2 | 19-21 | 120 | **54.17** | **52.99** | 2.82 | 18.26 | 100.66 |
| High | 9-12 | 237 | 42.94 | 40.07 | 1.93 | 36.00 | 150.0 |

As can be observed, there is no significant correlation between the area of living and the stable AhR ligands in plasma of volunteers living in those areas. However, there is a significant difference (one-way ANOVA, p=0.0008) between the level of stable AhR ligands in plasma of subjects from clusters 19, 20 and 21 as compared to all other clusters analyzed. These clusters include Irno and Sabato Valleys. Figure 20 reports a box-plot representation of the DR CALUX® results in plasma vs. clusters.
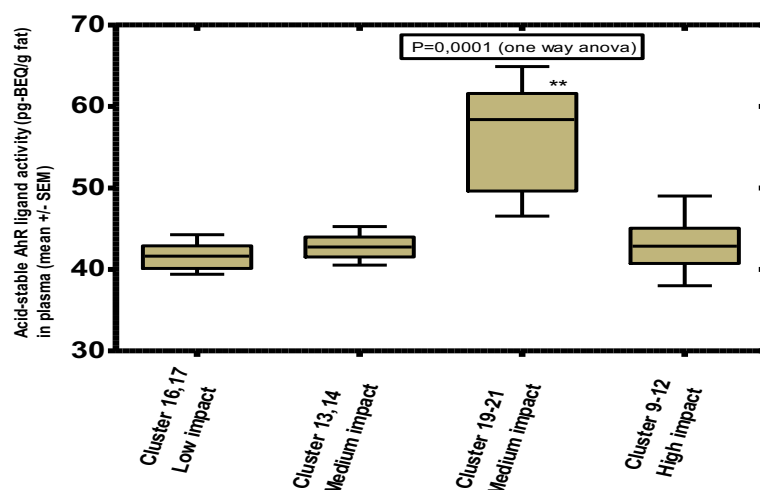
**Figure 20.** Box-Plot representation of Acid stable AhR ligands measured by DR CALUX® in plasma samples, in relation to clusters and impact areas.

The analysis of plasma samples on the PAH CALUX® bioassay resulted in measurable PAHs and PAH-like responses in all samples analyzed.

A wide range of PAH-like activities were observed in the plasma samples with a mean value of 86.00 ng BaP-EQ/ml, a minimum of 0.3 ng BaP-EQ/ml and a maximum of 7900.00 ng BaP-EQ/ml.

In Table 21 the PAH CALUX® activity in plasma (ngBaP-EQ/ml) is presented per impact areas and clusters. Medium impact area is divided in two groups, in order to better highlight differences among clusters.

As can be observed there is a wide range of PAH activities spanning 3-4 orders of magnitude between the lowest and the highest values per clusters. Overall, there is no relationship between PAH CALUX® levels in plasma and area and cluster of living. Apparently, other factors than the area of living may be more important to explain the internal exposure levels to PAH in ng BaP-EQ/ml as measured with the PAH CALUX® method.

**Table 21.** PAH CALUX® results (ng BaP-EQ/ml) compared to areas and cluster of living. SEM = standard error of mean.

| Impact Area | Cluster (grouped) | N. subjects | Mean | Median | SEM | Min. | Max. |
|:---:|:---:|:---:|:---:|:---:|:---:|:---:|:---:|
| Low | 16-17 | 120 | 86.00 | 50.00 | 13.1 | 0.4 | 1400.00 |
| Medium 1 | 13-14 | 120 | 114.00 | 29.00 | 65.7 | 0.3 | 7900.00 |
| Medium 2 | 19-21 | 120 | 87.00 | 31.00 | 27.4 | 0.1 | 3200.00 |
| High | 9-12 | 238 | 58.00 | 15.00 | 15.1 | 0.3 | 3300.00 |

The analysis on the ERα CALUX® bioassay resulted in measurable total estrogenic and estrogen-like responses (expressed in pg-EEQ/ml) in all plasma samples analyzed.

In Table 22, the total estrogenic activity data (pg-EEQ/ml) are compared to the main endogenous estrogenic hormone, 17-β-estradiol (17-β) levels measured in plasma of female volunteers from the SPES subgroup selected. Medium impact area is divided in two groups, in order to better highlight differences among clusters.

**Table 22.** Concentrations of 17-β-estradiol (17-β pg/ml) and total estrogenic activity (pg-EEQ/ml) in female volunteers of the SPES subgroup compared to cluster and area of living. In bold are represented values significantly higher as compared to 17-β value for same cluster (p-value<0.002, Mann-Whitney). SEM = standard error of mean.

| Impact Area | Cluster (grouped) | Analyte | N. subjects | Mean | Median | SEM | Min. | Max. |
|---|---|---|---|---|---|---|---|---|
| Low | 16-17 | 17-β | 60 | 21.22 | 21.15 | 0.77 | 10.00 | 53.9 |
| Medium 1 | 13-14 | 17-β | 60 | 22.91 | 21.80 | 0.93 | 6.80 | 51.00 |
| Medium 2 | 19-21 | 17-β | 57 | 22.61 | 22.00 | 1.51 | 7.00 | 70.00 |
| High | 9-12 | 17-β | 120 | 22.28 | 21.18 | 0.66 | 11.00 | 68.70 |
| Low | 16-17 | EEQ | 60 | **45.85** | 27.50 | 6.84 | 2.10 | 240.00 |
| Medium 1 | 13-14 | EEQ | 60 | **94.26** * | 59.50 | 12.30 | 2.10 | 510.00 |
| Medium 2 | 19-21 | EEQ | 57 | **85.44** * | 57.00 | 8.93 | 8.40 | 290.00 |
| High | 9-12 | EEQ | 120 | **109.39** ** | 73.00 | 8.43 | 2.10 | 510.00 |

*Significantly higher as compared to Low Impact Area, Cluster 16-17 (p-value = 0.007, Mann-Whitney).
** significantly higher (p-value= 0.0005, one-way Anova) as compared to all other clusters.

A wide range of total estrogenic activity was observed ranging from 2.10 to 510.00 pg-EEQ/ml. As can be observed there is a significant 3 to 5-fold difference in total estrogenic activity levels measured by the ERα-CALUX® reporter gene assay in comparison to the levels of the natural endogenous hormone 17-β Estradiol. This suggests the presence of endocrine active compounds, that activate the estrogen receptor-alpha in plasma of females, that can to a large extend not be explained by natural endogenous hormone levels and may thus indicate the presence of endocrine active compounds (EACs) in samples.

In addition, the total estrogenic activity levels are significantly higher (p-value = 0.0005, one-way Anova) in clusters of high and medium impact areas (Fig. 21).

Conversely, the plasma levels of 17-β estradiol in females do not differ significantly between volunteers from the various areas of living, suggesting that the additional estrogenic activity may arise from external sources.
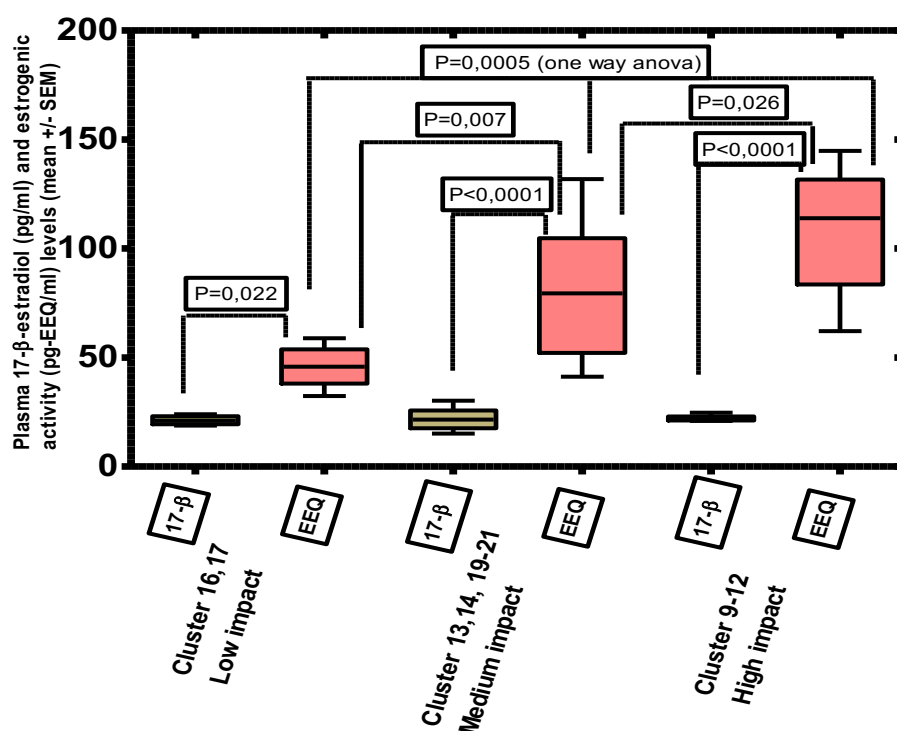
**Figure 21.** Boxplot of female plasma concentrations of 17-β-estradiol (pg/ml) and total estrogenic activity (pg-EEQ/ml) measured by ERα-CALUX®. Statistical differences were analyzed using one-way Anova for comparisons between clusters, and Mann Whitney test of significance between pairs of boxplots. Level of significance was set at p-value< 0.05 (17-β: 17-β-estradiol; EEQ: estrogen equivalents).

Similarly, it is reported the total estrogenic activity in plasma by the ERα-CALUX® reporter gene assay in male volunteers. These levels have been compared with concentrations of 17-β-estradiol in plasma of the same male individuals (Table 23). The range of total estrogenic activity levels in male plasma is much lower than in female individuals and shows a smaller range of differences i.e., ranging from 1.7 to 150.00 pg-EEQ/ml. Also the differences between the levels of the total estrogenic activity (EEQs) and the natural 17-β estrogen concentrations is much less pronounced, and variable, with a significantly lower EEQ level than 17-β levels in the low impacted area of living and significantly higher EEQ levels than 17-β in the high impacted areas of living (Fig. 22).

| Category | Cluster | Analyte | N | Mean | Median | SEM | Min | Max |
|----------|---------|---------|---|------|--------|-----|-----|-----|

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Low | 16-17 | 17-β | 60 | 19.59 | 18.35 | 0.96 | 10.00 | 52.00 |
| Medium 1 | 13,14 | 17-β | 60 | 19.23 | 18.20 | 0.88 | 10.00 | 51.00 |
| Medium 2 | 19-21 | 17-β | 60 | 20.35 | 18.15 | 1.14 | 7.20 | 44.00 |
| High | 9-12 | 17-β | 127 | 18.24 | 18.13 | 0.36 | 11.10 | 40.00 |
| Low | 16-17 | EEQ | 60 | **12.72** | 15.30 | 1.51 | 1.70 | 37.00 |
| Medium 1 | 13-14 | EEQ | 60 | 24.25* | 25.00 | 1.49 | 6.80 | 61.00 |
| Medium 2 | 19-21 | EEQ | 60 | 21.62* | 22.50 | 2.05 | 6.10 | 110.00 |
| High | 9-12 | EEQ | 117 | 27.88**, *** | 25.00 | 2.30 | 5.00 | 150.00 |

**Table 23.** Concentrations of 17-β-estradiol (17-β pg/ml) and total estrogenic activity (pg-EEQ/ml) in male volunteers of the SPES subgroup compared to cluster and area of living. In bold are represented values significantly lower as compared to 17-β value for same cluster (p-value = 0.002, Mann-Whitney).

SEM = standard error of mean.

* significantly higher as compared to Low Impact Area, Cluster 16-17 (P= 0.0072, Mann-Whitney).
** significantly higher as compared to 17-β value for same cluster (P<0.0001);
*** significantly higher (P-value: 0,0005, one-way Anova) as compared to all other clusters.
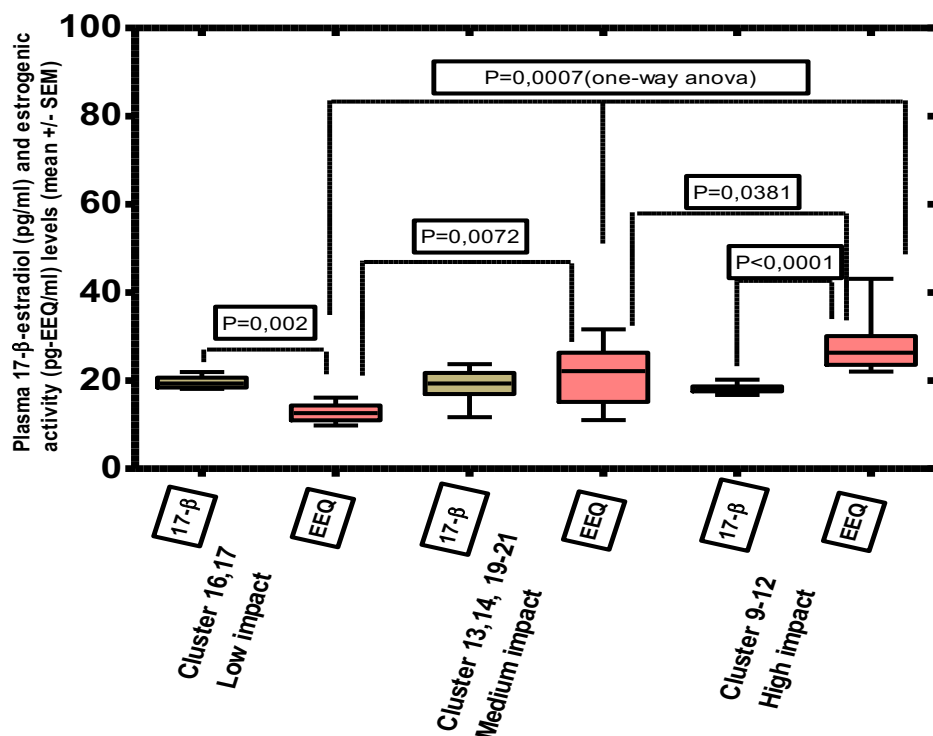


**Figure 22.** Boxplot of male plasma concentrations of 17-β-estradiol (pg/ml) and total estrogenic activity (pg-EEQ/ml) measured by ERα-CALUX®. Statistical differences were analyzed using one-way Anova for comparisons between clusters, and Mann Whitney test of significance between pairs of boxplots. Level of significance was set at p-value< 0.05 (17-β: 17-β-estradiol; EEQ: estrogen equivalents).

Therefore, also in male subjects the total estrogenic activity levels are significantly higher (p-value = 0.0007, one-way Anova) in clusters of high and medium impact areas (Fig. 22). The plasma levels of the natural hormone, 17-β-estradiol, in males do not differ significantly

between volunteers from the diverse areas of living, suggesting that the additional estrogenic activity observed in the high impacted area may arise from external sources. Overall, it is observed a significant higher level of estrogenic activity in plasma from human volunteers as compared to natural endogenous 17-β-estradiol levels, which increased with higher impacted areas of living. This may suggest that the contamination areas give rise to elevated exposure to contaminants with possible endocrine activity, so called Endocrine-disrupting chemicals (EDCs).

Figure 23 shows the geographical distribution per area of living of the EEQ measured by ERα-CALUX® assay on female cohort. Higher values are detected in women from high and medium impact areas. Notably, as previously shown by table22 and figure 21, among medium impact areas, the higher values are geolocated in Irno and Sabato valleys.
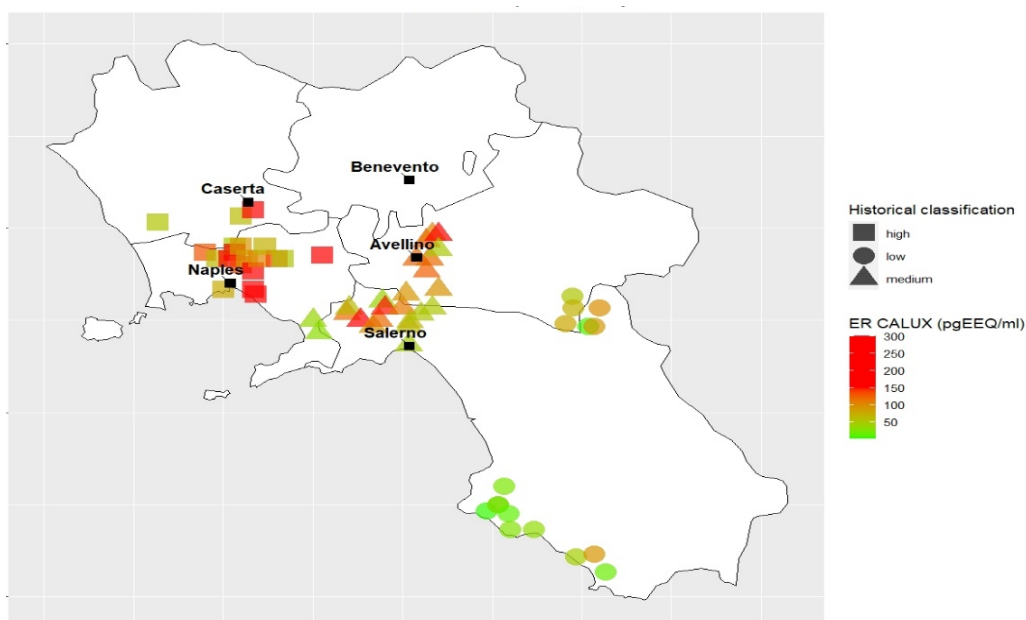


**Figure 23.** Campania Region maps representing hotspot areas of living for female subjects at municipal level, with their respective total estrogenic activity detected by ERα-CALUX® reporter gene assay (pg EEQ/ml) in plasma.

## 4.4 Effect Biomarkers

### 4.4.1 DNA methylation Array Preliminary Analysis

Methylation array data analysis was firstly performed through a quality control step, secondly with a global overview on methylation differences among the three impact areas and, finally, by means of a regression model method to explore the correlation between the exposure biomarkers assessed and the epigenetic profile.

As mentioned in paragraph 3.3.2, DNA methylation array analysis consists of a preliminary control quality check, with following filtering and normalization steps.

Samples with a lot of missing probes and probes with poor quality were removed from the analysis. Finally, 592 of 600 samples and 677886 of 865918 probes were included in the analysis.

