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THE IMPACT OF ENVIRONMENTAL CONTAMINANTS ON HUMAN DNA METHYLATION VARIABILITY: THE ITALIAN CASE OF BUSSI SUL TIRINO

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ABSTRACT

DNA methylation (DNAm) is an epigenetic mechanism that regulates gene expression and is involved in many biological processes, conferring plasticity to the organism. Given the centrality of DNAm in several biological functions, it is well known that its alteration can contribute to the onset of genomic instability and different disease states.

In recent decades, rapid technological progress and increasing industrialization have progressively exposed humans to contaminants never before encountered in nature.

The study of the effects of new pollutants on human variability and health is a growing theme in the field of evolutionary and personalized medicine. A large amount of scientific literature in fact has demonstrated the potential of environmental contaminants to alter DNAm profiles leading to significant impacts on human biology and health, especially in terms of reproductive health, biological aging, and neurological diseases. However, this emerging issue is still highly debated. Therefore, this dissertation focused primarily on a case study of great national public relevance: the Site of National Interest (SNI) of Bussi sul Tirino (2008, Abruzzo, Italy). This area has been exposed for decades to various pollutants (mercury, lead, organochlorinated compounds) due to the improper and illegal disposal of industrial waste in the soil and groundwater of the site. Previous studies conducted in the Bussi sul Tirino SNI have produced conflicting results, and the available data on the effects of contamination on humans in this area are limited. Given all these premises, the hypothesis of this PhD thesis was that prolonged exposure to pollutants could have affected the DNAm variability and health of the population of the Bussi sul Tirino SNI. Therefore, the aim of this study was to investigate the effect of exposure to contaminants on the natural variability and health status of the local population, particularly in terms of DNAm variability.

For this purpose, 61 individuals residing in the area were sampled via buccal swab and divided into two groups according to their degree of exposure to contamination. The study was conducted by analyzing the samples following two different approaches: epigenome-wide and candidate gene. DNAm levels and genetic variability were measured in a subgroup of 33 individuals using the Illumina MethylationEPIC BeadChip (850k) and the Illumina HumanOmniExpress BeadChip (720k), respectively. Meanwhile, the DNAm levels of three repetitive elements in the human genome (LINE-1, Alu, rDNA) were measured in all 61 samples through bisulfite sequencing (Illumina MiSeq). Subsequently, two age- and sex-adjusted linear regression models were implemented: the first revealed 622 CpG sites with different DNAm levels between the two groups, associated with several biological pathways related to development and immune response; the second identified 1,138 CpG sites with differential variability between the two groups, showing reduced variability and

convergence towards similar DNAm values in the high-exposure group. A meQTL analysis conducted on the 622 differentially methylated CpG sites revealed that the differences observed between the two groups were not related to the genetic background, suggesting the central role of the contaminated environment in shaping the DNAm profiles of the local population. The comparison between the two groups regarding the number of mutations and epigenetic lesions did not yield statistically significant results. Similarly, analysis of the epigenetic clocks showed no differences between the two groups, suggesting the contaminants had no effect on the biological aging of the SNI inhabitants. Finally, the analysis of repetitive elements showed differences in DNAm levels between the two groups and a general trend towards hypomethylation in the LINE-1 element of high-exposure individuals, suggesting a potential role of contaminants in generating genomic instability and contributing to the onset of certain diseases.

The presence of environmental pollutants in the Bussi sul Tirino area thus appears to cause variability in the DNAm profiles of the local population, with possible consequences for human biodiversity and health. Future studies will be needed to further investigate these results.

Finally, the purpose of this thesis was also the application of innovative multidisciplinary approaches to promote health safety, environmental protection, and collaboration building. Therefore, two preliminary studies are presented that may find future application in the fields of applied hydrogeology and precision environmental health, as they contribute to increasing knowledge on innovative DNA-based methods for tracking contaminants in groundwater and the role of genetic variability in detoxification from atmospheric contaminants.

1. INTRODUCTION

1.1. Epigenetics and DNA methylation

The term ‘epigenetics’ was coined by British biologist Conrad Hal Waddington (Evesham 1905 - Edinburgh 1975) in 1942 and literally means ‘above (from the Greek epi-) genetics’ (Waddington, 2012). Currently, the term epigenetics refers to a branch of Molecular Biology that studies a series of mechanisms that are triggered by various stimuli - such as diet, chemical and physical environmental factors, aging, and lifestyle in general - that regulate gene expression without altering the DNA sequence (Holliday, 2006; Kobow and Khan, 2024; Lieb et al., 2006). Therefore, epigenetic modifications drive the level of gene transcription and consequently the level of proteins in different tissues, resulting in changes in the phenotype, including the onset of diseases and adaptation to the environment. Furthermore, epigenetic modifications can be transmitted both from the mother cell to daughter cells and between different generations of individuals.

Among the main epigenetic mechanisms are DNA methylation (DNAm), histone modification, and non-coding RNAs (Li, 2021). DNAm is one of the most studied: there are 119,384 papers that have DNAm as a topic in the Web of Science Core Collection as of September 2024.

In mammals, the DNAm reaction involves the covalent addition of a methyl group ($-CH_3$) to the fifth carbon of a cytosine with the formation of 5-methylcytosine (5mC). Addition of the methyl group occurs at the level of CpG (cytosine-phosphate-guanine) dinucleotide by DNA methyltransferase (DNMT), which is an enzyme that employs S-adenosyl-l-methionine (SAM) as a methyl group donor (Chen et al., 2022). The DNAm process can be of two types: I) de novo methylation, which is operated by DNMT3A and DNMT3B, II) maintenance methylation during cell division, promoted by DNMT1 (Okano et al., 1999; Tammen et al., 2013; Zhang et al., 2023), which is important in preserving tissue-specific gene expression profiles.

In the human genome there are approximately 30 million CpG sites, of which 60-90% are estimated to be methylated (Kader and Ghai, 2015). In addition, there are regions 300-3000 bp long, called "CpG islands," that are rich in CpG sites (Kader and Ghai, 2015). However, such regions are associated with 70% of gene promoters (Hughes et al., 2020) and their methylation status tends to change throughout life, with consequences for gene expression. Increased and decreased levels of DNAm at CpG islands located in the gene promoter region can lead to decreased and increased gene transcription, respectively, with important consequences for the organism.

In general, gene promoters can be low in CpG (LCG) or high in CpG (HCG). LCG promoters tend to be hypermethylated and associated with tissue-specific genes, whereas HCG promoters tend to be

hypomethylated and related to broadly expressed genes. It has been shown that this division between LCG promoters and HCG promoters is conserved in several distantly related vertebrate taxa. Therefore, it is possible that the broadly expressed genes selectively avoided DNAm as their silencing would result in disastrous consequences (Elango and Yi, 2008).

Modulation of the DNAm level of intragenic and intergenic regions is also important in regulating gene expression (Tammen et al., 2013). It has been shown that "CpG island shores" - tissue-specific regions up to 2kb distant from CpG islands whose DNAm level is tissue-specific and strongly related to gene expression - in colon cancer tend to exhibit the most DNAm alterations, rather than promoters or CpG islands (Irizarry et al., 2009).