In order to have an overview of the dataset, the most variable 1000 probes were graphically represented in a multidimensional plot (MDS-plot) before and after normalization step (Fig. 24 a/b), as well as all probes raw data were represented in a density plot (Fig. 25 a/b).
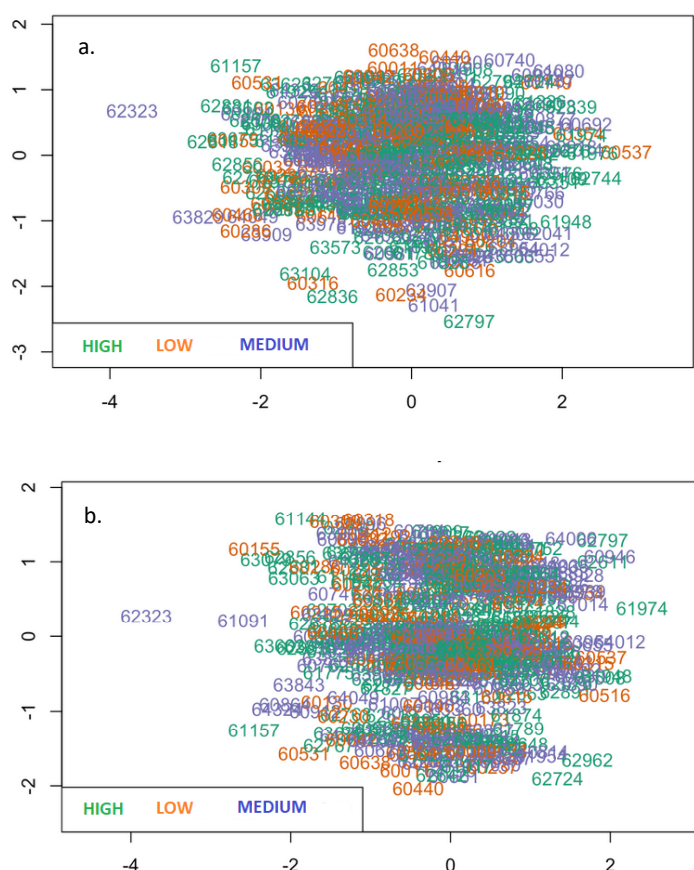


**Figure 24.** 1000 most variable probes represented in a multidimensional plot (MDS), before (a) and after (b) the normalization step. The 1000 probes were selected computing variance in the beta value matrix and sorting them in descending order. The figures were obtained by means a multidimensional scaling approach.
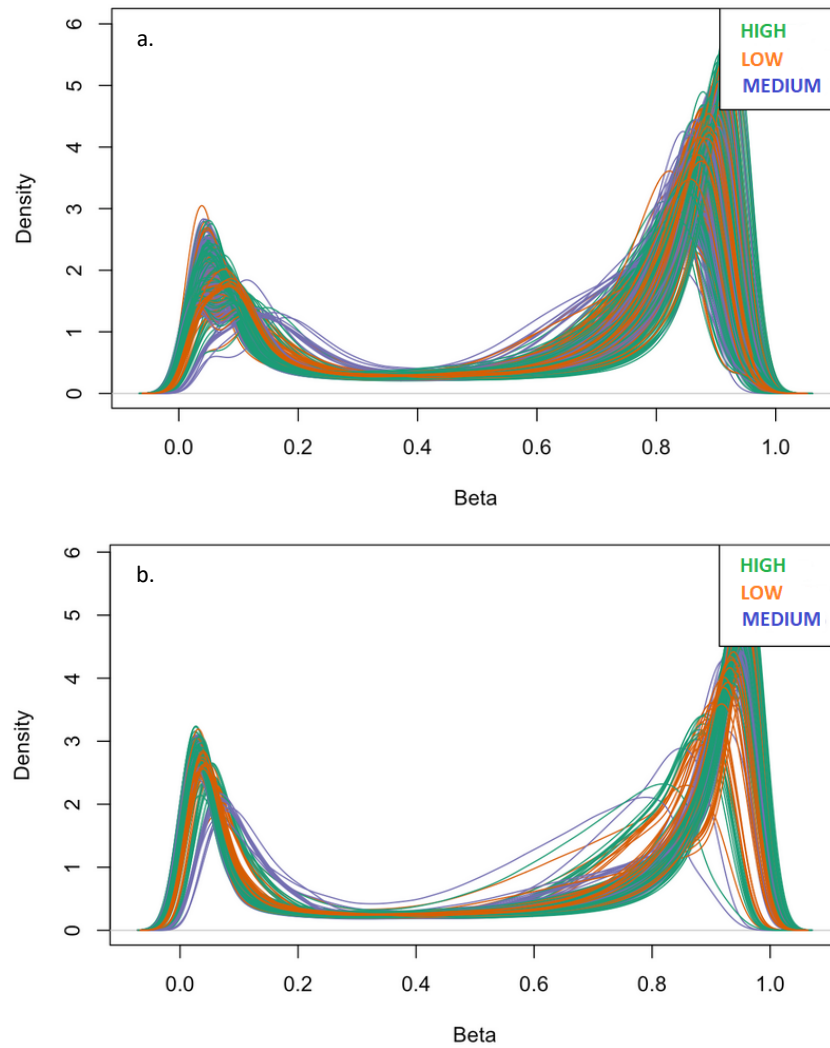
**Figure 25.** Density plot of raw data (677886 probes) before (a) and after (b) the normalization step.

It can be observed a better probe distribution after the normalization step, even though it is not completely satisfying. A total number of 188032 probes were removed through the normalization step. Profiles are consistent with reference methylation dataset distribution of beta values between 0.0 and 1.0. The following "cell heterogeneity" step improves the probes distribution from a qualitative point of view.

Then a Singular Value Decomposition Analysis (SVD) was performed in order to allow the batch correction (Fig. 26 a/b). Among the most important confounding variables it can be highlighted Run_ID, Slide, Cluster, Age and Gender. Run_ID and Slide are linked to the analytical process, while Cluster, Age and Gender are linked to the population features.
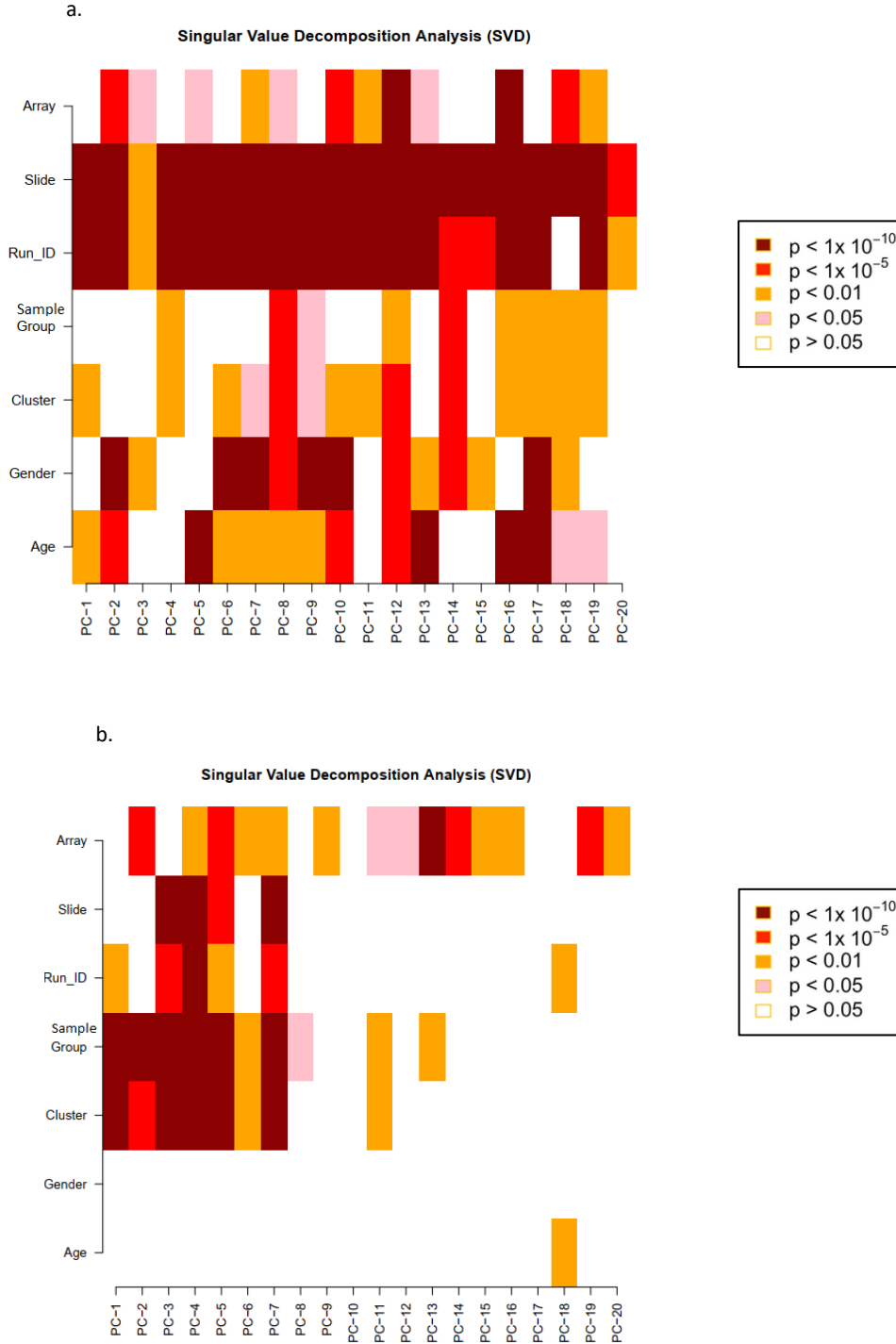
**Figure 26.** Singular Value Decomposition Analysis (SVD) before (a) and after (b) the batch correction considering Slide, Age and Gender as factors to correct.

Considering the nature of samples (genomic DNA from whole blood), it becomes fundamental a step of cell heterogeneity correction (Houseman et al., 2012).

In the Figure 27 a/b, the Multidimensional Plot on the most variables 1000 probes and the Density Plot on the whole dataset after batch and cell heterogeneity corrections, show a notably improvement of distribution from a qualitative point of view.
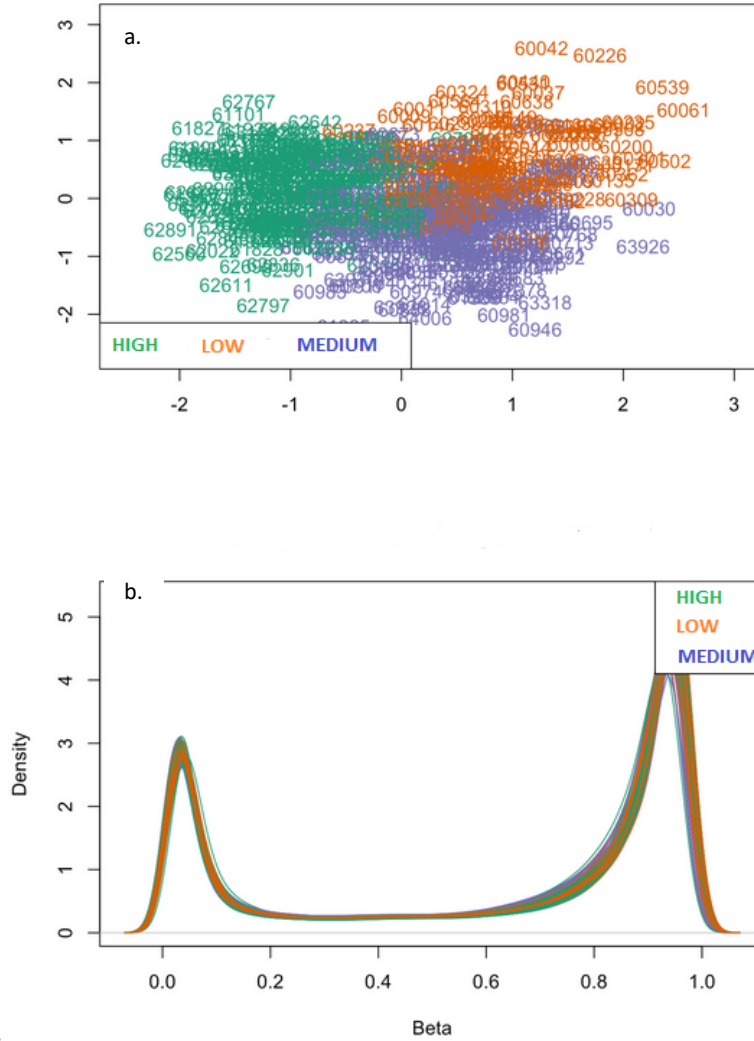
**Figure 2** ........................................................................................................ ter normalization, batch
and cell heterogeneity correction steps. (b) Density plot of raw data (677886 probes) after normalization, batch and
cell heterogeneity correction steps.

## 4.4.2 Differentially Methylated Probes and Differentially Methylated Regions

Differentially Methylated Probes (DMPs) were identified in the following comparisons,
among impact areas:

1. High impact area vs Low impact area;
2. Medium impact area vs High impact area;
3. Medium impact area vs Low impact area.

The following Volcano plots (Fig. 28 a/b/c) show an overview of the global methylation,
representing on the x the delta beta values ($\Delta\beta$) between the two impact areas observed in each
comparison, and on the y the statistical significance. A strict cut-off was selected for adjusted
p-value (0.00001) for the selection of the probes.
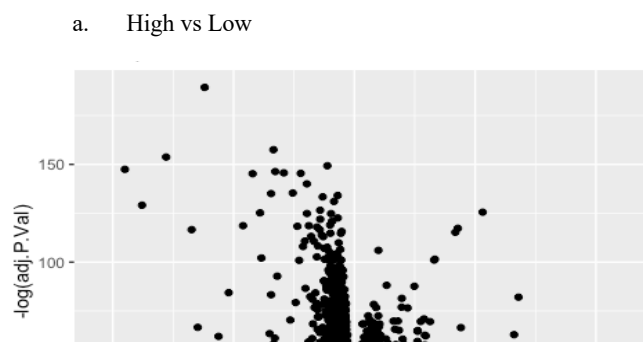
a.    High vs Low
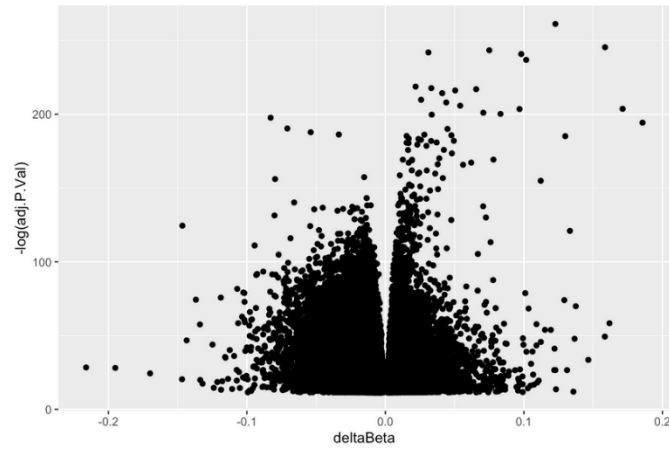
b.     Medium vs High



c.     Medium vs Low
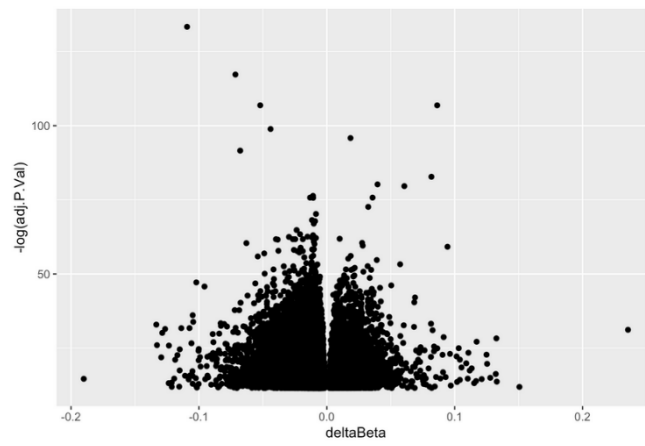


**Figure 28.** Volcano plot for DMP comparison among impact areas (a) high vs low; $\Delta\beta>0$ means higher value for high impact area; (b) medium vs high; $\Delta\beta>0$ means higher value for medium impact area; (c) medium vs low; $\Delta\beta>0$ means higher value for medium impact area.

Volcano plot 28a shows relevant statistical significance of global hypomethylation of high impact area compared to the low one.

Less visible difference on global methylation is shown in Fig. 28b and 28c: slight hypomethylation for high impact area vs medium impact area, slight global hypomethylation for medium impact area vs low impact area.

Frequently the delta beta values are not very high, and this means a difficulty in biological understanding of the phenomena. For the purposes of this dissertation the focus is particularly on DMPs mapping on promoters' regions.

All analysis were performed considering the M-values (the logit transformation of the beta-values), even if the results are shown in tables as β-values, in order to have an easier interpretation.

Tables below report DMPs filtered from impact areas comparisons, considering a $\Delta\beta \geq 0.10$.

Table 24a shows DMPs positioned on proximal promoter region (5'UTR, TSS200, TSS1500, 1stExon), comparing low vs high impact areas.

Table 24b report the same object referring to comparison between medium vs low impact areas. For both tables, hypermethylated CpGs in areas of increasing pollution risk were selected. Interestingly, among DMPs in table 24a, mapping on promotor regions in low vs high comparison, it is possible to list the following genes involved:

- RHOJ, a GTP binding protein, regulating angiogenesis and involved in insulin signaling pathway;
- MYCBPAP, encoding for a MYCBP associated protein, which is concern for spermatogenesis;
- WBSCR26, encoding for a lncRNA ABHD11-AS1, which play a role in gastric and bladder cancer;
- LOXL3, encoding for a Lysil Oxidase Like 3, which has a role in copper ion binding, oxidoreductase activity and regulation of inflammatory response;
- CLDN14, playing a role in cell-to-cell adhesion in epithelial or endothelial cell sheets;
- CLEC4C, implied in cell adhesion, cell-to-cell signaling, inflammation and immune responses;
- COQ5, with its methyltransferase activity;
- COX7A1, encoding for Cytochrome C Oxidase subunit 7A1;
- FAT1, encoding for adhesion molecule and signaling receptor, with a role in development processes and cell communication;
- PTPRE, encoding for a tyrosine phosphatase protein family member, which has a role in signaling regulating cell growth, differentiation, mitotic cycle and oncogenic transformation.

Considering table 24b, reporting the medium vs low impact area comparison, beyond RHOJ and LOXL3 genes, in common with the results of high vs low comparison, it is possible to count:

- NRIP1 gene, encoding a nuclear protein that modulate the transcriptional activity of the estrogen receptor, and has also a role in inflammation pathway lead by NF-kB;
- S100A13 gene, which encodes for a S100 family protein, involved in cell cycle progression and differentiation. Its expression was documented by Azimi et al., in 2014, in chemotherapy resistance in melanoma.