In addition, DNAm is also important for other biological processes, such as genomic imprinting, X-chromosome inactivation, and transposon regulation (Jones and Takai, 2001).

Changes in DNAm levels can be studied at site-specific, region (Differentially Methylated Regions - DMRs), and epigenome-wide levels. At the level of single CpG sites, we can observe Stochastic Epigenetic Mutations (SEMs), or more simply, epimutations. Epimutations are defined as extreme values (outliers) of a reference DNAm interval and are a powerful tool for assessing epigenetic drift typical of aging and DNA damage. Regions rich in epimutations are referred to as epigenetic lesions (Gentilini et al., 2023, 2015). In addition, analysis of the DNAm level of single CpG sites located on Repeat Elements (REs), such as ribosomal DNA tandem repeats (rDNA), Long Interspersed Element-1 (LINE-1), and short interspersed sequence Alu, can be used to study the global methylation level of an individual, as these elements are present in numerous copies in the genome and are located on almost all chromosomes. Specifically, approximately 600,000 LINE-1 repeats, 1,000,000 Alu repeats, and 400 rDNA repeats (coding for RNA45S, the precursor to 18S, 5.8S, and 28S ribosomal RNAs) are found in the human genome (Marson et al., 2023).

Finally, population studies have identified genetic variants (Single Nucleotide Polymorphisms (SNPs)) associated with the DNAm levels of some CpG sites, named methylation Quantitative Trait Loci (meQTLs) (Kassam et al., 2021; Min et al., 2021), which can be used to study the interplay between genome and epigenome.

In this regard, it is important to mention that DNAm can also interfere with the mutation rate in the genome. The normal deamination reaction of cytosines in fact converts unmethylated cytosines into uracils (C → U), which are then removed by the enzyme uracil-DNA glycosylase. However, this process does not succeed in the presence of methylated cytosines (meC), which are instead converted to thymines (meC → T) that cannot be removed from the sequence. Furthermore, the conversion of methylated cytosines (meC → T) occurs at a rate 2-3 times higher than that of unmethylated cytosines

(C → U), making CpG sites methylation one of the main causes of the increased rate of C → T point mutations (Agarwal and Przeworski, 2021; Coulondre et al., 1978; Duncan and Miller, 1980; Xia et al., 2012; Zhou et al., 2020). In addition, the mutation rate appears to be higher for CpG sites with a low or intermediate DNAm level and more evident in intergenic and intronic regions compared with promoter regions and CpG islands (Xia et al., 2012). Therefore, changing DNAm levels may contribute to increasing the mutation rate in the genome.

1.2. DNA methylation and environmental contaminants

Like all other living beings, *Homo sapiens* also had to implement various survival strategies to cope with the challenges of the environment. In the course of its short evolutionary history, it has been exposed to dust from the formation of deserts and savannas, smoke from the use of fire, toxic substances caused by thermal processes related to food cooking, fecal aerosols, pollen from changes in flora, and various pathogens (Dupont, 2003; Trumble and Finch, 2019; Z. Zhang et al., 2014). Epigenetic mechanisms, and in particular DNAm, have been shown to be sensitive to environmental exposures. Interestingly, DNAm seems to be a key player in recent human history. This molecular mechanism in fact operates as a medium-term response to the environment, pending the activation of slower, longer-lasting genetic mechanisms (Giuliani et al., 2015; Klironomos et al., 2013). The advent of the industrial revolution and the advancement of technology, decade after decade, have exposed modern humans to substances never encountered before, such as microplastics, solvents, pesticides, cigarette smoke, engineered nanomaterials, and air pollution, which appear to alter DNAm profiles (Crews and Gore, 2012; Trumble and Finch, 2019). For example, changes in DNAm levels have been observed in individuals exposed to 2,3,7,8-tetrachlorodibenzo-paradioxin (TCDD), Agent Orange (a chemical used during the Vietnam War), cigarette smoke, diesel, glyphosate (a pesticide), and arsenic (As) (Alegría-Torres et al., 2016; Galanter et al., 2017; Giuliani et al., 2018; Gonzalez-Cortes et al., 2017; Lucia et al., 2022; Rojas et al., 2015).

There are different studies, mainly on humans and mice, that have explored the interaction between new environmental agents and DNAm variability, investigating their implications for human health, especially in terms of (I) reproductive health, (II) epigenetic aging, and (III) nervous system disorders (Bolognesi et al., 2022; Martin and Fry, 2018). In the following sections, the available literature on these three topics will be explored. However, it is important to note that studies relating environmental contaminants to DNAm variability and each of these three different types of health impacts are still limited.

1.2.1. DNA methylation, environmental contaminants and human health

Altered gene expression patterns regulated by DNAm can lead to the development of autoimmune diseases, cancers and many other pathologies (Zhang et al., 2020). In the last decade, several studies have shown that toxic substances tend to alter DNAm mechanism leading to greater genomic instability with pathological consequences. For example, a 2017 study on lung cancer induced by air pollution with high levels of **benzo(a)pyrene** highlights the latter's contribution in generating abnormal DNAm with consequences for lung cancer development and progression (Jiang et al., 2017). A recent study on dyslipidemia, a disorder characterized by alterations in blood lipid levels (triglycerides and cholesterol), suggested that the risk of dyslipidemia associated with **cadmium** (Cd) exposure could be mediated by DNAm (Zhang et al., 2024). A study by Zhang and colleagues on arsenism, a pathological condition caused by chronic exposure to **As**, reported promoter methylation and exon 5 methylation rates of the p53 gene to be positively and negatively correlated, respectively, with the degree of arsenism in the individuals studied. Furthermore, it was observed that the mutation rate of exon 5 was positively correlated with the degree of arsenism and associated with hypermethylation of the gene promoter and hypomethylation of exon 5 (Zhang et al., 2011). Furthermore, a study on the interaction between **metals**, such as As and Cd, and human health has shown alterations in methylation profiles in biological pathways related to cancer and cardiovascular diseases (Wang et al., 2023). Another study investigated the effect of fetal exposure to **air pollution** reporting a positive correlation between a high mutation rate in Alu sequence, hypermethylation of the promoters of seven DNA repair and anti-cancer genes, and exposure to fine particulate matter ($PM_{2.5}$). Therefore, it is possible that variation in the DNAm levels of genes important for DNA repair may cause an alteration in the body's defence mechanisms, increasing the overall mutation rate in the genome (Alu) (Neven et al., 2018). Finally, a study on the exposure of petrol station workers to the mixture of Benzene, Toluene, Ethylbenzene and Xylene (**BTEX**) contained in petrol revealed hypermethylation of the p16INK4A gene in exposed individuals, which correlated with chromosomal abnormalities and miscarriage (Silvestre et al., 2020).

Collectively, these findings highlight the interdependence between genetic background and DNAm, describing an unstable scenario in which DNAm, influenced by exogenous factors such as environmental contaminants, tends to vary with potential consequences for human health.