**Table 24a.** High impact areas vs low impact area. Differential Methylated Probes with delta beta value ≥ 0.10, positioned on promoter region. Statistical significance is expressed as FDR (False Discovery Rate) adjusted p-value (adj.P.Val). Δβ > 0 means the methylation is higher in high impact areas.

| CpG | adj.P.Val | deltaBeta | gene | feature | cgi | UCSC_Islands_Name |
|-----|-----------|-----------|------|---------|-----|-------------------|
| cg11775521 | 1.13E-51 | 0.0857 | KCNE3 | TSS200 | island | chr11:74178175-74178801 |
| cg18297203 | 1.34E-44 | 0.0661 | LPCAT2 | TSS200 | shore | chr16:55542920-55543274 |
| cg20094837 | 6.36E-31 | 0.0627 | NBEA | TSS1500 | opensea | |
| cg08370546 | 3.20E-25 | 0.0843 | PHKG1 | TSS200 | opensea | |
| cg01216565 | 4.55E-22 | 0.0813 | WDR45B | TSS1500 | shore | chr17:80605301-80606324 |
| cg23980942 | 1.05E-21 | 0.0606 | SEMA6D | 5'UTR | shore | chr15:47476369-47477499 |
| cg20833838 | 2.25E-21 | 0.0649 | GPRIN1 | 5'UTR | shore | chr5:176036458-176037557 |
| cg25432232 | 5.89E-19 | 0.0861 | AURKC | 5'UTR | island | chr19:57741955-57742457 |
| cg01668281 | 7.77E-19 | 0.0605 | CLDN14 | 5'UTR | opensea | |
| cg09340198 | 3.73E-18 | 0.0677 | ANKRD28 | TSS1500 | shore | chr3:15900497-15901990 |
| cg21695771 | 3.85E-18 | 0.0735 | COX7A1 | TSS200 | shore | chr19:36642327-36643585 |
| cg12569497 | 7.14E-18 | 0.06 | COL21A1 | TSS1500 | shore | chr6:56112188-56112444 |
| cg11659652 | 1.51E-17 | 0.0708 | PTPRE | 5'UTR | opensea | |
| cg16443366 | 5.89E-17 | 0.0617 | SOGA3 | 5'UTR | shore | chr6:127836730-127837706 |
| cg09548084 | 3.20E-16 | 0.06 | SLC35B3 | TSS1500 | shore | chr6:8435314-8436141 |
| cg02176678 | 8.71E-16 | 0.0814 | TTLL4 | 5'UTR | shore | chr2:219575448-219576080 |
| cg19603903 | 1.12E-15 | 0.0893 | AURKC | TSS200 | island | chr19:57741955-57742457 |
| cg15176213 | 1.20E-15 | 0.0673 | COX7A1 | 5'UTR | island | chr19:36642327-36643585 |
| cg05524354 | 1.94E-15 | 0.071 | PTPRE | 5'UTR | opensea | |
| cg06643849 | 3.26E-15 | 0.085 | AURKC | 5'UTR | island | chr19:57741955-57742457 |
| cg05275307 | 1.27E-14 | 0.064 | CLDN14 | TSS200 | opensea | |
| cg19052272 | 3.00E-14 | 0.1095 | ALLC | TSS1500 | opensea | |
| cg24824686 | 2.23E-13 | 0.0615 | PTPRE | 5'UTR | opensea | |
| cg00161556 | 2.38E-13 | 0.0711 | ZDHHC13 | TSS1500 | shore | chr11:19138497-19139216 |
| cg25932599 | 3.48E-13 | 0.0669 | FGFRL1 | TSS1500 | island | chr4:1003106-1005455 |
| cg08161306 | 5.39E-13 | 0.127 | TBC1D22A | TSS1500 | opensea | |
| cg00255882 | 8.73E-13 | 0.0729 | WDR33 | TSS1500 | shore | chr2:128568321-128569064 |
| cg19825600 | 1.04E-12 | 0.0681 | ALLC | TSS1500 | opensea | |
| cg00999904 | 2.67E-12 | 0.0768 | ALLC | TSS1500 | opensea | |
| cg21183455 | 2.71E-12 | 0.0626 | C22orf34 | TSS1500 | opensea | |
| cg24335895 | 3.86E-12 | 0.0693 | COX7A1 | TSS200 | shore | chr19:36642327-36643585 |
| cg14409029 | 4.17E-12 | 0.0837 | ZDHHC13 | TSS1500 | shore | chr11:19138497-19139216 |
| cg06888460 | 7.22E-12 | 0.0693 | LDHAL6A | TSS200 | island | chr11:18477298-18477756 |
| cg23786580 | 8.73E-12 | 0.0723 | HECW1 | 5'UTR | opensea | |
| cg26740318 | 9.84E-12 | 0.0608 | LDHAL6A | TSS1500 | shore | chr11:18477298-18477756 |
| cg16168363 | 1.44E-11 | 0.0609 | FAT1 | 5'UTR | shore | chr4:187644319-187648253 |
| cg07889765 | 1.47E-11 | 0.0864 | NOC3L | TSS1500 | shore | chr10:96122540-96122904 |
| cg21517946 | 2.67E-11 | 0.0985 | FKBP5 | TSS1500 | shore | chr6:35699166-35699953 |
| cg24527560 | 3.28E-11 | 0.0915 | LOC441666 | TSS200 | shore | chr10:42862953-42863216 |
| cg25605888 | 4.06E-11 | 0.0618 | COQ5 | TSS1500 | shore | chr12:120966788-120967038 |
| cg21042336 | 7.07E-11 | 0.0794 | OSBPL1A | TSS1500 | shore | chr18:21977275-21978110 |
| cg17240725 | 7.94E-11 | 0.118 | WBSCR26 | TSS200 | shelf | chr7:73152564-73153716 |

| | | | | | | |
|---|---|---|---|---|---|---|
| cg01485177 | 3.12E-10 | 0.0611 | LOC441666 | TSS200 | shore | chr10:42862953-42863216 |
| cg04413036 | 3.37E-10 | 0.0626 | PLA2G6 | 5'UTR | opensea | |
| cg07157030 | 5.19E-10 | 0.1457 | RHOJ | 5'UTR | opensea | |
| cg19148731 | 9.85E-10 | 0.1126 | LOXL3 | 5'UTR | shore | chr2:74781494-74782685 |
| cg14259717 | 9.95E-10 | 0.0841 | LDHC | TSS200 | opensea | |
| cg12759523 | 1.14E-09 | 0.0707 | CYFIP1 | 5'UTR | opensea | |
| cg07189587 | 1.24E-09 | 0.1532 | RHOJ | 5'UTR | opensea | |
| cg02511570 | 1.30E-09 | 0.0788 | THCAT158 | TSS1500 | shore | chr17:45400874-45401440 |
| cg10645314 | 2.26E-09 | 0.0701 | ALLC | TSS1500 | opensea | |
| cg20111217 | 2.27E-09 | 0.0992 | MYCBPAP | TSS1500 | shore | chr17:48585385-48586167 |
| cg09408571 | 3.36E-09 | 0.0916 | GPR88 | TSS200 | shore | chr1:101004471-101005885 |
| cg00901687 | 4.44E-09 | 0.1182 | MYCBPAP | TSS1500 | shore | chr17:48585385-48586167 |
| cg19272349 | 4.71E-09 | 0.0793 | CSNK1A1L | TSS1500 | opensea | |
| cg21471707 | 1.26E-08 | 0.068 | LDHC | TSS200 | opensea | |
| cg11440486 | 1.62E-08 | 0.0902 | MYCBPAP | TSS1500 | shore | chr17:48585385-48586167 |
| cg25251562 | 2.44E-08 | 0.076 | ALLC | TSS1500 | opensea | |
| cg03343083 | 6.77E-08 | 0.0888 | LEPREL1 | 5'UTR | opensea | |
| cg11474464 | 7.46E-08 | 0.06 | GABRB3 | TSS200 | island | chr15:26874097-26874528 |
| cg21367232 | 2.05E-07 | 0.064 | SCAMP2 | TSS1500 | shore | chr15:75165373-75165903 |
| cg19149522 | 2.09E-07 | 0.0609 | ZDHHC4 | TSS1500 | shore | chr7:6616918-6617277 |
| cg00614959 | 3.08E-07 | 0.0681 | PCGF3 | 5'UTR | shelf | chr4:724335-726070 |
| cg11079896 | 3.43E-07 | 0.0912 | RHOJ | 5'UTR | opensea | |
| cg24480555 | 8.18E-07 | 0.0656 | C8orf33 | TSS1500 | shore | chr8:146277801-146278396 |
| cg07195891 | 2.48E-06 | 0.0981 | CLEC4C | 5'UTR | opensea | |
| cg24521141 | 2.91E-06 | 0.0791 | LOC101929596 | TSS1500 | opensea | |

**Table 24b.** Medium impact areas vs low impact area. Differential Methylated Probes with delta beta value ≥ 0.10, positioned on promoter region. Statistical significance is expressed as FDR (False Discovery Rate) adjusted p-value (adj.P.Val). Δβ > 0 means the methylation is higher in medium impact areas.

| CpG | adj.P.Val | deltaBeta | gene | feature | cgi | UCSC_Islands_Name |
|---|---|---|---|---|---|---|
| cg08370546 | 2.02E-26 | 0.0944 | PHKG1 | TSS200 | opensea | |
| cg01708150 | 3.46E-13 | 0.0914 | RHOJ | 5'UTR | opensea | |
| cg11079896 | 5.28E-13 | 0.1326 | RHOJ | 5'UTR | opensea | |
| cg16102102 | 1.43E-11 | 0.1032 | HAPLN1 | TSS1500 | shore | chr5:83017894-83018358 |
| cg04526399 | 2.93E-11 | 0.0765 | HAPLN1 | TSS1500 | shore | chr5:83017894-83018358 |
| cg16287373 | 6.85E-11 | 0.0723 | NRIP1 | 5'UTR | opensea | |
| cg00318109 | 6.89E-10 | 0.1041 | MYH7 | 5'UTR | opensea | |
| cg16406611 | 8.01E-09 | 0.0688 | IFITM5 | TSS1500 | shore | chr11:299389-299635 |
| cg19148731 | 9.59E-09 | 0.1054 | LOXL3 | 5'UTR | shore | chr2:74781494-74782685 |
| cg01347250 | 1.20E-08 | 0.0624 | S100A13 | TSS200 | opensea | |
| cg24443494 | 4.74E-08 | 0.0909 | CHST15 | 5'UTR | opensea | |
| cg07189587 | 3.11E-07 | 0.1279 | RHOJ | 5'UTR | opensea | |
| cg07157030 | 9.40E-07 | 0.1167 | RHOJ | 5'UTR | opensea | |
| cg18008345 | 1.89E-06 | 0.1153 | POLR2E | TSS1500 | shore | chr19:1094797-1095649 |
| cg08296601 | 3.01E-06 | 0.0777 | MRPL28 | 5'UTR | island | chr16:419860-420256 |
| cg22110428 | 4.01E-06 | 0.0655 | CEACAM18 | TSS1500 | opensea | |
| cg17422692 | 6.31E-06 | 0.0804 | MRPL28 | 5'UTR | island | chr16:419860-420256 |

The analysis was carried out through the identification of the Differentially Methylated Regions (DMRs), with the same comparisons among impact areas used for DMPs:

1. High impact area vs Low impact area;
2. Medium impact area vs High impact area;
3. Medium impact area vs Low impact area.

The DMRs identification was performed by means of two independent methods (Bumpunther and DMRcate, implemented on R software), then only the "consensus DMRs", reached out with both two methods, were selected: 392 consensus DMRs were identified for high impact vs low impact comparison, 1082 for medium vs high, 537 for medium vs low.

In order to understand the biological implication given by the differential methylation levels for each impact areas, a gene set enrichment analysis was performed for both DMPs and DMRs. This analysis consists of a Gene Ontology (GO) by "gometh" method and a Pathway Analysis by "KEGG" method (Adjusted P-value for GO was set at 0.001, for KEGG at 0.05).

Table 25 is an extract of the GO annotated terms, showing the most significative molecular functions come out from each impact area comparison for DMPs. Among the most enriched: "metal ion binding", "heterocyclic compound binding", and "organic cyclic compound binding" were counted.

Otherwise, in Table 26, among biological processes enriched in the GO, it is possible to find "nitrogen compound metabolic process", "organonitrogen compound metabolic process", "cell development" and " nervous system development".

Figure 29 a/b/c and 30 a/b/c graphically represents GO most enriched terms for each impact area comparison for molecular function and biological process, respectively.

Fig. 29d and Fig. 30d show the comparing bar plots of common terms and number of DMPs genes involved.

**Table 25.** Gene Ontology for DMPs and most significant Molecular Function (MF) reported for each impact area comparison (High vs Low; Medium vs Low; Medium vs High). DE=number of genes from DMPs annotated; FDR=statistical significance of the term.

| High vs Low | | | | |
|---|---|---|---|---|
| **TermID** | **ONTOLOGY** | **TERM** | **DE** | **FDR** |
| GO:0005515 | MF | protein binding | 11033 | 2.64E-115 |
| GO:0005488 | MF | binding | 12817 | 5.66E-94 |
| GO:0043167 | MF | ion binding | 5154 | 2.26E-46 |
| GO:0003824 | MF | catalytic activity | 4641 | 5.20E-46 |
| GO:0019899 | MF | enzyme binding | 1985 | 8.51E-39 |
| GO:1901363 | MF | heterocyclic compound binding | 4941 | 2.23E-36 |
| GO:0097159 | MF | organic cyclic compound binding | 5007 | 4.81E-36 |
| GO:0043168 | MF | anion binding | 2393 | 6.76E-29 |
| GO:1901265 | MF | nucleoside phosphate binding | 1826 | 3.75E-28 |
| GO:0000166 | MF | nucleotide binding | 1825 | 4.11E-28 |
| GO:0016740 | MF | transferase activity | 1886 | 1.20E-25 |
| GO:0043169 | MF | cation binding | 3503 | 2.06E-25 |
| GO:0046872 | MF | metal ion binding | 3439 | 2.69E-25 |
| GO:0017076 | MF | purine nucleotide binding | 1621 | 1.64E-24 |
| GO:0032555 | MF | purine ribonucleotide binding | 1610 | 1.64E-24 |
| GO:0032553 | MF | ribonucleotide binding | 1624 | 2.22E-24 |
| GO:0035639 | MF | purine ribonucleoside triphosphate binding | 1554 | 2.98E-24 |
| GO:0140096 | MF | catalytic activity, acting on a protein | 1828 | 6.86E-23 |
| **Medium vs Low** | | | | |
| **TermID** | **ONTOLOGY** | **TERM** | **DE** | **FDR** |
| GO:0005515 | MF | protein binding | 10657 | 1.15E-46 |
| GO:0005488 | MF | binding | 12442 | 3.31E-38 |
| GO:0043167 | MF | ion binding | 5000 | 1.84E-22 |
| GO:0003824 | MF | catalytic activity | 4482 | 3.14E-20 |
| GO:0043168 | MF | anion binding | 2331 | 2.32E-17 |
| GO:0008092 | MF | cytoskeletal protein binding | 852 | 2.34E-14 |
| GO:0019899 | MF | enzyme binding | 1891 | 2.36E-14 |
| GO:0036094 | MF | small molecule binding | 2073 | 2.63E-14 |
| GO:1901265 | MF | nucleoside phosphate binding | 1762 | 6.03E-14 |
| GO:0000166 | MF | nucleotide binding | 1761 | 6.42E-14 |
| GO:0005524 | MF | ATP binding | 1248 | 7.49E-14 |
| GO:0140096 | MF | catalytic activity, acting on a protein | 1780 | 7.49E-14 |
| GO:0097367 | MF | carbohydrate derivative binding | 1846 | 1.83E-13 |
| GO:0043169 | MF | cation binding | 3408 | 2.85E-13 |
| GO:0046872 | MF | metal ion binding | 3344 | 5.00E-13 |
| GO:0030554 | MF | adenyl nucleotide binding | 1302 | 5.50E-13 |
| GO:0032559 | MF | adenyl ribonucleotide binding | 1292 | 5.71E-13 |
| GO:0035639 | MF | purine ribonucleoside triphosphate binding | 1503 | 8.16E-13 |
| GO:0032555 | MF | purine ribonucleotide binding | 1555 | 2.01E-12 |

| Medium vs High | | | | |
|---|---|---|---|---|
| TermID | ONTOLOGY | TERM | DE | FDR |
| GO:0005488 | MF | binding | 14711 | 6.19E-213 |
| GO:0005515 | MF | protein binding | 12497 | 8.44E-215 |
| GO:0043167 | MF | ion binding | 5791 | 2.32E-69 |
| GO:0097159 | MF | organic cyclic compound binding | 5676 | 1.53E-42 |
| GO:1901363 | MF | heterocyclic compound binding | 5593 | 8.61E-41 |
| GO:0003824 | MF | catalytic activity | 5215 | 8.32E-73 |
| GO:0043169 | MF | cation binding | 3964 | 1.05E-41 |
| GO:0046872 | MF | metal ion binding | 3889 | 1.52E-40 |
| GO:0003676 | MF | nucleic acid binding | 3811 | 6.71E-13 |
| GO:0098772 | MF | molecular function regulator | 3444 | 8.37E-29 |
| GO:0043168 | MF | anion binding | 2640 | 1.09E-34 |
| GO:0036094 | MF | small molecule binding | 2361 | 5.05E-32 |
| GO:0003677 | MF | DNA binding | 2269 | 1.70E-13 |
| GO:0019899 | MF | enzyme binding | 2139 | 1.84E-29 |
| GO:0097367 | MF | carbohydrate derivative binding | 2091 | 3.39E-18 |
| GO:0016740 | MF | transferase activity | 2078 | 1.07E-28 |
| GO:0140096 | MF | catalytic activity, acting on a protein | 2021 | 3.26E-30 |
| GO:1901265 | MF | nucleoside phosphate binding | 1992 | 4.77E-28 |
| GO:0140110 | MF | transcription regulator activity | 1824 | 7.05E-13 |

**Table 26.** Gene Ontology for DMPs and most significant Biologic Process (BP) reported for each impact area comparison (High vs Low; Medium vs Low; Medium vs High). DE=number of genes from DMPs annotated; FDR=statistical significance of the term.

| High vs Low | | | | |
|---|---|---|---|---|
| TermID | ONTOLOGY | TERM | DE | FDR |
| GO:0006996 | BP | organelle organization | 3445 | 9.06E-61 |
| GO:0044237 | BP | cellular metabolic process | 8880 | 9.07E-57 |
| GO:0048522 | BP | positive regulation of cellular process | 4708 | 1.25E-52 |
| GO:0050794 | BP | regulation of cellular process | 8839 | 1.19E-50 |
| GO:1901576 | BP | organic substance biosynthetic process | 5051 | 2.44E-50 |
| GO:0044267 | BP | cellular protein metabolic process | 4400 | 3.95E-50 |
| GO:0043412 | BP | macromolecule modification | 3696 | 4.56E-50 |
| GO:0006464 | BP | cellular protein modification process | 3520 | 7.09E-50 |
| GO:0036211 | BP | protein modification process | 3520 | 7.09E-50 |
| GO:0031323 | BP | regulation of cellular metabolic process | 5195 | 7.92E-50 |
| GO:1901564 | BP | organonitrogen compound metabolic process | 5566 | 1.44E-49 |
| GO:0006807 | BP | nitrogen compound metabolic process | 8244 | 2.65E-48 |
| GO:0048518 | BP | positive regulation of biological process | 5133 | 1.60E-46 |
| GO:0019538 | BP | protein metabolic process | 4808 | 1.88E-46 |
| GO:0009059 | BP | macromolecule biosynthetic process | 4158 | 6.74E-46 |
| GO:0051171 | BP | regulation of nitrogen compound metabolic process | 4852 | 1.49E-45 |
| GO:0080090 | BP | regulation of primary metabolic process | 5006 | 3.42E-45 |
| GO:0034645 | BP | cellular macromolecule biosynthetic process | 4057 | 2.20E-44 |

| TermID | ONTOLOGY | TERM | DE | FDR |
|---|---|---|---|---|
| GO:0044085 | BP | cellular component biogenesis | 2738 | 1.06E-43 |
| GO:0007399 | BP | nervous system development | 2141 | 7.97E-43 |

**Medium vs Low**

| TermID | ONTOLOGY | TERM | DE | FDR |
|---|---|---|---|---|
| GO:0016043 | BP | cellular component organization | 5214 | 4.28E-33 |
| GO:0071840 | BP | cellular component organization or biogenesis | 5356 | 7.17E-32 |
| GO:0032502 | BP | developmental process | 5277 | 8.76E-24 |
| GO:0051179 | BP | localization | 5399 | 1.62E-23 |
| GO:1901564 | BP | organonitrogen compound metabolic process | 5389 | 1.84E-22 |
| GO:0048856 | BP | anatomical structure development | 4891 | 3.74E-22 |
| GO:0048518 | BP | positive regulation of biological process | 4970 | 5.64E-21 |
| GO:0051128 | BP | regulation of cellular component organization | 2045 | 7.35E-21 |
| GO:0048468 | BP | cell development | 1877 | 2.06E-20 |
| GO:0048523 | BP | negative regulation of cellular process | 4078 | 5.42E-20 |
| GO:0120036 | BP | plasma membrane bounded cell projection organization | 1334 | 1.09E-19 |
| GO:0048522 | BP | positive regulation of cellular process | 4524 | 1.09E-19 |
| GO:0044260 | BP | cellular macromolecule metabolic process | 6518 | 1.35E-19 |
| GO:0030030 | BP | cell projection organization | 1368 | 2.13E-19 |
| GO:0007275 | BP | multicellular organism development | 4495 | 2.79E-19 |
| GO:0019538 | BP | protein metabolic process | 4638 | 3.34E-19 |
| GO:0051234 | BP | establishment of localization | 4206 | 6.23E-19 |
| GO:0009653 | BP | anatomical structure morphogenesis | 2349 | 1.08E-18 |
| GO:0000902 | BP | cell morphogenesis | 916 | 2.16E-18 |
| GO:0007399 | BP | nervous system development | 2053 | 2.16E-18 |

**Medium vs High**

| TermID | ONTOLOGY | TERM | DE | FDR |
|---|---|---|---|---|
| GO:0071840 | BP | cellular component organization or biogenesis | 6170 | 7.01E-100 |
| GO:0016043 | BP | cellular component organization | 5989 | 9.49E-97 |
| GO:0009987 | BP | cellular process | 15580 | 5.87E-96 |
| GO:0044260 | BP | cellular macromolecule metabolic process | 7660 | 1.26E-88 |
| GO:0050794 | BP | regulation of cellular process | 10131 | 4.10E-81 |
| GO:1901564 | BP | organonitrogen compound metabolic process | 6287 | 2.87E-75 |
| GO:0065007 | BP | biological regulation | 11442 | 2.47E-69 |
| GO:0044237 | BP | cellular metabolic process | 10151 | 3.15E-69 |
| GO:0051179 | BP | localization | 6254 | 6.77E-68 |
| GO:0032502 | BP | developmental process | 6111 | 2.24E-65 |
| GO:0044238 | BP | primary metabolic process | 9898 | 3.69E-64 |
| GO:0048518 | BP | positive regulation of biological process | 5756 | 7.13E-61 |
| GO:0006807 | BP | nitrogen compound metabolic process | 9441 | 1.22E-60 |
| GO:0050789 | BP | regulation of biological process | 10843 | 6.15E-60 |
| GO:0048522 | BP | positive regulation of cellular process | 5232 | 3.44E-59 |
| GO:0019538 | BP | protein metabolic process | 5402 | 8.02E-59 |
| GO:0048856 | BP | anatomical structure development | 5657 | 1.97E-58 |
| GO:1901576 | BP | organic substance biosynthetic process | 5657 | 1.06E-55 |
| GO:0009058 | BP | biosynthetic process | 5734 | 1.87E-55 |
| GO:0006996 | BP | organelle organization | 3758 | 2.36E-54 |

**Figure 29.** GO most enriched terms for Molecular Function in (a) high vs low area, (b) medium vs low area, (c) medium vs high area. On the x the number of genes from DMPs annotated. (d) Common GO terms and number of DMPs annotated genes for each area comparison.

**Figure 30.** GO most enriched terms for Biological Process in (a) low vs high areas, (b) medium vs low area, (c) medium vs high area. On the x the number of genes from DMPs annotated. (d) Common GO terms and number of DMPs annotated genes for each area comparison.



a.

High vs Low

| Term | Value |
|---|---|
| cellular metabolic process | 8880 |
| regulation of cellular process | 8839 |
| nitrogen compound metabolic process | 8244 |
| organonitrogen compound metabolic process | 5566 |
| regulation of cellular metabolic process | 5195 |
| positive regulation of biological process | 5133 |
| organic substance biosynthetic process | 5051 |
| regulation of primary metabolic process | 5006 |
| regulation of nitrogen compound metabolic process | 4852 |
| protein metabolic process | 4808 |
| positive regulation of cellular process | 4708 |
| cellular protein metabolic process | 4400 |
| macromolecule biosynthetic process | 4158 |
| cellular macromolecule biosynthetic process | 4057 |
| macromolecule modification | 3696 |
| protein modification process | 3520 |
| cellular protein modification process | 3520 |
| organelle organization | 3445 |
| cellular component biogenesis | 2738 |
| nervous system development | 2141 |

b.

Medium vs Low

| Term | Value |
|---|---|
| cellular macromolecule metabolic process | 6518 |
| localization | 5399 |
| organonitrogen compound metabolic process | 5389 |
| cellular component organization or biogenesis | 5356 |
| developmental process | 5277 |
| cellular component organization | 5214 |
| positive regulation of biological process | 4970 |
| anatomical structure development | 4891 |
| protein metabolic process | 4638 |
| positive regulation of cellular process | 4524 |
| multicellular organism development | 4495 |
| establishment of localization | 4206 |
| negative regulation of cellular process | 4078 |
| anatomical structure morphogenesis | 2349 |
| nervous system development | 2053 |
| regulation of cellular component organization | 2045 |
| cell development | 1877 |
| cell projection organization | 1368 |
| plasma membrane bounded cell projection… | 1334 |
| cell morphogenesis | 916 |

**Figure 30. (**Continuing from previous page).

c.

Medium vs High

| Term | Value |
|---|---|
| cellular process | 15580 |
| biological regulation | 11442 |

Tables 27 a/b/c report the KEGG pathway analysis on DMPs, performed for each impact area comparison.

Noteworthy, it is possible to underline for high and medium impact area vs low one the enrichment of gene-set involved in metabolic and cancer pathways, transduction signaling and inflammation pathways, as well as small and non-small cell lung cancer, pancreatic and prostate cancer, cell cycle and p53 signaling pathways. For all conditions breast cancer and gastric cancer pathways are enriched. Interestingly, for high vs low and for medium vs high are counted some neurodegenerative disorders pathways (e.g. Alzheimer and Parkinson diseases), as well as a series of infectious disease pathways. Medium vs low comparison shows, in addition, enrichment for estrogenic signaling and thyroid hormone pathways, as well as endocrine resistance one.

Considering the DMRs consensus, the GO and the KEGG analysis show significant gene-set enrichment evidence only for medium vs high and medium vs low impact areas comparison (Table 28). All terms reported involve gene silencing processes and post-transcriptional regulation.

The "neuroactive ligand receptor interaction" is the only pathway that resulted enriched in medium vs high comparison by KEGG analysis on DMRs consensus (Table 29).

**Table 27a.** KEGG for DMPs reported for High vs Low impact area comparison. DE=number of genes from DMPs annotated; FDR=statistical significance of the pathway.

| High vs Low | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| TermID | Description | DE | FDR | TermID | Description | DE | FDR |
| path:hsa01100 | Metabolic pathways | 1206 | 1.56E-06 | path:hsa03040 | Spliceosome | 116 | 4.46E-03 |
| path:hsa05200 | Pathways in cancer | 468 | 1.29E-10 | path:hsa04110 | Cell cycle | 115 | 2.11E-04 |
| path:hsa05022 | Pathways of neurodegeneration - multiple diseases | 408 | 2.19E-07 | path:hsa04926 | Relaxin signaling pathway | 112 | 3.13E-02 |
| path:hsa05010 | Alzheimer disease | 313 | 6.13E-06 | path:hsa04071 | Sphingolipid signaling pathway | 111 | 1.17E-03 |
| path:hsa05014 | Amyotrophic lateral sclerosis | 308 | 4.46E-06 | path:hsa04611 | Platelet activation | 111 | 3.13E-02 |
| path:hsa04151 | PI3K-Akt signaling pathway | 302 | 2.78E-05 | path:hsa04142 | Lysosome | 110 | 3.71E-02 |
| path:hsa05165 | Human papillomavirus infection | 293 | 2.99E-06 | path:hsa04722 | Neurotrophin signaling pathway | 108 | 8.57E-04 |
| path:hsa04010 | MAPK signaling pathway | 262 | 6.11E-06 | path:hsa04919 | Thyroid hormone signaling pathway | 108 | 1.19E-02 |
| path:hsa05016 | Huntington disease | 255 | 2.89E-04 | path:hsa03010 | Ribosome | 108 | 4.50E-02 |
| path:hsa04144 | Endocytosis | 232 | 8.93E-09 | path:hsa04935 | Growth hormone synthesis, secretion and action | 105 | 1.33E-02 |
| path:hsa05020 | Prion disease | 222 | 2.53E-03 | path:hsa04725 | Cholinergic synapse | 103 | 1.19E-02 |
| path:hsa05132 | Salmonella infection | 217 | 2.13E-04 | path:hsa04114 | Oocyte meiosis | 103 | 4.96E-02 |
| path:hsa05131 | Shigellosis | 215 | 8.19E-05 | path:hsa04668 | TNF signaling pathway | 98 | 2.91E-02 |
| path:hsa04014 | Ras signaling pathway | 203 | 8.57E-04 | path:hsa04066 | HIF-1 signaling pathway | 97 | 9.79E-03 |
| path:hsa05012 | Parkinson disease | 202 | 2.78E-03 | path:hsa04916 | Melanogenesis | 92 | 2.82E-02 |
| path:hsa04810 | Regulation of actin cytoskeleton | 197 | 1.94E-06 | path:hsa01522 | Endocrine resistance | 90 | 2.78E-03 |
| path:hsa04015 | Rap1 signaling pathway | 196 | 6.13E-06 | path:hsa04713 | Circadian entrainment | 90 | 5.77E-03 |
| path:hsa05205 | Proteoglycans in cancer | 188 | 7.34E-06 | path:hsa04750 | Inflammatory mediator regulation of TRP channels | 90 | 1.38E-02 |
| path:hsa05163 | Human cytomegalovirus infection | 187 | 1.41E-02 | path:hsa04660 | T cell receptor signaling pathway | 90 | 3.08E-02 |
| path:hsa05166 | Human T-cell leukemia virus 1 infection | 184 | 3.29E-02 | path:hsa04070 | Phosphatidylinositol signaling system | 89 | 3.28E-04 |
| path:hsa04510 | Focal adhesion | 183 | 1.50E-05 | path:hsa05215 | Prostate cancer | 89 | 2.78E-03 |
| path:hsa04024 | cAMP signaling pathway | 183 | 4.34E-02 | path:hsa04933 | AGE-RAGE signaling pathway in diabetic complications | 88 | 1.38E-02 |
| path:hsa04020 | Calcium signaling pathway | 176 | 8.57E-04 | path:hsa05231 | Choline metabolism in cancer | 88 | 1.75E-02 |
| path:hsa05130 | Pathogenic Escherichia coli infection | 167 | 1.22E-02 | path:hsa05142 | Chagas disease | 88 | 3.75E-02 |

| path:hsa05169 | Epstein-Barr virus infection | 166 | 1.91E-02 | path:hsa04350 | TGF-beta signaling pathway | 87 | 1.11E-02 |
|---|---|---|---|---|---|---|---|
| path:hsa05203 | Viral carcinogenesis | 164 | 4.51E-03 | path:hsa04925 | Aldosterone synthesis and secretion | 86 | 3.08E-02 |
| path:hsa04360 | Axon guidance | 163 | 3.38E-04 | path:hsa05222 | Small cell lung cancer | 84 | 2.06E-03 |
| path:hsa05167 | Kaposi sarcoma-associated herpesvirus infection | 161 | 3.66E-02 | path:hsa04666 | Fc gamma R-mediated phagocytosis | 84 | 2.16E-02 |
| path:hsa04141 | Protein processing in endoplasmic reticulum | 155 | 1.50E-05 | path:hsa05032 | Morphine addiction | 82 | 1.24E-02 |
| path:hsa05225 | Hepatocellular carcinoma | 154 | 2.99E-06 | path:hsa05210 | Colorectal cancer | 81 | 8.57E-04 |
| path:hsa04022 | cGMP-PKG signaling pathway | 151 | 2.11E-04 | path:hsa04914 | Progesterone-mediated oocyte maturation | 81 | 1.09E-02 |
| path:hsa04390 | Hippo signaling pathway | 148 | 1.10E-05 | path:hsa04512 | ECM-receptor interaction | 80 | 1.81E-02 |
| path:hsa04310 | Wnt signaling pathway | 145 | 4.43E-04 | path:hsa05235 | PD-L1 expression and PD-1 checkpoint pathway in cancer | 80 | 3.76E-02 |
| path:hsa04934 | Cushing syndrome | 144 | 2.13E-04 | path:hsa04540 | Gap junction | 79 | 3.50E-02 |
| path:hsa04218 | Cellular senescence | 143 | 3.33E-04 | path:hsa03015 | mRNA surveillance pathway | 78 | 1.09E-02 |
| path:hsa05224 | Breast cancer | 139 | 1.80E-05 | path:hsa04727 | GABAergic synapse | 78 | 1.63E-02 |
| path:hsa05226 | Gastric cancer | 139 | 1.46E-04 | path:hsa04012 | ErbB signaling pathway | 75 | 1.75E-02 |
| path:hsa04921 | Oxytocin signaling pathway | 139 | 7.40E-03 | path:hsa05100 | Bacterial invasion of epithelial cells | 74 | 7.33E-04 |
| path:hsa04150 | mTOR signaling pathway | 138 | 3.34E-03 | path:hsa01521 | EGFR tyrosine kinase inhibitor resistance | 73 | 1.12E-02 |
| path:hsa04072 | Phospholipase D signaling pathway | 136 | 3.06E-04 | path:hsa04721 | Synaptic vesicle cycle | 73 | 1.41E-02 |
| path:hsa03013 | RNA transport | 135 | 6.39E-04 | path:hsa05214 | Glioma | 72 | 8.57E-04 |
| path:hsa05161 | Hepatitis B | 134 | 1.63E-02 | path:hsa05220 | Chronic myeloid leukemia | 72 | 8.57E-04 |
| path:hsa05017 | Spinocerebellar ataxia | 133 | 3.95E-05 | path:hsa04115 | p53 signaling pathway | 71 | 7.33E-04 |
| path:hsa04550 | Signaling pathways regulating pluripotency of stem cells | 132 | 9.99E-05 | path:hsa05223 | Non-small cell lung cancer | 70 | 3.28E-04 |
| path:hsa04261 | Adrenergic signaling in cardiomyocytes | 131 | 1.25E-02 | path:hsa05212 | Pancreatic cancer | 70 | 4.41E-03 |
| path:hsa04120 | Ubiquitin mediated proteolysis | 125 | 1.73E-04 | path:hsa05412 | Arrhythmogenic right ventricular cardiomyopathy | 70 | 1.38E-02 |
| path:hsa04140 | Autophagy - animal | 124 | 1.30E-04 | path:hsa00562 | Inositol phosphate metabolism | 68 | 3.45E-04 |
| path:hsa04723 | Retrograde endocannabinoid signaling | 122 | 8.71E-03 | path:hsa05218 | Melanoma | 67 | 4.51E-03 |
| path:hsa05418 | Fluid shear stress and atherosclerosis | 121 | 9.64E-04 | path:hsa04520 | Adherens junction | 67 | 7.00E-03 |
| path:hsa04728 | Dopaminergic synapse | 120 | 8.57E-04 | path:hsa05211 | Renal cell carcinoma | 62 | 1.19E-02 |
| path:hsa05135 | Yersinia infection | 120 | 4.41E-03 | path:hsa05217 | Basal cell carcinoma | 61 | 3.44E-03 |
| path:hsa04210 | Apoptosis | 119 | 1.41E-02 | path:hsa01524 | Platinum drug resistance | 61 | 1.28E-02 |

| TermID | Description | DE | FDR | TermID | Description | DE | FDR |
|---|---|---|---|---|---|---|---|
| path:hsa04270 | Vascular smooth muscle contraction | 118 | 2.32E-02 | path:hsa05031 | Amphetamine addiction | 61 | 4.34E-02 |
| path:hsa04910 | Insulin signaling pathway | 118 | 2.72E-02 | path:hsa05213 | Endometrial cancer | 55 | 2.50E-03 |
| path:hsa04068 | FoxO signaling pathway | 117 | 4.51E-03 | | | | |

**Table 27b.** KEGG for DMPs reported for Medium vs Low impact area comparison. DE=number of genes from DMPs annotated; FDR=statistical significance of the pathway.