1.2.1.1. DNA methylation, environmental contaminants and reproductive health

In recent years, an increase in infertility has been observed worldwide. The infertility phenomenon affects about 13% of females and 10% of males (Barbieri, 2019). In industrialized countries 17% of

couples need help because of infertility, while in developed countries between 80 and 90% of couples only manage to conceive after 1 year (Bhattacharya et al., 2010). According to data from the World Health Organization (WHO), in 2008 the rate of male infertility in infertile couples was around 30%, making male infertility one of the most common reproductive disorders (Agarwal et al., 2015; Luján et al., 2019; Zhang et al., 2019). Female infertility, on the other hand, is estimated to affect one in seven couples in the Western world and one in four in developing countries. In Central and Eastern Europe, Central and South Asia, the Middle East, North Africa, and some sub-Saharan African countries, female infertility rates reach 30% (Mascarenhas et al., 2012).
In this scenario, increasing exposure to chemical-toxic substances, such as endocrine disruptors, heavy metals, and cigarette smoking, resulting from the modern world's increasing industrialization has been associated with reproductive disorders (poor sperm quality, low sperm count, loss of the pre-implantation embryo, miscarriage). In this equation again the alteration of DNAm profiles, in all life stages, plays a central role.

Mature spermatozoa result from a series of molecular and morphological modifications of primordial germ cells, which also involve DNAm. Alterations in methylation profiles induced by the environment during spermatogenesis could impair reproductive success. Similarly, oogenesis works in females. However, differently from males, oocytes remain demethylated until puberty, making the whole period of childhood a critical phase for methylation profiles as they are susceptible to environmental exposures (Krajnik et al., 2023; Saftić Martinović et al., 2024).

Different studies have shown that there are several chemicals that can alter DNAm profiles, negatively interfering with the reproductive capacity of males and females. Below are some examples. Para-dichlorodiphenyltrichloroethane (**DDT**) is an organochlorine pesticide used in some developing countries to combat malaria (Aneck-Hahn et al., 2007). Although it is banned in many countries, DDT can still spread to other parts of the world, as it is carried over long distances by weather patterns and ocean currents (Lismer et al., 2024). This compound can cause negative effects on both male and female fertility. A study conducted on the exposure of pregnant rats (F0) to DDT for example observed in the F1 male offspring an increase in prostate disease and in the F3 male offspring (indirect exposure) an increase in testicular disease, apoptosis of sperm cells, reduced sperm count, and DMRs in spermatozoa (Skinner et al., 2013). The harmful effects of early DDT exposure have also been observed in female offspring, with increased polycystic ovary in the F1 generation and increased uterine infection in the F3 generation (Skinner et al., 2013).

Equally dangerous is DDT's metabolite, p,p‘-dichlorodiphenyloxydichloroethylene (**p,p’-DDE**), which can lead to hypomethylation of the IGF2 imprinted gene in spermatozoa of rats exposed in utero, resulting in altered testicular histology and male fertility (Song et al., 2014).

It has also been shown that two other metabolites of DDT, 2,2-bis(p-chlorophenyl)-acetic acid (**DDA**) and 1-chloro-2,2-bis-(p-chlorophenyl) ethylene (**DDMU**), can interfere with DNMT1 activity. Indeed, these compounds bind to DNMT1, changing the conformation of its catalytic domain and altering DNAm levels in the promoters of genes involved in sexual development. This interference also affects the expression of Sox9 and Oct4 genes in the embryo, possibly affecting the survival and health of the offspring (Hu et al., 2021).

The fungicide **Carbendazim** (CBZ), on the other hand, is used in agriculture. Low dosages of this compound are sufficient to cause interruption of spermatogenesis, leading to reduction in sperm number and motility. CBZ also causes a decrease in DNAm levels in the interstitial cells of the testes (Leydig cells), which are the main source of male testosterone (J. Liu et al., 2019).

Another fungicide, **Vinclozolin** (VCZ), causes DNAm modifications in male germ cells that appear to be related to several phenotypic abnormalities, such as prostatic lesions, prostatitis, sperm cell apoptosis, abnormal spermatogenesis, and male subfertility. Early exposure to VCZ also may alter DNAm levels in the imprinted genes H19, Gtl2, Peg1, Snrpn, and Peg3 (Anway et al., 2006; Stouder and Paoloni-Giacobino, 2010).

Bisphenol A (BPA) is used to produce polycarbonate plastics and thermosetting resins. Even for this compound, in utero or neonatal exposure has been shown to alter DNAm, interfering with reproductive function. Specifically, BPA causes hypermethylation of estrogen receptors (ERα and ERβ) and DNMT3A, with negative consequences on spermatogenesis and global DNAm levels (Doshi et al., 2011; El Henafy et al., 2020; Kundakovic et al., 2013; Li et al., 2018). Exposure to BPA can also alter DNAm levels of the proteins mybph (Myosin-Binding Protein H) and prkcd (Protein Kinase C Delta), and LINE-1, resulting in reduced spermatocyte number and motility and sperm quality, respectively (Miao et al., 2014; Yin et al., 2016). In the female setting, however, it has been observed that early exposure to BPA can reduce the DNAm level in the Hoxa10 gene. This gene regulates the development of the embryo's uterus and is necessary for implantation of the embryo into the adult endometrium, therefore an alteration of DNAm in this gene could impair the reproductive capacity of female offspring (Bromer et al., 2010).

Polychlorinated biphenyls (**PCBs**), chlorinated hydrocarbons used in industry, also have adverse effects on male and female fertility. In particular, prenatal exposure to 2,3′,4,4′,5-pentachlorobiphenyl (PCB118) can cause decreased expression of DNMT1, PCNA, and STRA8 (PCNA-dependent

regulatory factor of spermatogenesis) resulting in global DNA hypomethylation in the embryos' testes. These changes in DNAm level are maintained even following sexual maturity, possibly resulting in reduced fertility (Tao et al., 2021). PCB118 also affects female reproductive capacity leading to uterine malformations and embryo implantation failures. This compound is thought to increase the DNAm level, which in turn causes downregulation of the Homeobox A10 (Hoxa10) gene, involved in regulating the uterus' receptivity to the embryo. This results in a higher probability of preimplantation embryo loss and infertility (Qu et al., 2018).

The organochlorine insecticide and acaricide **Endosulfan** has also been shown to alter the DNAm of the Hoxa10 gene, leading to reduced Hoxa10 gene expression and contributing to increased risk of preimplantation embryo loss (Milesi et al., 2022).

In comparison, exposure to another pesticide, **Methoxychlor** (MXC), has been linked to altered DNAm of Estrogen Receptor genes (ERα and ERβ) in the ovaries. This exposure also resulted in increased expression of the Dnmt3b gene and elevated global DNAm levels. Early-life exposure to MXC may therefore increase the risk of developing ovarian dysfunction in adulthood (Zama and Uzumcu, 2013, 2009).