| Medium vs Low | | | | | | | |
|---|---|---|---|---|---|---|---|
| TermID | Description | DE | FDR | TermID | Description | DE | FDR |
| path:hsa01100 | Metabolic pathways | 1169 | 4.18E-03 | path:hsa04916 | Melanogenesis | 93 | 1.45E-02 |
| path:hsa05200 | Pathways in cancer | 443 | 7.15E-04 | path:hsa04625 | C-type lectin receptor signaling pathway | 93 | 2.40E-02 |
| path:hsa04151 | PI3K-Akt signaling pathway | 292 | 7.31E-03 | path:hsa05231 | Choline metabolism in cancer | 92 | 6.00E-04 |
| path:hsa04010 | MAPK signaling pathway | 259 | 1.21E-04 | path:hsa01522 | Endocrine resistance | 91 | 1.35E-03 |
| path:hsa04014 | Ras signaling pathway | 202 | 1.81E-03 | path:hsa04660 | T cell receptor signaling pathway | 90 | 3.25E-02 |
| path:hsa04015 | Rap1 signaling pathway | 194 | 1.21E-04 | path:hsa05146 | Amoebiasis | 88 | 2.19E-02 |
| path:hsa04810 | Regulation of actin cytoskeleton | 191 | 5.71E-04 | path:hsa04713 | Circadian entrainment | 88 | 3.14E-02 |
| path:hsa05166 | Human T-cell leukemia virus 1 infection | 184 | 2.82E-02 | path:hsa04925 | Aldosterone synthesis and secretion | 87 | 1.69E-02 |
| path:hsa04714 | Thermogenesis | 183 | 3.42E-02 | path:hsa05414 | Dilated cardiomyopathy | 86 | 1.12E-02 |
| path:hsa05205 | Proteoglycans in cancer | 180 | 5.59E-03 | path:hsa04070 | Phosphatidylinositol signaling system | 85 | 2.19E-02 |
| path:hsa04510 | Focal adhesion | 179 | 7.77E-04 | path:hsa05410 | Hypertrophic cardiomyopathy | 83 | 3.13E-03 |
| path:hsa04360 | Axon guidance | 164 | 4.65E-04 | path:hsa04512 | ECM-receptor interaction | 82 | 4.18E-03 |
| path:hsa05225 | Hepatocellular carcinoma | 148 | 1.35E-03 | path:hsa04914 | Progesterone-mediated oocyte maturation | 79 | 4.61E-02 |
| path:hsa04310 | Wnt signaling pathway | 146 | 4.65E-04 | path:hsa04012 | ErbB signaling pathway | 77 | 3.27E-03 |
| path:hsa04921 | Oxytocin signaling pathway | 139 | 7.51E-03 | path:hsa04911 | Insulin secretion | 76 | 4.61E-02 |
| path:hsa04934 | Cushing syndrome | 139 | 1.03E-02 | path:hsa05412 | Arrhythmogenic right ventricular cardiomyopathy | 73 | 6.00E-04 |
| path:hsa04072 | Phospholipase D signaling pathway | 135 | 1.19E-03 | path:hsa01521 | EGFR tyrosine kinase inhibitor resistance | 73 | 1.17E-02 |
| path:hsa05224 | Breast cancer | 134 | 3.13E-03 | path:hsa04971 | Gastric acid secretion | 71 | 1.12E-02 |

| path:hsa05226 | Gastric cancer | 134 | 8.78E-03 | path:hsa05212 | Pancreatic cancer | 70 | 5.75E-03 |
|---|---|---|---|---|---|---|---|
| path:hsa04261 | Adrenergic signaling in cardiomyocytes | 129 | 3.42E-02 | path:hsa05214 | Glioma | 70 | 1.17E-02 |
| path:hsa04915 | Estrogen signaling pathway | 122 | 1.17E-02 | path:hsa05220 | Chronic myeloid leukemia | 70 | 1.17E-02 |
| path:hsa04371 | Apelin signaling pathway | 121 | 4.09E-02 | path:hsa05100 | Bacterial invasion of epithelial cells | 70 | 4.84E-02 |
| path:hsa05135 | Yersinia infection | 118 | 1.58E-02 | path:hsa04520 | Adherens junction | 69 | 7.15E-04 |
| path:hsa04140 | Autophagy - animal | 117 | 3.25E-02 | path:hsa05223 | Non-small cell lung cancer | 67 | 1.92E-02 |
| path:hsa04270 | Vascular smooth muscle contraction | 117 | 3.69E-02 | path:hsa00562 | Inositol phosphate metabolism | 65 | 2.04E-02 |
| path:hsa04068 | FoxO signaling pathway | 116 | 9.90E-03 | path:hsa05218 | Melanoma | 65 | 3.32E-02 |
| path:hsa05418 | Fluid shear stress and atherosclerosis | 116 | 3.25E-02 | path:hsa04664 | Fc epsilon RI signaling pathway | 64 | 2.72E-03 |
| path:hsa04926 | Relaxin signaling pathway | 113 | 1.87E-02 | path:hsa04929 | GnRH secretion | 61 | 1.35E-03 |
| path:hsa04071 | Sphingolipid signaling pathway | 112 | 7.15E-04 | path:hsa04137 | Mitophagy - animal | 61 | 1.92E-02 |
| path:hsa04919 | Thyroid hormone signaling pathway | 109 | 6.67E-03 | path:hsa05211 | Renal cell carcinoma | 61 | 3.25E-02 |
| path:hsa04152 | AMPK signaling pathway | 108 | 1.00E-02 | path:hsa05221 | Acute myeloid leukemia | 59 | 3.37E-02 |
| path:hsa04724 | Glutamatergic synapse | 107 | 1.51E-03 | path:hsa04370 | VEGF signaling pathway | 57 | 7.48E-03 |
| path:hsa04935 | Growth hormone synthesis, secretion and action | 106 | 7.48E-03 | path:hsa04730 | Long-term depression | 56 | 1.00E-02 |
| path:hsa04722 | Neurotrophin signaling pathway | 105 | 1.17E-02 | path:hsa05213 | Endometrial cancer | 54 | 1.17E-02 |
| path:hsa04725 | Cholinergic synapse | 104 | 7.31E-03 | path:hsa04340 | Hedgehog signaling pathway | 48 | 2.79E-02 |
| path:hsa04066 | HIF-1 signaling pathway | 97 | 1.00E-02 | path:hsa04960 | Aldosterone-regulated sodium reabsorption | 36 | 8.78E-03 |

**Table 27c.** KEGG for DMPs reported for Medium vs High impact area comparison. DE=number of genes from DMPs annotated; FDR=statistical significance of the pathway.

| Medium vs High | | | | | | | |
|---|---|---|---|---|---|---|---|
| **TermID** | **Description** | **DE** | **FDR** | **TermID** | **Description** | **DE** | **FDR** |
| path:hsa01100 | Metabolic pathways | 1386 | 2.13E-13 | path:hsa05225 | Hepatocellular carcinoma | 159 | 1.95E-02 |
| path:hsa05200 | Pathways in cancer | 497 | 1.58E-04 | path:hsa04310 | Wnt signaling pathway | 155 | 5.65E-03 |
| path:hsa05022 | Pathways of neurodegeneration - multiple diseases | 445 | 6.62E-06 | path:hsa04390 | Hippo signaling pathway | 154 | 6.15E-03 |
| path:hsa05010 | Alzheimer disease | 343 | 9.29E-05 | path:hsa04934 | Cushing syndrome | 152 | 2.42E-02 |
| path:hsa05014 | Amyotrophic lateral sclerosis | 337 | 4.18E-04 | path:hsa04921 | Oxytocin signaling pathway | 151 | 2.42E-02 |
| path:hsa05165 | Human papillomavirus infection | 312 | 1.73E-02 | path:hsa04150 | mTOR signaling pathway | 149 | 2.42E-02 |
| path:hsa04080 | Neuroactive ligand-receptor interaction | 310 | 2.42E-02 | path:hsa05226 | Gastric cancer | 147 | 6.87E-03 |
| path:hsa05016 | Huntington disease | 281 | 6.87E-03 | path:hsa05224 | Breast cancer | 145 | 6.87E-03 |
| path:hsa04010 | MAPK signaling pathway | 280 | 9.75E-04 | path:hsa04072 | Phospholipase D signaling pathway | 143 | 3.06E-02 |
| path:hsa05020 | Prion disease | 248 | 1.95E-02 | path:hsa05017 | Spinocerebellar ataxia | 139 | 9.06E-03 |
| path:hsa04144 | Endocytosis | 240 | 4.85E-03 | path:hsa04550 | Signaling pathways regulating pluripotency of stem cells | 138 | 3.53E-02 |
| path:hsa05132 | Salmonella infection | 235 | 2.42E-02 | path:hsa04723 | Retrograde endocannabinoid signaling | 133 | 4.26E-02 |
| path:hsa05131 | Shigellosis | 231 | 3.06E-02 | path:hsa04210 | Apoptosis | 132 | 1.09E-02 |
| path:hsa05012 | Parkinson disease | 225 | 1.57E-02 | path:hsa04270 | Vascular smooth muscle contraction | 131 | 1.19E-02 |
| path:hsa04014 | Ras signaling pathway | 221 | 6.87E-03 | path:hsa04120 | Ubiquitin mediated proteolysis | 131 | 4.53E-02 |
| path:hsa04714 | Thermogenesis | 209 | 2.42E-02 | path:hsa04140 | Autophagy - animal | 129 | 4.89E-02 |
| path:hsa04015 | Rap1 signaling pathway | 207 | 6.19E-04 | path:hsa04071 | Sphingolipid signaling pathway | 118 | 1.95E-02 |
| path:hsa04810 | Regulation of actin cytoskeleton | 207 | 6.19E-04 | path:hsa01200 | Carbon metabolism | 108 | 2.42E-02 |
| path:hsa04024 | cAMP signaling pathway | 206 | 2.42E-02 | path:hsa04066 | HIF-1 signaling pathway | 105 | 2.85E-02 |
| path:hsa05205 | Proteoglycans in cancer | 198 | 5.65E-03 | path:hsa01522 | Endocrine resistance | 95 | 4.34E-02 |
| path:hsa04510 | Focal adhesion | 192 | 6.87E-03 | path:hsa05231 | Choline metabolism in cancer | 95 | 4.45E-02 |
| path:hsa04020 | Calcium signaling pathway | 189 | 2.34E-02 | path:hsa04350 | TGF-beta signaling pathway | 94 | 4.45E-02 |
| path:hsa04360 | Axon guidance | 174 | 2.94E-03 | path:hsa00564 | Glycerophospholipid metabolism | 93 | 4.53E-02 |
| path:hsa04141 | Protein processing in endoplasmic reticulum | 163 | 1.73E-02 | | | | |

**Table 28.** Gene Ontology for DMRs consensus, reported for the enriched comparison (Medium vs High and Medium vs Low). DE=number of genes from DMRs annotated; FDR=statistical significance of the term.

| Medium vs High | | | | |
|---|---|---|---|---|
| **TermID** | **ONTOLOGY** | **TERM** | **DE** | **FDR** |
| GO:0016458 | BP | gene silencing | 56 | 1.73E-05 |
| GO:0031047 | BP | gene silencing by RNA | 54 | 1.14E-06 |
| GO:0016441 | BP | posttranscriptional gene silencing | 50 | 1.86E-06 |
| GO:0035194 | BP | post-transcriptional gene silencing by RNA | 50 | 1.86E-06 |
| GO:0035195 | BP | gene silencing by miRNA | 49 | 1.86E-06 |
| GO:0150100 | MF | RNA binding involved in posttranscriptional gene silencing | 21 | 4.24E-04 |
| GO:1903231 | MF | mRNA binding involved in posttranscriptional gene silencing | 21 | 4.24E-04 |
| GO:0008191 | MF | metalloendopeptidase inhibitor activity | 7 | 2.89E-02 |
| **Medium vs Low** | | | | |
| **TermID** | **ONTOLOGY** | **TERM** | **DE** | **FDR** |
| GO:0007156 | BP | homophilic cell adhesion via plasma membrane adhesion molecules | 21 | 5.64E-04 |
| GO:0098742 | BP | cell-cell adhesion via plasma-membrane adhesion molecules | 25 | 1.19E-02 |

**Table 29.** KEGG for DMRs consensus reported for Medium vs High impact area comparison. DE=number of genes from DMRs annotated; FDR=statistical significance of the pathway.

| Medium vs High | | | |
|---|---|---|---|
| **TermID** | **Description** | **DE** | **FDR** |
| path:hsa04080 | Neuroactive ligand-receptor interaction | 38 | 9.54E-04 |

### 4.4.3 Regression analysis for methylation and exposure biomarkers correlation

In order to highlight a potential correlation between methylation levels and exposure biomarkers evaluated in the subgroup of 600 volunteers, data analysis proceeded through a "Robust Linear Multiple Regression Model", by M-value transformation from beta values, considering the following predictive parameters:

- exposure biomarkers;
- clusters;
- gender;
- age;
- BMI;
- Lymphocytes;
- Neutrophils.

Among the 19 heavy metals assessed as biomarkers of exposure, those showing a statistical significance in correlation to one of the cluster or to one of the impact areas in their independent data analysis were selected (Mercury – Hg202, Arsenic – As75, Cadmium – Cd111, Thallium – Tl205 (µg/L)).

For dioxins and dioxin-like compounds the sum of PCDD/F+DL-PCB (pg WHO-TEQ/g lipids) was considered, as an explorative example of cumulative effect of more congeners. For Calux gene reporter, all the three assays were included in the regression analysis.

The robust linear multiple regression model, for computing power reasons, was performed on the most variable 5000 differentially methylated probes.

Heatmaps below show the results of the regression model as estimate association between each probe and each exposure biomarker selected, represented by position on gene (Fig. 31a) and by relation to CpG features (Fig. 32a). The same is represented also with the indication of the statistical significance level (Adjusted P-value) (Fig. 31b and 32b).

The model explains how the methylation is affected by exposure biomarkers, given the contribute of the other factors selected as predictive parameters.

Estimate > 0 means that, with the increase of the measured biomarker, there is an increase in the methylation for the probe observed. Conversely, if estimate < 0, the increase of the biomarker causes a decrease in the methylation level for the probe observed.

The Adjusted P-value gives support in understanding if the contribute of the exposure biomarker on the methylation level for each probe has a statistical significance.

No particular patterns of hyper- or hypo-methylation are shown in the heatmaps, nor considering the link with gene position, neither the relation to the CpG features.
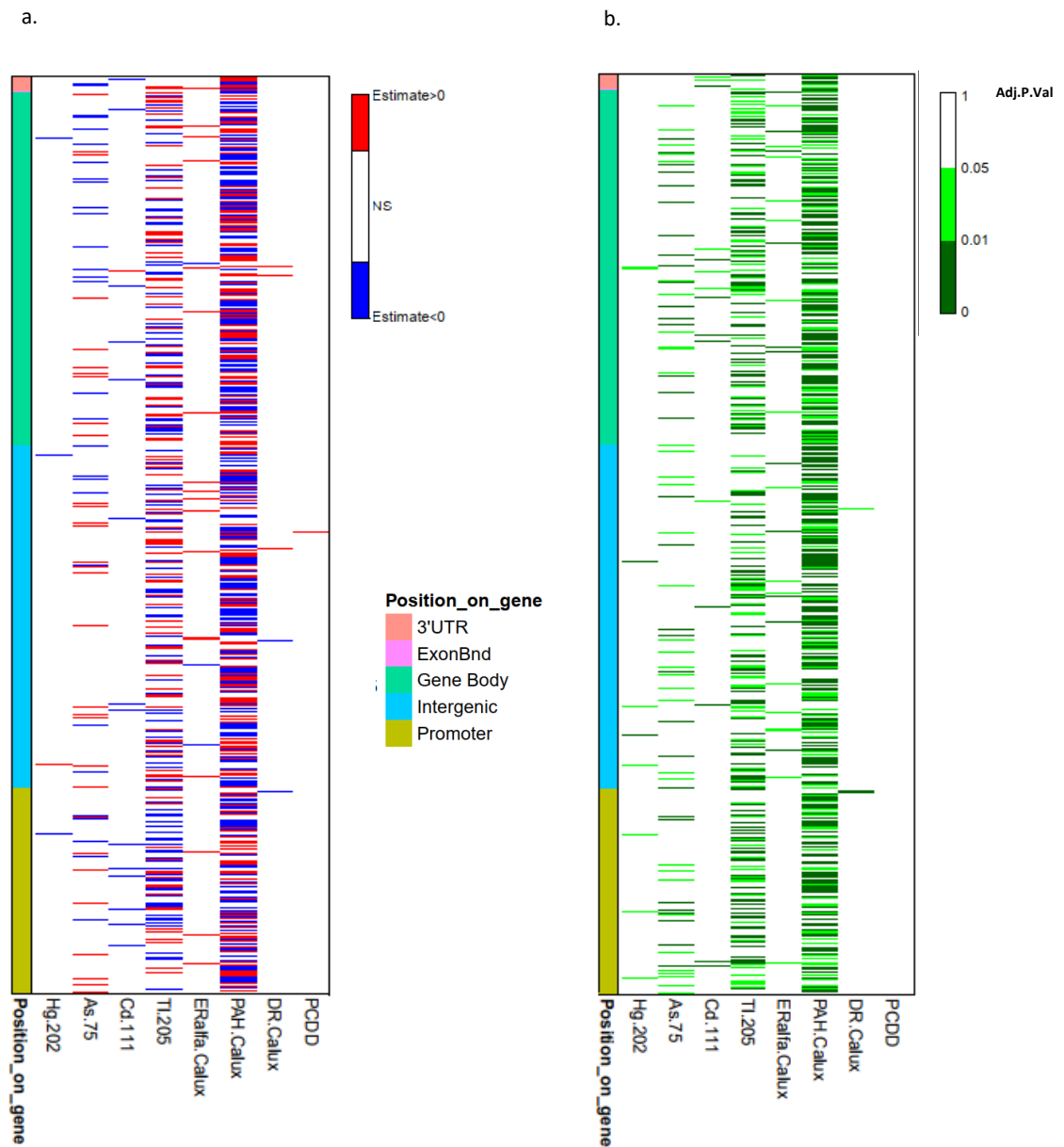
**Figure 31.** Heatmaps of robust linear multiple regression model linked to probe position on gene. (a) Estimate association between each probe and biomarkers of exposure. (b) Adjusted p-value as indicator of statistical significance for the correlation between the probe and the biomarkers of exposure.

Hg.202 = Mercury (µg/L); As.75 = Arsenic (µg/L); Cd.111 = Cadmium (µg/L); Tl.205 = Thallium (µg/L); ERalfa.Calux = Erα Calux assay (pg-EEQ/ml) ; PAH.Calux = PAH Calux assay (ngBaP-EQ/ml); DR.Calux = DR Calux assay (pg BEQ/g fats); PCDD = sum of PCDD/F+DL-PCB (pg WHO-TEQ/g fats).
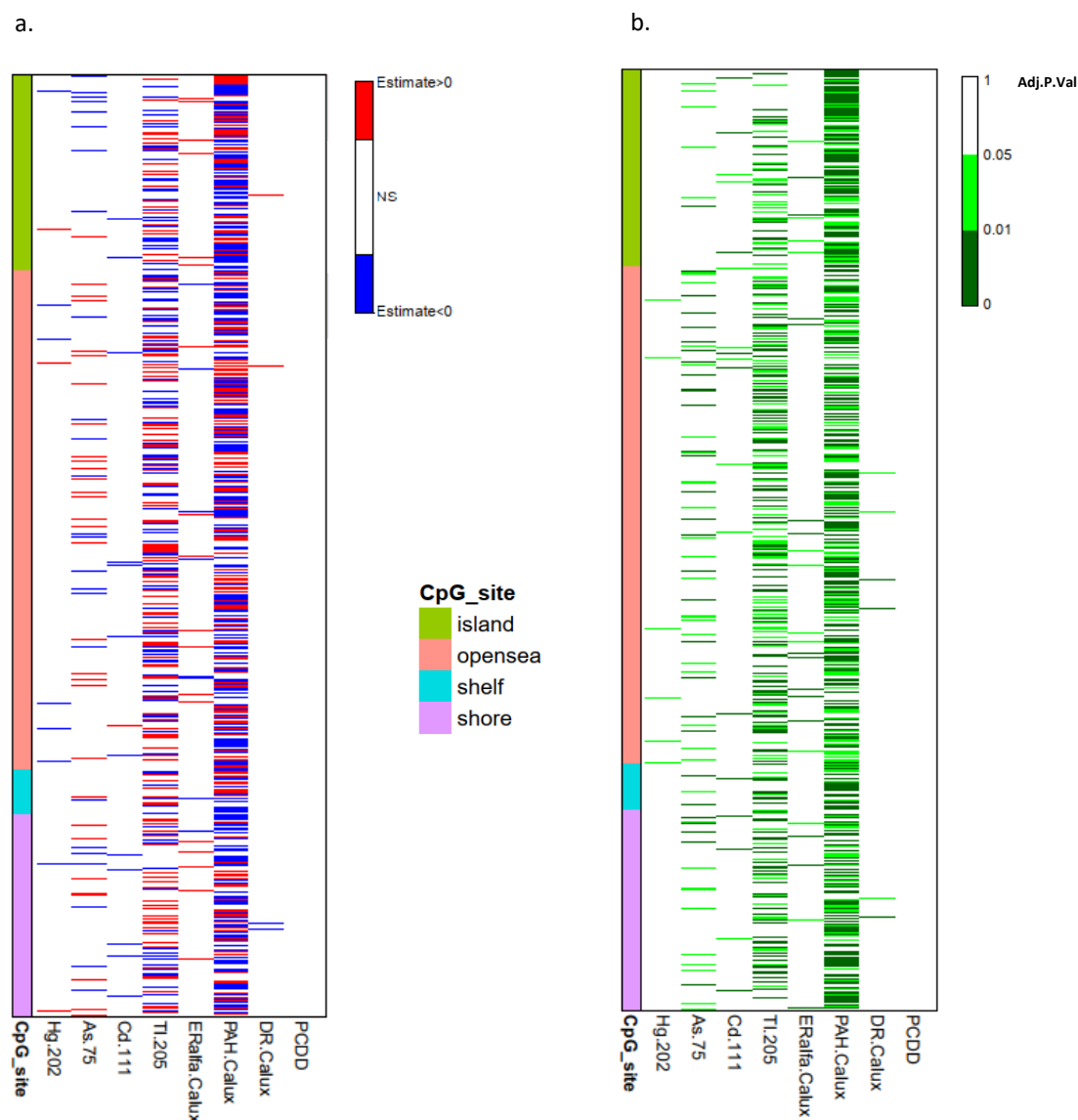
**Figure 32.** Heatmaps of robust linear multiple regression model linked to CpG features. (a) Estimate association between each probe and biomarkers of exposure. (b) Adjusted p-value as indicator of statistical significance for the correlation between the probe and the biomarker of exposure.

Hg.202 = Mercury (µg/L); As.75 = Arsenic (µg/L); Cd.111 = Cadmium (µg/L); Tl.205 = Thallium (µg/L); ERalfa.Calux = Erα Calux assay (pg-EEQ/ml) ; PAH.Calux = PAH Calux assay (ngBaP-EQ/ml); DR.Calux = DR Calux assay (pg BEQ/g fats); PCDD = sum of PCDD/F+DL-PCB (pg WHO-TEQ/g fats).

In the regression model for the mercury (Hg202), the contribute of the metal to explain the change in methylation is significant for the probes showed in table 30. The focus is on those located on promoter regions, linked to CpG islands and shores.

As reported in 2016 by Martin and Fry, the ATHL1 gene shows a mercury-associated hypomethylation for probes located on CpG shore-promoter region. Likewise, probes mapping on RUNX1 gene (leukemia linked gene) and RASGEF1C show mercury-associated hypomethylation on promoter proximal region. RASGEF1C was linked to differentially methylated site in Alzheimer's disease by Li et al. in 2020. SLC43A2 gene, associated to the transport of aminoacids, shows mercury-associated hypermethylation in promoter region, that means transcriptional repression.

**Table 30.** Top significant probes in robust linear regression model for Mercury (Hg202). Metal.Estimate > 0 means that with the increase of the measured biomarker, there is an increase in the methylation for the probe observed. Metal.Pr expresses the statistical significance $\leq 0.05$.

| CpG | gene | feature | cgi | UCSC_Islands_0.00me | metal.Estimate | metal.Pr |
|-----|------|---------|-----|---------------------|----------------|----------|
| cg26423139 | SLC43A2 | TSS200 | shore | chr17:1509882-1510101 | 2.05E+14 | 0.0370 |
| cg15747436 | ATHL1 | TSS1500 | shore | chr11:289771-290010 | -1.39E+14 | 0.0190 |
| cg08829299 | ATHL1 | TSS1500 | shore | chr11:288847-289239 | -2.31E+14 | 0.0510 |
| cg18727742 | ATHL1 | TSS1500 | shore | chr11:288847-289239 | -2.67E+14 | 0.0330 |
| cg12955789 | RASGEF1C | 5'UTR | island | chr5:179588400-179588773 | -1.13E+14 | 0.0500 |
| cg06758350 | RUNX1 | 5'UTR | island | chr21:36258952-36259472 | -1.20E+14 | 0.0500 |
| cg12477880 | RUNX1 | 1stExon | island | chr21:36258952-36259472 | -1.69E+14 | 0.0300 |

In the regression model for the arsenic (As75), the contribute of the metal to explain the change in methylation is significant for probes showed in table 31. The focus is on those located on promoter regions, linked to CpG islands and shores. Interestingly, among the genes involved, those showing arsenic-related hypermethylation for multiple probes are:

- C1orf109, which encode for a CK2 (casein-kinase 2) substrate that is involved in cell cycle control (G1 to S phase transition) and DNA repair;
- MRPS18A, with its mitochondrial function;
- PIWIL1, which plays a role in gene silencing by RNA; Sellitto et al. in 2019 reported that the PIWIL1-piRNA pathway actively contribute to the clinic-pathological features of the colorectal cancers. Also Amaar and Reeves in 2020 documented the RASSF1C-PIWIL1-piRNA pathway involvement in modulation of key oncogenes and tumor suppressors;
- NAV1 involved in neuronal development;
- CYP1A1, encoding for a member of the Cytochrome P450 family; its expression is induced by some polycyclic aromatic hydrocarbons (PAHs) and has been associated with lung cancer risk.

Conversely, PON1 and SDHAP3 genes show arsenic-related hypomethylation. PON1 encodes for an enzyme able to bind "paraoxon", a metabolite of Parathion, a toxic organophosphorus pesticide. SDHAP3 is linked to pancreatic cancer (Wolpin et al., 2014). Noteworthy, these results report a total arsenic estimation that need to be careful interpreted because of the presence of non-toxic organic arsenic for the food intake, which could hide the association between the toxic inorganic arsenic species. Indeed, over the purposes of the present dissertation, the SPES protocol have considered the speciation of Arsenic in the biological samples (urine) collected in the study.

**Table 31.** Top significant probes in robust linear regression model for Arsenic (As75). Metal.Estimate > 0 means that with the increase of the measured biomarker, there is an increase in the methylation for the probe observed. Metal.Pr expresses the statistical significance ≤0.05.

| CpG | gene | feature | cgi | UCSC_Islands_Name | metal.Estimate | metal.Pr |
|---|---|---|---|---|---|---|
| cg26052728 | ADAP1 | TSS1500 | shore | chr7:960048-960351 | 5.88E+14 | 0.03236 |
| cg14644031 | APOB | TSS1500 | shore | chr2:21266669-21266961 | 3.02E+14 | 0.01887 |
| cg24088508 | C1orf109 | TSS1500 | shore | chr1:38157295-38158586 | 1.21E+14 | 0.00065 |
| cg06917450 | C1orf109 | TSS1500 | shore | chr1:38157295-38158586 | 4.02E+13 | 0.00445 |
| cg04944931 | COMTD1 | TSS1500 | shore | chr10:76993892-76995953 | 2.49E+14 | 0.03363 |
| cg22549041 | CYP1A1 | TSS1500 | island | chr15:75018186-75019336 | 2.45E+14 | 0.00374 |
| cg14644378 | DNAJB5 | TSS1500 | shore | chr9:34989555-34989875 | 1.97E+14 | 0.01310 |
| cg26038582 | HIVEP3 | 1stExon | shore | chr1:42383640-42384003 | 2.75E+14 | 0.00008 |
| cg20290983 | MRPS18A | 1stExon | island | chr6:43655271-43655618 | 5.62E+14 | 0.01002 |
| cg04842962 | MRPS18A | 1stExon | island | chr6:43655271-43655618 | 4.22E+14 | 0.01002 |
| cg04124281 | MTHFS | TSS1500 | shore | chr15:80189006-80189695 | 2.09E+14 | 0.01887 |
| cg14920846 | NAV1 | 1stExon | island | chr1:201617041-201619788 | 1.54E+14 | 0.04120 |
| cg08010094 | NXPH2 | TSS1500 | shore | chr2:139537692-139538650 | 4.11E+14 | 0.01599 |
| cg18782210 | PAMR1 | TSS1500 | shore | chr11:35547139-35547396 | 1.82E+14 | 0.00158 |
| cg24838063 | PIWIL1 | TSS200 | island | chr12:130822360-130822696 | 5.04E+14 | 0.01111 |
| cg19424457 | PIWIL1 | TSS200 | shore | chr12:130823502-130824083 | 4.21E+14 | 0.00313 |
| cg26677194 | PIWIL1 | TSS200 | island | chr12:130822360-130822696 | 3.97E+14 | 0.00290 |
| cg27630820 | PIWIL1 | TSS200 | shore | chr12:130823502-130824083 | 3.53E+14 | 0.01635 |
| cg11931211 | PIWIL1 | TSS200 | island | chr12:130822360-130822696 | 3.33E+14 | 0.03954 |
| cg11649415 | PTPRS | 5'UTR | shore | chr19:5335044-5335261 | 2.98E+14 | 0.04571 |
| cg03760072 | SRPRB | TSS200 | island | chr3:133502561-133502963 | 2.46E+14 | 0.04261 |
| cg10426581 | SRRT | TSS1500 | island | chr7:100472375-100473393 | 4.18E+14 | 0.02306 |
| cg25093797 | UBASH3B | TSS1500 | shore | chr11:122526278-122527479 | 1.25E+14 | 0.05157 |
| cg03012280 | ZFYVE19 | TSS1500 | shore | chr15:41099243-41099878 | 1.45E+14 | 0.00008 |
| cg15721243 | ZNF468 | TSS1500 | shore | chr19:53360201-53360460 | 3.56E+14 | 0.03373 |
| cg01147067 | RNF213 | TSS1500 | shore | chr17:78233888-78235283 | -2.74E+13 | 0.02352 |
| cg22355889 | ELMOD1 | TSS1500 | shore | chr11:107461743-107462648 | -3.07E+14 | 0.03847 |
| cg25605443 | METTL24 | TSS1500 | shore | chr6:110679417-110679833 | -1.89E+14 | 0.01846 |
| cg00785941 | OR2L13 | 1stExon | island | chr1:248100325-248100726 | -5.78E+13 | 0.00516 |
| cg09506600 | OR2L13 | TSS1500 | shore | chr1:248100325-248100726 | -2.13E+14 | 0.02507 |
| cg08944170 | OR2L13 | 1stExon | island | chr1:248100325-248100726 | -7.47E+14 | 0.02352 |
| cg22190023 | PCDHB7 | 1stExon | shore | chr5:140553681-140554637 | -2.03E+14 | 0.00430 |
| cg18008345 | POLR2E | TSS1500 | shore | chr19:1094797-1095649 | -4.80E+14 | 0.00537 |
| cg19678392 | PON1 | 1stExon | island | chr7:94953769-94953971 | -3.38E+14 | 0.00179 |
| cg20119798 | PON1 | TSS1500 | shore | chr7:94953769-94953971 | -3.61E+14 | 0.00013 |
| cg17330251 | PON1 | TSS200 | island | chr7:94953769-94953971 | -3.72E+14 | 0.02789 |
| cg01874867 | PON1 | TSS200 | shore | chr7:94953769-94953971 | -3.91E+14 | 0.00818 |
| cg15571646 | RAMP1 | TSS1500 | shore | chr2:238768073-238768831 | -3.97E+14 | 0.00088 |
| cg06665890 | RNF213 | TSS1500 | shore | chr17:78233888-78235283 | -3.28E+14 | 0.01640 |
| cg24960960 | SDHAP3 | TSS200 | island | chr5:1594238-1595027 | -4.50E+14 | 0.02820 |
| cg21931717 | SDHAP3 | TSS200 | island | chr5:1594238-1595027 | -4.51E+14 | 0.04767 |
| cg19397885 | VWDE | TSS1500 | shore | chr7:12443163-12443705 | -3.07E+14 | 0.00528 |
| cg03579179 | VWDE | TSS1500 | shore | chr7:12443163-12443705 | -3.12E+14 | 0.00820 |

In the regression model for the cadmium (Cd111), the contribute of the metal to explain the change in methylation is significant for probes showed in table 32. The focus is on those located on promoter regions, linked to CpG islands and shores. Among genes showing cadmium-related hypermethylation C1orf109, the same listed for arsenic regression model, which is involved in cell proliferation by control on G1-S phase transition.

Instead, the HNF1B gene, involved in type II diabetes mellitus pathway and with altered expression in some cancer types, shows cadmium-related hypomethylation.

**Table 32.** Top significant probes in robust linear regression model for Cadmium (Cd111). Metal.Estimate > 0 means that with the increase of the measured biomarker, there is an increase in the methylation for the probe observed. Metal.Pr expresses the statistical significance ≤ 0.05.

| CpG | gene | feature | cgi | UCSC_Islands_Name | metal.Estimate | metal.Pr |
|---|---|---|---|---|---|---|
| cg22355889 | ELMOD1 | TSS1500 | shore | chr11:107461743-107462648 | 5.31583E+14 | 0.0048 |
| cg24088508 | C1orf109 | TSS1500 | shore | chr1:38157295-38158586 | 5.08453E+14 | 0.0085 |
| cg15655868 | DZIP1L | TSS1500 | shore | chr3:137833848-137834592 | -2.18796E+13 | 0.0495 |
| cg04198914 | HNF1B | TSS1500 | shore | chr17:36102033-36104766 | -1.70354E+14 | 0.0100 |
| cg17729891 | SNAR-F | TSS1500 | island | chr19:51107425-51107742 | -1.90419E+14 | 1.69E-05 |
| cg07107916 | PPP2R5A | TSS1500 | shore | chr1:212457967-212459694 | -1.96994E+14 | 0.0184 |
| cg21140145 | ZC2HC1A | TSS1500 | shore | chr8:79578112-79578667 | -3.67212E+14 | 0.0002 |
| cg03983883 | ZC2HC1A | TSS1500 | shore | chr8:79578112-79578667 | -4.28353E+14 | 0.0002 |
| cg04842962 | MRPS18A | 1stExon | island | chr6:43655271-43655618 | -6.09795E+14 | 1.69E-05 |

In the regression model for the Thallium (Tl205), the contribute of the metal to explain the change in methylation is significant for probes showed in table 33. The focus is on those located on promoter regions, linked to CpG islands and shores.

Among thallium related CpGs, those showing multiple hypermethylated probes within promoter regions, involve genes as:

- COLEC11, playing a role in innate immunity, apoptosis and embryogenesis;
- PRDM8, encoding a protein belonging to histone methyltransferase family, which acts as negative regulators of transcription. It is involved in steroidogenesis and neural development, a particular interesting function considering that thallium exposure could affect nervous system.

Considering the hypermethylation related genes:

- DUSP22, activating the JNK signaling pathway;
- NOC3L, involved in adipogenesis, gastric and colorectal cancers;
- PON1, encoding the enzyme able to bind paraoxon, a metabolite of Parathion, a toxic organophosphorus pesticide, then with a role in toxic compounds response;
- RRAS2, with its important role in activating signal transduction pathways controlling cell proliferation.

**Table 33.** Top significant probes in robust linear regression model for Thallium (Tl205). Metal.Estimate > 0 means that with the increase of the measured biomarker, there is an increase in the methylation for the probe observed. Metal.Pr expresses the statistical significance ≤ 0.05.

| CpG | gene | feature | cgi | UCSC_Islands_Name | metal.Estimate | Metal.Pr |
|------|------|---------|-----|-------------------|----------------|----------|
| cg13286116 | ARNTL | 5'UTR | shore | chr11:13298796-13300735 | 4.63E+14 | 1.18E-03 |
| cg08837037 | C22orf27 | TSS200 | shore | chr22:31318228-31318489 | 1.54E+14 | 8.57E-05 |
| cg19867917 | COLEC11 | TSS200 | island | chr2:3642547-3642751 | 8.93E+14 | 5.31E-05 |
| cg12135269 | COLEC11 | 1stExon | island | chr2:3642547-3642751 | 6.76E+14 | 5.56E-05 |
| cg18032014 | DYNC1I1 | 5'UTR | island | chr7:95401691-95402432 | 1.08E+14 | 8.37E-04 |
| cg16631618 | GPR150 | TSS1500 | shore | chr5:94955630-94957244 | 2.16E+14 | 3.83E-12 |
| cg02104434 | LOC148824 | TSS1500 | island | chr1:247694035-247694501 | 2.14E+14 | 2.74E-04 |
| cg12758973 | LOC148824 | TSS200 | island | chr1:247694035-247694501 | 1.72E+14 | 5.06E-06 |
| cg12754571 | LOC148824 | TSS200 | island | chr1:247694035-247694501 | 1.86E+13 | 8.22E-06 |
| cg00112952 | OR2B11 | TSS1500 | shore | chr1:247614695-247614944 | 8.33E+14 | 7.54E-04 |
| cg14830002 | OR2B11 | TSS1500 | shore | chr1:247614695-247614944 | 5.61E+14 | 4.32E-04 |
| cg20717585 | PCDHGA2 | 1stExon | island | chr5:140720138-140720829 | 1.75E+14 | 1.90E-04 |
| cg05059566 | PRDM8 | TSS200 | shore | chr4:81119095-81119391 | 1.88E+14 | 1.58E-04 |
| cg05452645 | PRDM8 | TSS1500 | shore | chr4:81118137-81118603 | 1.77E+14 | 3.09E-04 |
| cg26299084 | PRDM8 | 5'UTR | island | chr4:81118137-81118603 | 1.66E+14 | 1.18E-03 |
| cg10694470 | PRDM8 | TSS200 | island | chr4:81118137-81118603 | 1.59E+14 | 3.40E-06 |
| cg06373870 | PRDM8 | TSS1500 | shore | chr4:81118137-81118603 | 1.44E+14 | 1.61E-04 |
| cg06307913 | PRDM8 | 5'UTR | shore | chr4:81119095-81119391 | 1.33E+14 | 5.70E-04 |
| cg09419670 | PSMD5 | TSS1500 | shore | chr9:123605043-123605585 | 3.69E+14 | 1.21E-05 |
| cg10411850 | TDRD10 | TSS1500 | shore | chr1:154474107-154475699 | 2.45E+14 | 6.22E-10 |
| cg07732735 | TTC23 | TSS200 | shore | chr15:99791328-99792042 | 2.01E+14 | 1.90E-04 |
| cg22618164 | WDR66 | TSS200 | island | chr12:122356315-122356853 | 6.03E+14 | 4.32E-04 |
| cg04074835 | ACCS | 5'UTR | shore | chr11:44087518-44088165 | -5.44E+14 | 1.93E-05 |
| cg08742575 | C21orf56 | 5'UTR | shore | chr21:47602431-47602740 | -9.14E+14 | 1.23E-04 |
| cg19427746 | CAMTA2 | 5'UTR | shore | chr17:4890282-4890991 | -2.00E+14 | 5.37E-08 |
| cg24324837 | CCDC155 | 1stExon | island | chr19:49891269-49891604 | -1.51E+14 | 2.50E-04 |
| cg02656474 | CMAS | TSS1500 | shore | chr12:22199062-22199589 | -2.09E+13 | 2.76E-09 |
| cg03680517 | CSGALNACT2 | TSS1500 | island | chr10:43632966-43634222 | -1.55E+14 | 1.12E-03 |
| cg05185784 | DEF8 | 5'UTR | island | chr16:90015551-90016120 | -1.84E+14 | 1.17E-03 |
| cg03395511 | DUSP22 | TSS200 | shore | chr6:291948-292839 | -2.05E+13 | 1.00E-04 |
| cg05064044 | DUSP22 | 1stExon | island | chr6:291948-292839 | -1.18E+14 | 7.68E-04 |
| cg18110333 | DUSP22 | 1stExon | island | chr6:291948-292839 | -2.00E+14 | 3.17E-04 |
| cg21548813 | DUSP22 | TSS1500 | shore | chr6:291948-292839 | -2.15E+14 | 3.62E-06 |
| cg11245928 | EIF3L | TSS1500 | shore | chr22:38245341-38245681 | -1.41E+14 | 5.30E-05 |
| cg24578493 | EIF3L | TSS1500 | shore | chr22:38245341-38245681 | -1.87E+14 | 1.69E-07 |
| cg01359822 | ETS2 | TSS1500 | shore | chr21:40177002-40178667 | -1.77E+13 | 5.23E-06 |
| cg14748380 | FAM163A | 5'UTR | island | chr1:179711947-179713951 | -1.36E+14 | 2.43E-05 |
| cg13607226 | GGTA1 | TSS1500 | shore | chr9:124261806-124262587 | -1.33E+14 | 9.20E-04 |
| cg04945312 | GLB1L | 5'UTR | shore | chr2:220107844-220108348 | -1.88E+14 | 6.18E-04 |
| cg10602248 | GLB1L | 1stExon | island | chr2:220107844-220108348 | -2.46E+14 | 1.86E-08 |
| cg24061197 | GLB1L | 5'UTR | shore | chr2:220107844-220108348 | -2.46E+14 | 7.38E-05 |
| cg24076236 | ICE2 | TSS1500 | shore | chr15:60771068-60771558 | -2.10E+14 | 2.33E-04 |
| cg07520074 | KCTD11 | TSS1500 | shore | chr17:7253298-7253764 | -2.76E+14 | 1.84E-04 |
| cg03185704 | LRRC61 | TSS200 | island | chr7:150019950-150020752 | -2.51E+14 | 2.99E-05 |
| cg06099315 | MIR8078 | TSS200 | island | chr18:112309-112529 | -1.29E+14 | 3.20E-04 |