Contaminants interfere with DNAm profiles not only individually but also by acting synergistically. Pre- or neo-natal exposure to Persistent Organic Pollutants (**POPs**), for example, causes altered DNAm levels leading to reduced testosterone levels and low sperm quality. In addition, a reduction in the number of offspring per pregnancy and an increase in pre-implantation embryo loss was also observed (Maurice et al., 2021).

A study on the consumption of food contaminated with a mixture of chemicals, such as **methylmercury**, **PCBs**, **hexachlorobenzene**, **DDT**, **DDE**, and **perfluoroalkyl substances**, observed an association between these substances and genome-wide DNAm level (Leung et al., 2018).

Air pollution can also be considered as a mixture of different pollutants, as it is composed of coarse and fine particulate matter (PM_{10} and $PM_{2.5}$), exhaust gases (e.g., CO_2, SO_2, and NO_2), tropospheric ozone (O_3), heavy metals, and Volatile Organic Compounds (VOCs). Prolonged exposure to PM_{10}, $PM_{2.5}$, SO_2 and NO_2 was associated with reduced sperm motility. In particular, PM_{10} showed a correlation with DNAm loss in sperm (Cheng et al., 2022).

All these data highlight the wide variety of pollutants capable of altering DNAm profiles, causing harmful effects on both male and female reproductive health (Figure 1).

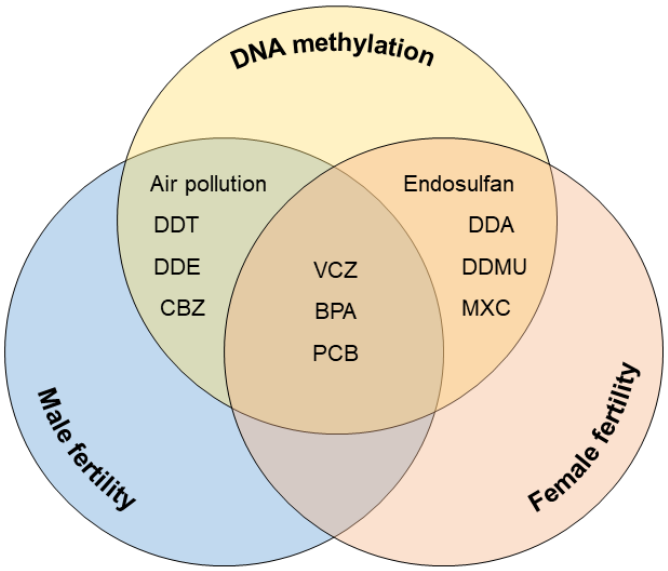

Figure 1. Venn diagram summarizing the principal contaminants mentioned in the text which cause variability in DNAm levels with consequent impact on male and/or female fertility.
DDT: para-dichlorodiphenyltrichloroethane; DDE: p,p'-dichlorodiphenyloxydichloroethylene; CBZ: carbendazim; VCZ: vinclozolin; BPA: bisphenol A; PCB: polychlorinated biphenyl; DDA: 2,2-bis(p-chlorophenyl)-acetic acid; DDMU: 1-chloro-2,2-bis-(p-chlorophenyl) ethylene; MXC: methoxychlor.

1.2.1.2. DNA methylation, environmental contaminants and epigenetic aging

Recently, research has increasingly focused on studying biological aging and preventing the risk of developing age-related diseases. The aging process involves numerous alterations at the cellular and molecular levels, which have been widely documented (López-Otín et al., 2016), leading to the accumulation of damage in tissues. Among these changes, DNAm has emerged as a powerful biomarker of biological aging (Bolognesi et al., 2022). Alterations in DNAm profiles can result from intrinsic factors, such as stochastic and genetic influences, but also from extrinsic factors, such as environmental exposure (Duan et al., 2022).

One of the most effective and popular tools for measuring biological age from DNAm levels are so-called "epigenetic clocks", mathematical algorithms that combine DNAm values from specific CpG sites that vary predictably during the lifespan (Bolognesi et al., 2022; Roberts et al., 2021). The first epigenetic clock was developed in 2013 (Horvath, 2013), followed by several subsequent models (Chen et al., 2016; Horvath et al., 2018; Levine et al., 2018; Lu et al., 2019; Sabbatinelli et al., 2024). Specifically, there are different generations of epigenetic clocks, each characterized by different methods, approaches and applications. First-generation epigenetic clocks such as those developed by Horvath and Hannum are based on models that aim to predict chronological age (Ferreri et al., 2024;

Hannum et al., 2013; Horvath, 2013). Second-generation clocks such as *GrimAge* and *PhenoAge*, on the other hand, are evolutions of earlier clocks based on more complex models that consider biological and environmental factors associated with aging other than chronological age, such as mortality or the incidence of chronic diseases (Ferreri et al., 2024; Levine et al., 2018; Lu et al., 2019). Finally, third-generation clocks, such as *DunedinPACE*, estimate the pace of biological aging - years of epigenetic aging per chronological year, and not a static biological age relative to the time of measurement - using models that consider the rate of physiological decline over time caused by age-related diseases, socio-economic conditions and lack of geroprotective interventions (Aroke et al., 2024; Belsky et al., 2020; Ferreri et al., 2024).

Each biological tissue has a tissue-specific methylation profile that can be affected differently by aging processes. Therefore, epigenetic clocks, being built on methylation data, are also suitable for certain tissues. Horvath's clock, for example, is considered a pan-tissue epigenetic clock, i.e. a clock applicable to all tissue types (Horvath, 2013). Hannum's clock (Hannum et al., 2013) and the *PhenoAge* (Levine et al., 2018), *GrimAge* (Lu et al., 2019) and *DunedinPACE* (Belsky et al., 2020) clocks, on the other hand, are better suited to blood samples. Finally, a second clock developed by Horvath, called the *skin & blood clock*, works best when applied to data from cultured cells (Horvath et al., 2018).

Recently, several studies have evaluated the impact of various environmental contaminants on biological aging, using different epigenetic clocks.

Industrial solvents such as **benzene** and trichloroethylene (**TCE**), for example, have been associated with DNAm changes in blood, resulting in accelerated biological age according to the Hannum and Skin-Blood clocks (Van Der Laan et al., 2022). Similarly, **$PM_{2.5}$**, a major component of air pollution, has been shown to affect epigenetic age in blood samples. Specifically, through the Levine epigenetic clock, an acceleration of aging of about 6 years was observed following exposure to crustal-rich $PM_{2.5}$, an acceleration of about 2 years with sulfur-poor $PM_{2.5}$, and a deceleration of about 1 year with nitrate-poor $PM_{2.5}$ (White et al., 2019). Other components of air pollution, such as carbon monoxide (**CO**), **PM_{10}**, and **O_3**, also appear to accelerate epigenetic age, as evidenced by the Horvath and Skin and Blood epigenetic clocks in blood samples (Lee et al., 2024). In addition, a recent study of serum samples from a Taiwanese cohort showed the acceleration of epigenetic age following exposure to $PM_{2.5}$ and O_3 (Huang et al., 2025). Similarly, **As** exposure resulted in accelerated epigenetic age, as measured by the Hannum and Skin and Blood clocks (Bozack et al., 2022). In addition, exposure to Polycyclic Aromatic Hydrocarbons (**PAHs**), carcinogenic organic compounds resulting from incomplete combustion of solid fuels used for domestic purposes, was also associated with

acceleration of biological age in blood samples, as detected via the GrimAge clock (Blechter et al., 2023). Furthermore, exposure to polybrominated biphenyl-153 (**PBB-153**), an endocrine disruptor used as a flame retardant in plastics, has been shown to accelerate epigenetic aging according to the next-generation CheekAge clock (Shokhirev and Johnson, 2025). Finally, a correlation has been reported also between exposure to **organochlorine pesticides**, in particular p,p′-DDE and transnonachlor, and an increase in epigenetic aging, as calculated by the Hannum clock in blood cells (Lind et al., 2018).