| cg21537187 | MTPN | TSS1500 | shore | chr7:135661841-135662272 | -3.16E+14 | 3.55E-07 |
|---|---|---|---|---|---|---|
| cg19968001 | NFKBIB | 5'UTR | shore | chr19:39390033-39390895 | -2.83E+14 | 1.11E-06 |
| cg27514038 | NIPA2 | TSS1500 | shore | chr15:23033933-23034710 | -1.69E+14 | 3.58E-04 |
| cg07889765 | NOC3L | TSS1500 | shore | chr10:96122540-96122904 | -2.14E+14 | 4.17E-05 |
| cg08923894 | NOC3L | TSS1500 | shore | chr10:96122540-96122904 | -2.68E+14 | 5.70E-04 |
| cg15157241 | PKNOX1 | 5'UTR | shore | chr21:44394084-44395850 | -1.23E+14 | 3.66E-04 |
| cg02802029 | PLOD2 | TSS1500 | shore | chr3:145878430-145879287 | -1.85E+14 | 1.75E-04 |
| cg17330251 | PON1 | TSS200 | island | chr7:94953769-94953971 | -2.79E+14 | 1.76E-04 |
| cg07107916 | PPP2R5A | TSS1500 | shore | chr1:212457967-212459694 | -1.96E+14 | 1.28E-07 |
| cg13824270 | PRPF4B | TSS1500 | shore | chr6:4021444-4022004 | -4.37E+14 | 7.00E-04 |
| cg09293560 | REPIN1 | TSS200 | shore | chr7:150068756-150070051 | -1.59E+14 | 4.25E-09 |
| cg19336497 | RRAS2 | TSS1500 | shore | chr11:14379808-14380737 | -1.59E+14 | 5.96E-04 |
| cg15356281 | SEPT2 | 5'UTR | shore | chr2:242254299-242256026 | -1.24E+13 | 6.58E-04 |
| cg08602346 | TUBGCP3 | TSS1500 | shore | chr13:113241711-113241925 | -1.32E+14 | 1.51E-04 |
| cg14853367 | ZNF329 | TSS1500 | shore | chr19:58661737-58662287 | -1.71E+14 | 1.56E-04 |
| cg21464363 | ZSWIM4 | TSS1500 | shore | chr19:13905810-13906649 | -2.48E+14 | 5.31E-05 |

In the regression model for the DRCalux, the contribute of the biomarker to explain the change in methylation is significant for probes showed in table 34. The focus is on those located on promoter regions, linked to CpG islands and shores.

The only two probes with significant hypomethylation DR Calux-linked, are cg00321850 and cg00523161, with no particular documented links with dioxin compounds exposure. However, because its role in fatty acids metabolism, LP1N1 gene could potentially be related to the biological processes involved, given the lipophilic nature of the dioxins.

**Table 34.** Top significant probes in robust linear regression model for DRCalux. Calux.Estimate > 0 means that with the increase of the measured biomarker, there is an increase in the methylation for the probe observed. Calux.Pr expresses the statistical significance ≤ 0.05.

| CpG | gene | feature | cgi | UCSC_Islands_Name | calux.Estimate | calux.Pr |
|---|---|---|---|---|---|---|
| cg00321850 | KIAA0040 | TSS200 | shore | chr1:175161900-175162364 | -2.88E+14 | 0.0012 |
| cg00523161 | LPIN1 | 5'UTR | shore | chr2:11886249-11887207 | -2.84E+14 | 0.0046 |

In the regression model for the ERαCalux, the contribute of the biomarker to explain the change in methylation is significant for probes showed in table 35. The focus is on those located on promoter regions, linked to CpG islands and shores. Genes with ErαCalux related hypermethylation are:

- C21orf56 (SPATC1L), associated with Protein Kinase A regulatory subunit and male infertility (Kim et al., 2018);
- PLSCR2, with its role in blood coagulation and apoptosis;
- RUNX1, leukemia linked gene.

Among hypomethylated:

- DDX4 which encodes for a DEAD box protein, involved in embryogenesis, spermatogenesis, cellular growth and division;
- ZNF714 which plays a role in transcriptional regulation of zinc finger proteins.

**Table 35.** Top significant probes in robust linear regression model for ERαCalux. Calux.Estimate > 0 means that with the increase of the measured biomarker, there is an increase in the methylation for the probe observed. Calux.Pr expresses the statistical significance ≤ 0.05.

| CpG | gene | feature | cgi | UCSC_Islands_Name | calux.Estimate | calux.Pr |
|-----|------|---------|-----|-------------------|----------------|----------|
| cg07747299 | C21orf56 | 5'UTR | shore | chr21:47602431-47602740 | 7.06E+14 | 0.018 |
| cg12016809 | C21orf56 | 5'UTR | shore | chr21:47602431-47602740 | 6.60E+14 | 0.024 |
| cg22355889 | ELMOD1 | TSS1500 | shore | chr11:107461743-107462648 | 1.65E+14 | 0.041 |
| cg24005645 | PLSCR2 | TSS200 | island | chr3:146187108-146187710 | 5.89E+14 | 0.038 |
| cg14937633 | RUNX1 | 1stExon | island | chr21:36258952-36259472 | 5.17E+14 | 0.022 |
| cg20418711 | RUNX1 | 1stExon | island | chr21:36258952-36259472 | 4.85E+14 | 0.027 |
| cg17729891 | SNAR-F | TSS1500 | island | chr19:51107425-51107742 | 2.40E+14 | 0.041 |
| cg09352518 | ZNF714 | 5'UTR | island | chr19:21265164-21265433 | -1.05E+14 | 0.011 |
| cg05202629 | C17orf97 | TSS1500 | shore | chr17:259754-260306 | -5.42E+14 | 0.016 |
| cg27560391 | DDX24 | 5'UTR | shore | chr14:94547001-94547665 | -8.89E+14 | 4.09E-06 |
| cg06238316 | ZNF714 | 5'UTR | island | chr19:21265164-21265433 | -7.17E+14 | 0.034 |

In the regression model for the PAHCalux, the contribute of the biomarker to explain the change in methylation is significant for probes showed in table 36. The focus is on those located on promoter regions, linked to CpG islands and shores. Genes with PAHCalux related hypermethylation are:

- AURKC, encoding for a member of Aurora subfamily of serine/threonine protein kinase. It is overexpressed in several cancer lines, suggesting a role in oncogenic signal transduction;
- GPR88 with its role in cognitive disorders;
- PF4 with its role in platelet aggregation;
- RNF213 involved in wnt signaling;

- UHMK1 encoding a serine/threonine protein kinase that promotes cell cycle progression through G1, by phosphorylation of the cyclin-dependent kinase inhibitor 1B. The encoded protein has also a role in the adult nervous system.

Among hypomethylated:

- CDKN1A, regulator of cell cycle progression at G1 (expression of gene controlled by p53) and DNA damage;

- HOX4, with its role in gene expression, morphogenesis and differentiation, for instance during embryonic development;

- MIR886, pre-miRNA, direct inhibitor of protein kinase R, plays an important role in regulation of cell growth. It is repressed in some cancers.

- TACSTD2, encoding a carcinoma-associated antigen, a cell receptor that transduces calcium signals.

- TEKT4, which contributes to sperm motility, being a structural component of the sperm flagellum.

**Table 36.** Top significant probes in robust linear regression model for PAHCalux. Calux.Estimate > 0 means that with the increase of the measured biomarker, there is an increase in the methylation for the probe observed. Calux.Pr expresses the statistical significance ≤ $1.0^{-5}$.

| CpG | gene | feature | cgi | UCSC_Islands_Name | calux.Estimate | calux.Pr. |
|---|---|---|---|---|---|---|
| cg04450003 | AQP5 | 1stExon | island | chr12:50354839-50356163 | 3.23E+08 | 2.00E-10 |
| cg18315870 | ATG101 | TSS1500 | shore | chr12:52463348-52464202 | 4.74E+08 | 5.88E-06 |
| cg07314988 | ATHL1 | 5'UTR | island | chr11:289771-290010 | 7.57E+08 | 2.58E-07 |
| cg14370847 | AURKC | TSS200 | island | chr19:57741955-57742457 | 4.25E+08 | 3.17E-06 |
| cg02349264 | CARHSP1 | 5'UTR | shore | chr16:8962189-8963190 | 2.83E+08 | 4.03E-07 |
| cg03651525 | CCDC25 | TSS1500 | shore | chr8:27629753-27630234 | 4.18E+08 | 6.08E-06 |
| cg07187855 | DDR1 | 5'UTR | shore | chr6:30852102-30852676 | 3.25E+08 | 1.65E-05 |
| cg05185784 | DEF8 | 5'UTR | island | chr16:90015551-90016120 | 2.67E+08 | 4.33E-05 |
| cg27638615 | FZD7 | 1stExon | shore | chr2:202897386-202901046 | 5.92E+08 | 5.27E-11 |
| cg06223162 | GPR88 | TSS200 | shore | chr1:101004471-101005885 | 1.04E+09 | 1.13E-09 |
| cg07412545 | GPR88 | 1stExon | shore | chr1:101004471-101005885 | 1.03E+09 | 1.23E-12 |
| cg09408571 | GPR88 | TSS200 | shore | chr1:101004471-101005885 | 8.07E+08 | 1.29E-08 |
| cg19978674 | HOPX | 5'UTR | shore | chr4:57521621-57522703 | 6.65E+08 | 8.84E-05 |
| cg16406611 | IFITM5 | TSS1500 | shore | chr11:299389-299635 | 4.70E+08 | 8.30E-09 |
| cg00944873 | KIAA0319 | TSS1500 | shore | chr6:24645931-24646591 | 5.13E+08 | 3.50E-05 |
| cg18049933 | LOC100996579 | TSS1500 | shore | chr2:162101439-162101650 | 3.54E+08 | 3.71E-07 |
| cg25755428 | MRI1 | TSS1500 | island | chr19:13875044-13875951 | 1.39E+09 | 1.06E-06 |
| cg08989084 | P2RX2 | TSS1500 | shore | chr12:133195922-133196556 | 3.75E+08 | 2.26E-05 |
| cg05509609 | PF4 | TSS200 | island | chr4:74847528-74847830 | 6.10E+08 | 1.65E-09 |
| cg06834998 | PF4 | TSS200 | island | chr4:74847528-74847830 | 5.51E+08 | 4.95E-10 |
| cg02530824 | PF4 | TSS200 | island | chr4:74847528-74847830 | 5.12E+08 | 2.65E-08 |
| cg16072462 | PF4 | TSS200 | island | chr4:74847528-74847830 | 4.67E+08 | 5.90E-09 |
| cg15398841 | PF4 | TSS200 | island | chr4:74847528-74847830 | 4.57E+08 | 4.96E-09 |
| cg21043213 | PF4 | 1stExon | island | chr4:74847528-74847830 | 4.11E+08 | 1.92E-08 |
| cg11649415 | PTPRS | 5'UTR | shore | chr19:5335044-5335261 | 4.53E+08 | 2.03E-06 |
| cg08742290 | RNASET2 | TSS1500 | shore | chr6:167369439-167370588 | 4.42E+08 | 2.31E-07 |
| cg06665890 | RNF213 | TSS1500 | shore | chr17:78233888-78235283 | 4.69E+08 | 5.92E-06 |
| cg01147067 | RNF213 | TSS1500 | shore | chr17:78233888-78235283 | 4.48E+08 | 4.16E-07 |
| cg22198044 | SH3BP2 | TSS1500 | island | chr4:2819499-2820429 | 2.48E+07 | 7.25E-07 |
| cg26390081 | TMEM204 | 5'UTR | shore | chr16:1583809-1584641 | 3.97E+08 | 1.49E-07 |
| cg27594616 | TMEM204 | 5'UTR | shore | chr16:1583809-1584641 | 2.90E+08 | 8.56E-05 |
| cg09777637 | TMEM229A | TSS1500 | shore | chr7:123672063-123673691 | 3.28E+07 | 2.03E-09 |
| cg06179011 | TSPAN4 | TSS1500 | shore | chr11:842293-843396 | 3.11E+08 | 1.61E-05 |
| cg19814518 | UHMK1 | TSS1500 | shore | chr1:162467599-162468027 | 6.61E+08 | 1.44E-14 |
| cg00859178 | UHMK1 | TSS1500 | shore | chr1:162467599-162468027 | 5.80E+08 | 6.98E-14 |
| cg03012280 | ZFYVE19 | TSS1500 | shore | chr15:41099243-41099878 | 5.98E+08 | 3.33E-09 |
| cg26751094 | ABCC4 | TSS1500 | shore | chr13:95953337-95954211 | -2.60E+08 | 4.44E-05 |
| cg01324343 | ABCC5 | 5'UTR | shore | chr3:183735183-183736103 | -5.58E+08 | 7.36E-08 |
| cg18349420 | AQP11 | TSS1500 | shore | chr11:77300360-77301391 | -4.24E+07 | 3.44E-12 |
| cg06061081 | AQP11 | TSS1500 | shore | chr11:77300360-77301391 | -3.70E+08 | 2.02E-08 |
| cg04518186 | AQP11 | TSS1500 | shore | chr11:77300360-77301391 | -4.83E+08 | 1.01E-08 |
| cg15747436 | ATHL1 | TSS1500 | shore | chr11:289771-290010 | -3.74E+08 | 1.67E-10 |
| cg08829299 | ATHL1 | TSS1500 | shore | chr11:288847-289239 | -5.64E+08 | 5.71E-09 |
| cg18727742 | ATHL1 | TSS1500 | shore | chr11:288847-289239 | -6.52E+08 | 3.98E-08 |
| cg04567952 | C2orf69 | TSS1500 | shore | chr2:200775746-200776548 | -6.12E+08 | 1.41E-06 |
| cg15855900 | CA7 | TSS1500 | shore | chr16:66878172-66879072 | -2.42E+08 | 6.91E-05 |
| cg24425727 | CDKN1A | TSS1500 | shore | chr6:36646244-36648465 | -2.34E+08 | 8.19E-05 |
| cg02452435 | COG1 | TSS1500 | shore | chr17:71188407-71189458 | -4.84E+08 | 1.55E-07 |
| cg02271943 | COMTD1 | TSS1500 | shore | chr10:76993892-76995953 | -1.19E+09 | 8.54E-70 |
| cg27560391 | DDX24 | 5'UTR | shore | chr14:94547001-94547665 | -8.17E+08 | 9.01E-17 |