Taken together, these findings suggest that exposure to toxic substances can significantly influence the epigenetic aging process. However, it is essential to consider that the various epigenetic clocks developed and the type of tissue analyzed may lead to variations in the results obtained, as they show different aspects of the biological aging process.

1.2.1.3. DNA methylation, environmental contaminants and nervous system disorders

A recent study published in The Lancet Neurology reveals that in 2021, over 3 billion people worldwide were living with a neurological condition. More than 80% of deaths and health losses caused by neurological diseases occur in low- and middle-income countries. Additionally, in high-income countries, the number of neurologists per 100,000 people is up to 70 times higher than in low- and middle-income countries, thus ensuring greater access to care (Steinmetz et al., 2024).

Nervous system disorders are varied and include: neurodevelopmental disorders, such as attention deficit hyperactivity disorder, autism spectrum disorder, and idiopathic intellectual disability; age-related neurodegeneration, such as Alzheimer’s disease, dementia, stroke, and Parkinson’s disease; and recently emerged conditions, such as post-COVID-19 cognitive decline (Steinmetz et al., 2024). DNAm is essential for human brain development, influencing synaptic plasticity, learning, and memory processes, and is also associated with the onset of various neurological disorders, especially following exposure to toxic substances (Feng et al., 2007; Lister et al., 2013). In fact, although it is difficult to identify clear causal associations between exposure to pollutants and the onset of neurological diseases through the alteration of epigenetic mechanisms such as DNAm, an increasing number of studies are observing possible correlations.

One of the most studied neurodegenerative diseases is Parkinson's disease, a condition that leads to the death of dopaminergic neurons resulting in loss of control of movement and balance. Prenatal exposure to **dieldrin**, an organochlorine pesticide, has been shown to alter DNAm levels of the Nr4a2 and Lmx1b genes, which are involved in the development of dopaminergic neurons. Therefore, the early exposure to this toxicant appears to increase the risk of developing Parkinson's disease in

adulthood (Kochmanski et al., 2019). According to a recent study, the risk of developing Parkinson's could also be associated with changes in DNAm levels caused by exposure to **lead** (Pb), a neurotoxic heavy metal that can cross the blood-brain barrier and enter neurons and neuroglia, thus interfering with neurotransmitter release and energy metabolism (Paul et al., 2021).

Pb exposure also appears to influence the onset of Alzheimer's disease, which is the most common neurodegenerative disease in the world. It is estimated that around 55 million people worldwide are affected by Alzheimer's disease today and that this number is expected to increase dramatically (D'Cruz and Banerjee, 2021). Alzheimer's disease is characterized by the accumulation of beta-amyloid plaques, aggregations of the hyperphosphorylated tau protein in neurofibrillary tangles within neurons, and loss of synapses, resulting in memory loss and personality changes (Bihaqi et al., 2014). It has been observed that some genes related to Alzheimer's disease are overexpressed in later life as a result of exposure to Pb in youth. These genes are rich in CpG sites, therefore it is possible that their overexpression is caused by a loss of DNAm triggered by exposure to the contaminant (Bihaqi et al., 2014).

Some studies suggest that changes in DNAm profiles caused by **Pb** exposure may contribute to the onset of autism spectrum disorders, which are neurological developmental disorders characterized by difficulties in social interactions and repetitive, stereotypical behaviors (Keil and Lein, 2016; Nazeer and Ghaziuddin, 2012; Senut et al., 2014; Sherman et al., 2022). Indeed, it appears that exposure of embryonic stem cells to this toxicant leads to reduced expression of the PAX6 and MSI1 genes that regulate brain development and altered DNAm levels in more than 1000 genomic regions important for neurodevelopment, calcium ion uptake, actin cytoskeleton and neuronal projections, resulting in abnormalities of neuronal connectivity (Keil and Lein, 2016; Penzes et al., 2011; Senut et al., 2014; Stamou et al., 2013). Similarly, it has been shown that maternal exposure to high concentrations of di-(2-ethylhexyl) phthalate (**DEHP**), a plasticizer used in the production of cosmetic and food packaging, can alter placental methylation profiles with the possibility of predisposing the fetus to neurological disorders, such as autism and dementia (Tran et al., 2023).

Another contaminant that has shown associations with neurological disorders through altered epigenetic mechanisms is **BPA**. Indeed, a study on mice has observed that prenatal exposure to this toxicant alters Dnmt1 and Dnmt3a levels in the brain, and increases the expression of the SLC1A1 gene, implicated in the regulation of the neurotransmitter glutamate, in the brains of female mice, possibly affecting behavior and social interactions (Wolstenholme et al., 2011).

Finally, a recent study on exposure to **air pollution** identified DNAm changes in genes associated with neuronal functions, the dopaminergic system and neurodegenerative diseases (Honkova et al., 2022).

Collectively, these studies emphasize the significant impact environmental contaminants can have on neurological health by altering DNAm levels, especially during the crucial early stages of development (Figure 2).

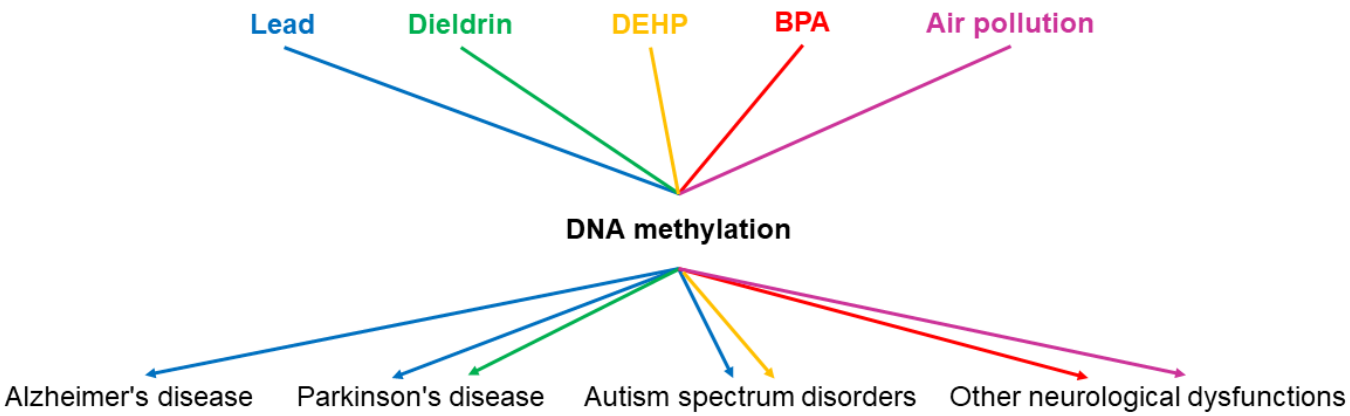

Figure 2. Schematic summary of the existing literature on the potential of certain contaminants to contribute to the onset of determined neurological disorders through alteration of DNAm. DEHP: di-(2-ethylhexyl) phthalate; BPA: bisphenol A.

1.3. The Italian case study of Bussi sul Tirino

Polluted sites represent one of the most serious aspects of the global environmental crisis. The accumulation of chemical-toxic contaminants in specific areas can compromise human health, biodiversity, and the quality of ecosystems for decades (Landrigan et al., 2023). In particular, heavy metals and POPs, when accumulated along the food chain or inhaled, can significantly increase the risk of developing serious health conditions.

There are numerous contaminated sites around the world. A famous example is Minamata Bay, Japan, which was severely contaminated in the 1950s by methylmercury in wastewater from the Chisso Corporation. Some fishing communities and domestic animals in the area have suffered from a serious central nervous system disease probably caused by long-term exposure to the contaminated site (Kitamura et al., 2020; Yoshino et al., 2020). Another example is the Niger Delta region of Nigeria, famous for oil extraction. This region over the years has been victim to massive crude oil spills and various anthropogenic activities that have led to severe environmental contamination, in particular by PAHs and heavy metals, such as As, Cd, Mercury (Hg), and Iron (Fe) (Udom et al., 2023; Umeoguaju

et al., 2023). Another well-known case is that of Guiyu, a city in south-eastern China that has been recognized since the late 1980s as a major site for the dismantling and recycling of electronic waste. Over time, the unregulated activities conducted in Guiyu have led to severe environmental pollution, especially by Pb, which appears to have contributed to several health disorders of the resident population (Huang et al., 2021). Finally, a pilot study on the community of Martinsville, a city in the state of Indiana (USA) characterized by groundwater contaminated mainly by tetrachloroethylene (PCE), TCE, and other VOCs, found PCE in both the tap water and the exhaled breath of the local population. Furthermore, according to this study, PCE was detected in at least another 949 of the 1854 United States Environmental Protection Agency (USEPA) Superfund sites, proving to be a complex and widespread problem (Liu et al., 2022).

In Europe, according to the European Environment Agency (EEA), around half of the industrial facilities responsible for the highest emissions of pollutants into the environment are located in the United Kingdom, Germany, Poland, and France (European Environment Agency).

In Italy, extended contaminated areas of high health and ecological risk are defined by the Ministry of Environment and Energy Security as "Sites of National Interest" (SNIs), recognizing the urgent need for remediation and safety measures. However, the process of land remediation is highly complex and requires significant financial investment and innovative solutions that consider the interconnection between the environment, health, and social development (Garg et al., 2022; Kumar et al., 2022; Narayanan and Ma, 2023; Roy et al., 2023).

As of December 2024, there are 42 SNIs in Italy, including the SNI of Bussi sul Tirino.

In fact, in 2008, the industrial site of Bussi sul Tirino, located in the province of Pescara, in the Abruzzo region of central Italy, was added to the list of SNIs (Italian Ministerial Decree of May 29, 2008) due to the high levels of contaminants found in the soil and groundwater (Guerranti et al., 2017; Ministero dell'Ambiente e della Sicurezza Energetica, n.d.; Vitali et al., 2021). The Bussi sul Tirino SNI spans across the Pescara River valley, which connects the central Apennines to the Adriatic Sea, and occupies an area of about 236 ha (Italian M.D. No. 49 of Jan. 27, 2021), embracing 11 municipalities: Bussi sul Tirino, Popoli, Tocco da Casauria, Castiglione a Casauria, Bolognano, Torre de' Passeri, Alanno, Scafa, Manoppello, Rosciano, and Chieti (Luchetti et al., 2021; Ministero dell'Ambiente e della Sicurezza Energetica, n.d.).

At the junction of the Tirino and Pescara rivers lies the industrial hub of Bussi sul Tirino, where various chemical industries have operated since the early 1900s (Di Molfetta and Fracassi, 2008). In the 1960s, after the post-WWII reconstruction, the Montecatini company (later Montedison) took control of the Bussi sul Tirino chemical complex. Ownership shifted in 2002 to the multinational

Solvay, and in 2016 to Società Chimica Bussi (Ministero dell'Ambiente e della Sicurezza Energetica, n.d.). Currently, the industrial pole is mostly dismissed, with some residual active processes, including a power plant and a chemical facility, which manufactures chlorinated compounds, fungicides, anti-knockout agents, and other chemicals (Zona et al., 2023).

Over the years, substantial quantities of industrial waste have been illegally disposed of and spread around the plants. Especially, in the Tirino river, in the Tre Monti dump, and in areas adjacent to the former Montedison and Montecatini plants. This has resulted in extensive soil and groundwater contamination (Guerranti et al., 2017; Milan et al., 2019; Ministero dell'Ambiente e della Sicurezza Energetica, n.d.). The hydrogeological conditions of the area further facilitated the spread of contaminants in groundwater, which eventually reached the main local drinking water source, namely the Colle Sant'Angelo well field, located 2.2 km downstream from the Tre Monti landfill (Di Curzio et al., 2018; Filippini et al., 2018). Between 1980 and 2007, these wells, connected to the Giardino aqueduct network, supplied water to approximately 700,000 inhabitants of the area, thereby exacerbating the contamination problem (Di Molfetta and Fracassi, 2008; Filippini et al., 2018; Vitali et al., 2021).

The first traces of contamination in drinking water, especially chlorinated solvents, were detected in 1992 through water analyses from the well field (Filippini et al., 2018). However, it is certain that chlorinated solvents were already present in the wells since their construction in the early 1980s, as industrial byproducts from the production of chloromethanes (chlorinated pitches) had been buried in the Tre Monti dump since the 1970s. Citizens in the province of Pescara thus unknowingly used contaminated water for about a decade before the contamination of the aqueduct was brought to light. Subsequent inspections identified the presence of various pollutants in both soil and water in the Bussi sul Tirino SNI. Specifically, we refer to water found under or near the chemical hub or landfills as "groundwater" and water found in aqueduct wells as "drinking water". Metals, such as **Fe**, **Hg**, **Pb**, **aluminum**, **As**, and substances like **PAHs**, **BTEX**, and **chlorinated aliphatic compounds** were found in both soil and groundwater. **Boron**, **dichloromethane**, **hexachloroethane**, **hydrocarbons** were found only in groundwater. **Carbon tetrachloride**, **PCE**, **TCE**, and **trichloromethane** were found in both groundwater and drinking water. Finally, **dioxins** have been identified only in soil and subsoil. The contaminants present in the highest concentrations in soil and subsurface layers were Pb and Hg, while in drinking water, the most abundant contaminant was TCE. In addition, carbon tetrachloride, dichloromethane, and hexachloroethane were present in amounts above the limits defined by the Italian National Institute of Health (Istituto Superiore di Sanità (ISS)) (Filippini et al., 2018; Ministero dell'Ambiente e della Sicurezza Energetica, n.d.; Vitali et al., 2021).