| | | | | | | |
|---|---|---|---|---|---|---|
| cg00481259 | DECR2 | TSS1500 | island | chr16:450834-451140 | -3.88E+08 | 2.63E-09 |
| cg07973095 | DECR2 | TSS1500 | island | chr16:450834-451140 | -5.33E+08 | 1.66E-05 |
| cg24578493 | EIF3L | TSS1500 | shore | chr22:38245341-38245681 | -3.80E+08 | 1.37E-10 |
| cg11245928 | EIF3L | TSS1500 | shore | chr22:38245341-38245681 | -4.35E+08 | 2.00E-11 |
| cg08291335 | FANCG | TSS1500 | shore | chr9:35079628-35080213 | -4.81E+08 | 6.40E-11 |
| cg14785464 | HLCS | TSS200 | island | chr21:38362015-38362868 | -3.25E+08 | 4.91E-06 |
| cg17457637 | HOXA4 | TSS1500 | shore | chr7:27169572-27170638 | -2.99E+08 | 6.91E-05 |
| cg07317062 | HOXA4 | 5'UTR | island | chr7:27169572-27170638 | -3.47E+08 | 3.28E-05 |
| cg24426391 | HTATIP2 | TSS1500 | shore | chr11:20385161-20385673 | -8.09E+08 | 1.18E-06 |
| cg19521832 | KCNE1 | TSS200 | island | chr21:35831697-35832365 | -3.71E+08 | 6.49E-07 |
| cg04177826 | KLHDC7B | TSS1500 | island | chr22:50984972-50988141 | -2.57E+08 | 2.20E-06 |
| cg15509177 | LOC100101938 | TSS200 | island | chr13:19918585-19919221 | -3.05E+07 | 2.52E-05 |
| cg15469871 | LOC387646 | TSS200 | island | chr10:27541297-27541883 | -3.74E+08 | 1.44E-05 |
| cg18806716 | MAP3K8 | TSS1500 | shore | chr10:30722378-30723707 | -4.23E+08 | 5.04E-05 |
| cg26896946 | MIR886 | TSS200 | island | chr5:135416204-135416475 | -1.07E+07 | 9.48E-38 |
| cg18797653 | MIR886 | TSS1500 | shore | chr5:135415069-135415307 | -9.63E+08 | 7.58E-30 |
| cg00124993 | MIR886 | TSS200 | island | chr5:135416204-135416475 | -1.15E+09 | 5.26E-22 |
| cg08745965 | MIR886 | TSS1500 | shore | chr5:135415069-135415307 | -1.20E+09 | 3.22E-48 |
| cg18678645 | MIR886 | TSS200 | island | chr5:135416204-135416475 | -1.30E+09 | 1.99E-33 |
| cg23605644 | MOBP | TSS200 | shore | chr3:39543771-39544223 | -3.91E+08 | 3.06E-06 |
| cg03054684 | MOBP | 5'UTR | shore | chr3:39543771-39544223 | -5.16E+08 | 1.09E-06 |
| cg07878407 | MOBP | 1stExon | shore | chr3:39543771-39544223 | -6.31E+08 | 2.02E-09 |
| cg05633900 | MOBP | 1stExon | shore | chr3:39543771-39544223 | -6.58E+08 | 2.13E-10 |
| cg14666699 | MRGPRG-AS1 | TSS1500 | shore | chr11:3239217-3239988 | -2.36E+08 | 1.42E-05 |
| cg18705301 | NDUFAF1 | TSS1500 | shore | chr15:41694493-41694756 | -5.54E+08 | 3.07E-05 |
| cg13348653 | PCDHB3 | TSS200 | shore | chr5:140481546-140482460 | -3.68E+08 | 2.24E-08 |
| cg03780733 | PCDHB7 | 1stExon | shore | chr5:140553681-140554637 | -1.69E+09 | 7.31E-05 |
| cg15157241 | PKNOX1 | 5'UTR | shore | chr21:44394084-44395850 | -5.91E+08 | 6.34E-12 |
| cg24530147 | PRR23C | TSS200 | island | chr3:138762689-138764113 | -3.42E+08 | 9.50E-09 |
| cg05895034 | PRSS22 | TSS1500 | shore | chr16:2907574-2907836 | -4.19E+08 | 2.66E-11 |
| cg09745688 | PRSS22 | TSS1500 | shore | chr16:2907574-2907836 | -4.67E+08 | 1.49E-05 |
| cg09309269 | PSMD11 | TSS1500 | island | chr17:30770960-30772137 | -3.24E+08 | 4.49E-06 |
| cg00871487 | RANBP17 | TSS200 | shore | chr5:170288879-170289737 | -5.18E+08 | 1.72E-07 |
| cg06091474 | SEMA3C | TSS1500 | shore | chr7:80548306-80548726 | -3.09E+08 | 1.95E-07 |
| cg10589385 | SETDB1 | TSS1500 | shore | chr1:150898619-150898839 | -2.73E+08 | 5.05E-05 |
| cg26748578 | SLC1A4 | 1stExon | island | chr2:65215598-65217212 | -3.65E+07 | 3.69E-06 |
| cg05845376 | SLC25A2 | TSS200 | island | chr5:140683195-140683773 | -2.93E+07 | 3.46E-05 |
| cg17729891 | SNAR-F | TSS1500 | island | chr19:51107425-51107742 | -8.53E+08 | 3.55E-06 |
| cg22023531 | SORD | TSS1500 | shore | chr15:45315201-45315543 | -4.42E+08 | 1.14E-09 |
| cg16699148 | TACSTD2 | TSS200 | island | chr1:59042013-59043295 | -3.67E+08 | 3.23E-06 |
| cg16080552 | TACSTD2 | TSS200 | island | chr1:59042013-59043295 | -6.19E+08 | 6.32E-05 |
| cg04863005 | TACSTD2 | TSS200 | island | chr1:59042013-59043295 | -6.40E+08 | 3.06E-06 |
| cg06282964 | TEKT4 | 1stExon | island | chr2:95537196-95537810 | -3.68E+07 | 8.82E-10 |
| cg05852568 | TEKT4 | 1stExon | island | chr2:95537196-95537810 | -4.24E+08 | 6.40E-09 |
| cg24354818 | TNFRSF6B | 5'UTR | island | chr20:62328013-62328558 | -3.82E+08 | 6.60E-05 |
| cg00152799 | TXNL1 | TSS1500 | shore | chr18:54305419-54306087 | -3.80E+08 | 2.17E-06 |
| cg17751872 | ZNF714 | 5'UTR | shore | chr19:21265164-21265433 | -4.13E+08 | 6.71E-05 |
| cg21464363 | ZSWIM4 | TSS1500 | shore | chr19:13905810-13906649 | -4.33E+08 | 2.78E-06 |

In the regression model for the sum of PCDD/F + DL-PCB, no significant probes on promoter regions and linked to CpG islands and shores were reported.

# 5. DISCUSSION

Human Biomonitoring can be considered as a form of public health surveillance (Salines, 2012). It represents an important and useful tool to investigate human exposure to environmental chemicals, providing a reliable measurement of the internal dose of pollutants, through different exposure pathways (Calafat et al., 2006).

Its methodological approach, aimed to estimate exposure importance and extension, is widely used in the risk assessment for occupational health and for monitoring of general population. According to EU in 2013, HB can serve the chemicals surveillance by providing "authorities with a more comprehensive view of actual exposure of the population to pollutants, […] and can provide better evidence from guiding appropriate responses" (Ganzleben et al., 2017).

Assessing chemicals in body tissues and fluids, HB can help to understand the toxic-kinetic profile of compounds and their interactions, the correlation with effects on biological systems and the potential health outcomes (CDC, 2005). Moreover, from an epidemiological point of view, it can help to define what is the total exposure to the substances from different sources, how does the chemical exposure evolve over time and among regions or population groups, and incidence and mortality rates of putative linked diseases. Thus, HB evidence is able to support both the advance of scientific knowledge and the effectiveness of policy and regulatory actions (Louro et al., 2019).

Despite of the great potential, the conventional HB protocols often suffer the lack of data integration and methodological sharing between different science fields, as well as the lack of a holistic approach oriented to a "one health" perspective (Frazzoli et al., 2015; Falzon et al., 2018; Sleeman et al., 2019).

To support a complete understanding of the phenomena, HB protocols need to integrate a single multilevel analysis. The environmental background characterization, the anamnestic and epidemiological data collection, together with the omics approach, become fundamental for the evaluation of the exposure risk and for depicting a framework of the real degree of contamination (Pierri et al., 2020). This means to set up a systemic overview, in which the multidimensionality and the complexity of the observations are solved by dividing the phenomenon in several levels of analysis that, in the final intersection, could provide the global reading and interpretation.

Especially considering the Campania Region and the Land of Fire case study, the controversy emerging from scientific literature (Cantoni, 2016; Mazza et al., 2018) does not allow to obtain a clear definition of the level of pollution in the regional territory.

For these reasons, the main purpose of the present PhD dissertation is to introduce and describe an innovative HB integrated approach, the basis of the SPES protocol, the observational study on Campania Region healthy population promoted by the IZSM. A

particular focus in the dissertation comes from exploring the role of epigenetic biomarkers, as the DNA methylation, and their integration with several levels of analysis by means statistical model of data elaboration.

Starting from the paradigm of the method (which considers three main elements: the identification of pollution sources, of pollution migration pathways and of final target organisms), the Campania environmental context has been characterized, highlighting sites of particular interest. By means of a "Pressure Index" (IRc), the environmental background provides a synthetic representation of the level of pressure exerted by the contamination sources on the municipalities. The three increasing levels of impact derived were consistent with areas with the already known reputation of "triangle of death" (Triassi et al., 2015), expression firstly coined by Senior and Mazza on The Lancet Oncology in 2004 and referred to area with documented illegal disposal of urban, toxic and industrial wastes. Interestingly, the IRc methods showed a medium impact area, where the presence of industrial plants gives an important contribute to the spreading of heavy metals and polycyclic aromatic hydrocarbons in the ecosystem. It is well known that workers surveillance and study monitoring on people indwelling areas close to industrial plants document adverse health effects linked to the spreading of pollutants. Scientific literature describes a series of renowned italian sites, as well as Taranto city, with its steel foundry, that is one of the most famous of the Southern Italy (Vimercati et al., 2017; Lovreglio et al., 2018; Lucchini et al., 2019). The medium impact areas, identified with the computed IRc in SPES protocol, consist of some sites of particular interest, given the presence of industrial plants: tanneries in Solofra area, a steel foundry in the Irno Valley, metallurgic, iron and steel industries, asbestos disposal plant in Sabato Valley and, finally, the area known as "agro-nocerino-sarnese", mainly characterized by groundwater contamination caused by landfill leachate and wide presence of compounds linked to the use of pesticides. This suggests other areas, over those included in the LOF, where industrial sources of contamination could represent a risk for residing people. Data acquired by HB support this hypothesis. The assessment of heavy metals in serum of healthy volunteers, resulting from SPES human biomonitoring, revealed that the average levels ($\mu$g/L) of cadmium (Cd111) and mercury (Hg202) were approximately 5 times higher in Irno and Sabato Valley clusters (Medium Impact Area), compared to the entire population assessed.

The high impact areas, where are present municipalities belonging to the so-called "Land of Fire", were only characterized by thallium (Tl205) higher values, probably due to the natural background levels of this compound in the soil, because of the volcanic origin of the region (Karbowska, 2016).

In line with these results, also organic compounds measurement showed a consistent statistical significance in medium impact area, especially for Irno and Sabato Valley clusters. Considering high resolution gas chromatography results, the median value of the

sum of PCDD/PCDF + DL-PCB (pg WHO-TEQ/g lipids) showed a sensitive increase for Sabato Valley cluster, compared to the overall median. A slighter increase is also evident for Irno Valley cluster and High 12 cluster. This last includes the municipalities of Acerra, Villa Literno and Naples, where the presence of a waste incinerator for Acerra, a busy commercial road (Nola-Villa Literno provincial road - SP333), and the complex urbanization of Naples metropolis, could explain the higher values of the sum of dioxins congeners (Marques and Domingo, 2019).

Comparable data were obtained considering the DR-Calux® bioassay, expression of stable ligands of Ahrilic receptor (e.g. dioxins congeners), which found a significant correlation between DR-Calux® median values (pgBEQ/g lipids) and Irno and Sabato Valley clusters. In addition, the bioassay showed an interesting evidence of total estrogenic and estrogenic-like responses by the ER-Calux® assessment (pgEEQ/ml), with higher values detected in females and males from high and medium impact areas. The total estrogenic activity observed in females is 3 to 5-fold increase compared to the levels of the natural endogenous hormone 17-β-estradiol, and this could suggest the presence of compounds with possible endocrine activity (Endocrine disrupting chemicals EDCs).

Morgan et al. in 2017 highlighted a link between environmental exposure to PCB138 and breast cancers, as well as a correlation with the sum of non-dioxin like PBCs and breast cancer. Calaf et al. in 2020 reviewed xenoestrogens compounds in relation to breast cancer incidence, showing that Bisphenol A, Dichlorodiphenyltrichloroethane (DDT) and PCBs are involved in alteration of cell proliferation and of cell death, are immunosuppressive agents and induce oxidative stress and epigenetic alterations.

The Report AIOM-AIRTUM of 2019 (www.salute.gov.it) estimated approximately 4050 new diagnosis of breast cancers among women in Campania Region, with an incidence rate of 140.5 per 100.000 residents (Friuli Venezia Giulia Region has the higher incidence rate at 203.9 per 100.000 people, while Calabria Region has the lower rate at 124 per 100.000 people). Fusco et al., in 2013, already showed an increasing trend in Campania for this type of cancer, as well as for lung cancer and skin melanoma in women, for colorectal cancer and skin melanoma for men.

Some studies reported a link between exposure to PCBs and risk of cutaneous melanoma (Zani et al., 2017; Boffetta et al., 2018; Cao et al., 2019).

Lung and colorectal cancers were also reported in literature as possible adverse outcome linked to pollution (de Groot et al., 2012; Juloski et al., 2020).

Certainly, the incidence trends from literature need to be explained also considering life habits, prevention culture and familiar history. Indeed, these anamnestic considerations are possible confounding factors.

This is the reason why in the SPES protocol the subgroup of 600 volunteers, selected for deepened investigation, were chosen according to inclusion criteria that allowed to avoid bias given by the confounding factors (see paragraph 3.1.5).

Data obtained with exposure biomarkers investigation are supported by the deep analysis of effect biomarkers. Considering the epigenetic profile resulted from methylation array data analysis, it is important to underline that the KEGG pathway analysis on the DMPs revealed, in medium impact areas vs low impact areas, an interesting enrichment for estrogenic signaling and thyroid hormone pathways, as well as for the endocrine resistance one. These results are consistent with the higher levels of organic compounds detected in the high and medium clusters.

The same KEGG pathway analysis has shown for high and medium impact areas vs low one, the enrichment of gene-set involved in metabolic and cancer pathways, transduction signaling and inflammation, as well as breast cancer, gastric cancer, small and non-small lung cancer, melanoma, cell cycle and p53 signaling.

The KEGG pathway analysis performed on the DMRs also revealed the enrichment of "neuroactive ligand receptor interaction", supported by biological processes terms enriched in the gene ontology analysis, "nervous system development", and by the KEGG neurodegenerative disorders pathways reported for DMPs in high and medium vs low impact area comparison.

As a matter of fact, there are a lot of scientific evidence that pre- and post-natal exposures to environmental factors could predispose to the onset of neurodegenerative diseases in later life (e.g. Alzheimer and Parkinson diseases). According to Chin-Chan et al., neurotoxic metals such as lead, mercury, aluminum, cadmium and arsenic, as well as some pesticides have involved in Alzheimer etiology, due to their ability to increase the formation of beta amyloids plaques. The exposure to lead, manganese, solvents and some pesticides has related to Parkinson disease. Epigenetic alteration is one of the involved mechanisms (Chin-Chan et al., 2015).

The use of epigenetics as a key to understand the molecular mechanism subtended to toxicity, gives the chance of characterize the biological response to environmental stressors and identify possible molecular biomarkers or patterns of exposure (Vineis et al., 2013).

From the epigenetic analysis, carried out for the purposes of the present dissertation, emerges a global hypomethylation in the high impact areas and in the medium one compared to the low impact areas. This is consistent with the exposure to several toxic compounds as benzene (Bollati et al., 2007), POPs persistent Organic Pollutants (Rusiecki et al., 2008), Particular Matters (Baccarelli et al., 2009; Plusquin et al., 2017), Endocrine Disruptors (La Rocca et al., 2014) and, at the same time, with genomic instability and a series of chronic and degenerative conditions (Van Togelen et al., 2017; Zang et al., 2020).

Additionally, the regression analysis performed to highlight possible correlations between DNA methylation levels and exposure biomarkers, revealed some promoter regions, linked to CpG island and shore, where hypermethylated sites could be involved in a transcriptional repression mechanisms, and could contribute to generate the final response of the biological system to the external stressors. Similarly, hypomethylated sites could contribute to generate a reaction to the external input.

As reported in the Results chapter, heavy metals included in the robust linear regression models (Hg, As, Cd and Tl) showed a significant link with genes involved in cell cycle regulation, carcinogenesis, neuronal development and this is consistent with the known possible effect of some of them (WHO, 2015).

Among them, an interesting correlation emerged between arsenic and PIWIL1 gene, which plays a role in gene silencing by RNA; as previously described the PIWIL1-piRNA pathway is involved in modulation of key oncogenes and tumor suppressors (Amaar and Reeves, 2020), and, as documented by Sellitto et al. in 2019, in colorectal cancer cells contributes to the clinic-pathological features of the disease. Noteworthy, as previously described (see paragraph 4.4.3), these results report a total arsenic estimation that need to be careful interpreted because of the presence of non-toxic organic arsenic for the food intake, which could hide the association between the toxic inorganic arsenic species. Indeed, over the purposes of the present dissertation, the SPES protocol have considered the speciation of Arsenic in the biological samples (urine) collected in the study.

Regarding arsenic and thallium, both the two metals show a correlation with the gene PON1: arsenic-related hypomethylation and thallium-related hypermethylation of its promoter region. This gene encodes for the enzyme able to bind the paraoxon, a metabolite of Parathion, a toxic organophosphorus pesticide. Marhooz et al. in 2019 described PON1 as a fundamental antiatherogenic and antioxidant enzyme in the circulation that has been associated with adverse health outcomes, particularly cardiovascular disease. It has also been analyzed by Cardenas et al., in 2017, in order to assess an association with the maternal prenatal mercury exposure in cord blood samples. According to their findings, DNA methylation at the PON1 locus could regulate the association between prenatal mercury exposure, cognitive development and other health outcomes in children. The researchers expressed that the persistent epigenetic disruption of the PON1 gene might serve as a biomarker of mercury exposure and disease susceptibility. Moreover, the PON1 activity shows a polymorphic distribution in patients with Alzheimer's disease (Saeidi et al., 2017), with different frequencies in group of people exposed or not to organophosphate pesticides (Javeres et al., 2020). This could suggest the methylation and the expression of PON1 gene as a biomarker of effect in response to heavy metals exposure, and as an early sentinel of health consequences, given its involvement in multiple biological processes.

Finally, the organic compounds included in the robust linear regression models (DR Calux, ER Calux, PAH Calux) showed a significant link with genes involved in fertility, embryogenesis, sperm motility, but also oncogenic signal transduction (AURCK), cell cycle progression (UHMK1 and CDKN1A) and cognitive disorders (GPR88).

These results are consistent with the possible action of the compounds as endocrine disruptors (Schug et al., 2011).

In view of these, the multilevel analysis carried out by means of a holistic approach, from the environmental background characterization, through human biomonitoring assessment, to epidemiological considerations, has been able to depict a frame of the regional state of contamination. The spatial and geographical reference given to pollutants allows to identify a new area of interest where compounds associated to industrial plants and the presence of a particular orography of the valleys, could represents a risk for residing people.

The hypothesis is supported by HB data and by an early epidemiological evaluation of incidence rates of diseases potentially related with.

Notwithstanding the preliminary results encourage the theory, the integration of the observational SPES dataset with *in vitro* validation of the effects of compounds, as well as a comparative meta-analysis with other HB or omics datasets, could be useful to consolidate and support the evidence, avoiding false positive correlations, potentially generating from multiple comparisons.

Furthermore, a potential contribute could come from integrative bio-statistics models of data elaboration, considering mixture of elements and their cumulative effects, together with other biomarkers of effects assessed and not object of the present dissertation. Indeed, as previously described (see paragraph 3.1.5), a set of other effect biomarkers are object of further investigations in the SPES protocol: inflammatory cytokines and redox status aimed to explore early evidence of phlogosis and immunity involvement, telomeric length and polymorphisms in order to observe genetic consequences of exposure and individual susceptibility, miRNA to deepen epigenetic alterations, gene expression profiling to identify transcriptomic variations in response to environmental exposure.

Model of big data analysis could be the right choice to implement the multiparametric evaluation, and the integrative approach could provide a fully detailed insight into the biological meaning of exposure to several compounds. Novel biomarkers of diagnostic, prognostic, predictive and therapeutic interest could be found out from this multi-level analysis.

## 5.1 Applications and future perspectives

Considering the long-term purpose of the study, all data collected with the SPES protocol and its integrated approach represent an important contribute to define a full and dynamic map of the environmental pollution of the Campania Region. This could constitute a

scientific support for public health policy and for decision making, giving to the model the chance to be translated in different contexts and for various applications.

Indeed, susceptible cohorts of people, or people residing in critical areas, involved in environmental emergencies for spreading of pollutants, or for the presence of a known source of contamination, could be investigated through this innovative approach.

The systemic overview, from environmental matrices, through biomonitoring and molecular insight, to epidemiology, could be used to characterize the context and acquire the real level of pressure exerted by pollutants in that area or on that population.

From a mere scientific and research point of view, the "one health" perspective, intrinsic in the novel human biomonitoring model, reveals the ability of the methodological approach in integrate different science fields, in order to gain an inter-disciplinary reading and interpretation of phenomena observed.

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