In 2017, a decade after the illegal dumping was discovered and following three levels of legal proceedings, the criminal court found the company responsible for the site guilty of unintentional environmental disaster and poisoning public water supplies. Currently, a civil lawsuit is underway between the Ministry of Environment and the company to seek compensation for the environmental damage.

Although a wide range of pollutants and a vast affected area are present, some studies on the Bussi sul Tirino SNI have reported low concentrations of pollutants or inconsistent results. Contaminants in food (eggs from free-range chickens, milk from grazing sheep and goats), in environmental matrices (water and outdoor air), and in the tissues of bioindicator species (earthworms, barbel, trout, and coot), for example, generally showed levels that comply with European standards or were lower than those reported in existing literature (Castellani et al., 2023, 2021; Guerranti et al., 2017; Vitali et al., 2021). However, barbel exhibited higher concentrations of MEHP (the primary metabolite of di-2-ethylhexyl phthalate), polybrominated diphenyl ethers (PBDEs), PAHs, Hg, and Pb, while earthworms showed elevated levels of PCBs and Cd (Guerranti et al., 2017). Furthermore, analysis of wild edible mushrooms revealed an average Hg concentration higher than those found in unpolluted or industrial areas, although still lower than levels recorded near a Hg mining site in Slovakia (Vitali et al., 2021). Lastly, urine tests performed on the local population showed slightly higher average levels of Pb and Hg compared to American populations, although Pb levels were consistent with those of the general Italian population (Vitali et al., 2021).

All these data collectively suggest a level of contamination that is still unclear and needs further investigation, particularly concerning the potential effects of past exposure on human biodiversity and health (Giuliani et al., 2018). In addition, it is important to consider that the samples used in the cited studies were collected between 2010 and 2018, which is several years after the site was secured in 2007, implying that the influence of pre-existing contamination that could have persistent effects on human biology cannot be excluded.

1.3.1. Epidemiology of the Bussi sul Tirino SNI

The SENTIERI Project (National Epidemiological Study of Territories and Settlements Exposed to Pollution Risk) is an innovative and informative project that since 2011 has provided information on the health status of a large part of the Italian population living in contaminated sites.

The most recent report on the Bussi sul Tirino area (SENTIERI 2023) reported a 5.5% (8.7 per 100,000 cases) increase in the rate of premature mortality from chronic diseases in the female population compared to the regional reference (Zona et al., 2023). Mortality from all types of cancers

has also increased in the female population, with an increase in stomach and breast cancers associated in the literature with the presence of chemical plants and landfills, respectively. In the male population, on the other hand, diseases of the respiratory system were in excess. For both genders, however, deaths from bladder cancer and non-Hodgkin's lymphomas associated with exposure to the polluted environment were increasing (Zona et al., 2023) (Table 1).

The hospitalization rate for diseases of the circulatory, urinary (particularly nephritis, nephrotic syndrome, nephrosis, and chronic renal failure), digestive, and respiratory systems was in excess among both males and females. Hospitalizations for colorectal cancer, lung cancer, and respiratory diseases associated with exposure to the contaminated site environment instead increased for both sexes. In contrast, for women only, there were excess hospitalizations for breast and bladder cancer (Zona et al., 2023) (Table 1).

Relative to the pediatric-adolescent-youth age category, three deaths between the ages of 20 and 29 from diseases of the circulatory system were recorded (Zona et al., 2023).

The number of hospitalizations for all natural causes in the first year of life instead was in excess compared with the regional population, with an increase also in morbid conditions of perinatal origin. In the pediatric age (0-19 years), there were an overall excess of malignant tumors and an excess of brain tumors and lymphomas in males only. The excess of malignant tumors persisted only for the male population in the pediatric-adolescent age group. Finally, in young age (20-29 years) there was an excess of hospitalizations for diseases of the circulatory system and cancers of the lymphohematopoietic system (non-Hodgkin's lymphomas in female and leukemias in males) in both sexes (Zona et al., 2023) (Table 1).

According to the SENTIERI report, considering the long latency period of many of these pathologies and the half-life of the pollutants present at the site, the excesses currently observed could be the result of past exposures. This highlights the importance of continuing to monitor the health status of the exposed population in order to identify potential groups of individuals who may still be at risk of developing specific pathologies, with particular reference to the areas served by the Giardino aqueduct (Zona et al., 2023).

Table 1. Standardized Mortality Ratio (SMR) and Standardized Hospitalization Ratio (SHR) (90% confidence interval (90% CI)) for diseases mentioned in the text, divided into pediatric age (0-19 years), young age (20-29 years), and general population (GP).

Pathology	SMR (90% CI)				SHR (90% CI)					
	20-29 yrs		GP		0-19 yrs		20-29 yrs		GP	
	F	M	F	M	F	M	F	M	F	M
Stomach cancer			106							
Breast cancer			120						103	
Diseases of the respiratory system				103					104	109
Bladder cancer			222	116					109	
Non-Hodgkin's lymphomas			102	118			301			
Diseases of the circulatory system	334						123		110	105
Diseases of the urinary system									110	111
Diseases of the digestive system									101	103
Colorectal cancer									102	107
Lung cancer									109	106
All natural causes					110					
Morbid conditions of perinatal origin					140					
Cancer					140					
Brain cancer						251				
Lymphomas						251				
Cancers of the lymphohematopoietic system							164			
Leukemia								498		

2. AIM OF THE STUDY

The emerging field of precision environmental health proposes an integrated approach that combines multiple pollutant analysis with multi-omics methodology in order to gain a wide and deeper understanding of the effects of contaminant exposure on human biological variability and health. As mentioned earlier, to date several studies have pointed to the potential of environmental contaminants to interfere with epigenetic mechanisms, and in particular with DNAm, at different stages of life and with different outcomes, including the onset of certain diseases (Di Criscio et al., 2023; Ho et al., 2006; Koestler et al., 2013; Martin and Fry, 2018; The BIOS consortium et al., 2019). In particular, some studies have focused on the variability of DNAm levels in REs, such as LINE-1 and Alu, which, being present in multiple copies in the human genome, can have a widespread impact when affected by environmental contaminants, with the probability of contributing to the onset of pathological conditions (Antelo et al., 2012; Chaiwongkot et al., 2022; Kitahara et al., 2020; Miao et al., 2014; Munnia et al., 2023; White et al., 2016; Yang, 2004; Ye et al., 2020).

Given the multiple effects of environmental pollutants on human biology (reproductive health, biological aging, nervous system disorders) and the limited and conflicting information currently available regarding their impact, particularly chlorinated solvents, on the residents of one of the most well-known contaminated sites in Italy, the hypothesis of this dissertation was that exposure to pollutants could have caused widespread alterations in human DNAm with possible implications for aging and, more generally, the health status of the population of the Bussi sul Tirino SNI. Therefore, the main aim of this study was to investigate the variability of DNAm profiles of the inhabitants of the Bussi sul Tirino SNI, specifically by studying DNAm at the **epigenome-wide** and **candidate genes** levels, thus contributing to a deeper understanding of the potential effects of pollutants improperly and illegally dumped in the area on local human biodiversity and health.

For this purpose, 61 volunteers from the Bussi sul Tirino SNI with different levels of exposure to pollutants were considered. The epigenome-wide analysis focused on differentially methylated positions and regions (DMPs, DMRs, gene ontology, meQTLs, DVPs), epigenetic aging, and epimutations/epigenetic lesions. The candidate genes analysis, on the other hand, focused on three different REs (rDNA, LINE-1, and Alu) (Figure 3).

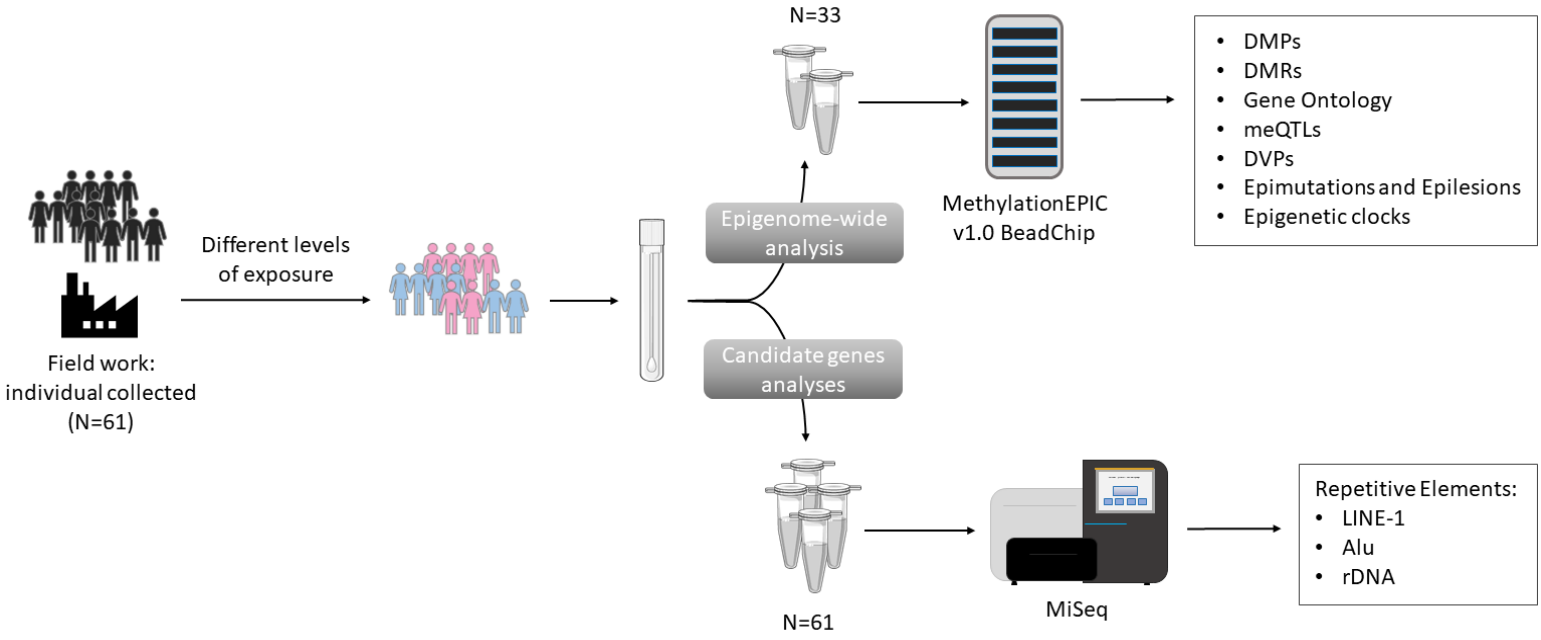

Figure 3. Flowchart of the analysis conducted to assess the impact of the contamination present in the Bussi sul Tirino SNI on the DNAm profiles of the local population, at the epigenomic and specific genes levels. DMPs = Differentially Methylated Positions; DMRs = Differentially Methylated Regions; meQTLs = methylation Quantitative Trait Loci; DVPs = Differentially Variable Positions; LINE-1 = Long Interspersed Nuclear Element-1; Alu = short interspersed sequence Alu; rDNA = ribosomal DNA tandem repeat. See the following chapters of the manuscript for further details. Figure assembled and adapted from SMART (https://smart.servier.com/) and FLATICON (https://www.flaticon.com/).

In order to identify the dynamics of contamination distribution and thus perform a more specific characterization of the individuals sampled, an experimental protocol for tracing groundwater flow through DNA tracers was also developed and tested in parallel with this pivotal study.

Finally, in order to investigate the close relationship between DNAm and the human genome in relation to environmental contaminants, an analysis of European genetic variability was performed in regard to exposure to air pollution, one of the most widespread and studied contaminants today.

3. MATERIALS AND METHODS

3.1. Samples description

In order to study the variability of DNAm profiles of the population exposed to contaminants in the Bussi sul Tirino SNI, oral mucosa cells of 61 individuals were sampled through buccal swabs. Specifically, in October 2019, 30 volunteers were recruited in the municipality of Rosciano (Pescara province, Italy), and in February 2020, 31 volunteers were recruited in the municipality of Torre de' Passeri (Pescara province, Italy) (Figure 4 and Figure S1). The sampled cohort consisted of 19 healthy female and 42 healthy male individuals, with a mean age of 51.95 years (± 13.26 SD). The average duration of exposure to contaminants was 18.68 years (± 4.08 SD), calculated considering that the Colle Sant'Angelo well field was active since the early 1980s and that a significant level of contaminants was certainly present in the wells until the early 2000s, as techniques to reduce contaminant concentrations (e.g., the use of activated carbon for water purification) were implemented at the turn of the century.

All study participants provided informed consent and completed a bio-demographic questionnaire designed to collect individuals' personal data and some information about their lifestyle.

Donor privacy was ensured by labeling the collected biological samples with alphanumeric codes.

Finally, the samples were stored at the Laboratory of Molecular Anthropology and Centre for Genome Biology of the University of Bologna and their use was approved by the Bioethics Committee of the University of Bologna (11/11/2015).

Individuals' DNA was then extracted from buccal cells using the QIAamp DNA Mini kit (QIAGEN, Germany) and quantified using the Qubit fluorometer (ThermoFisher Scientific, USA).

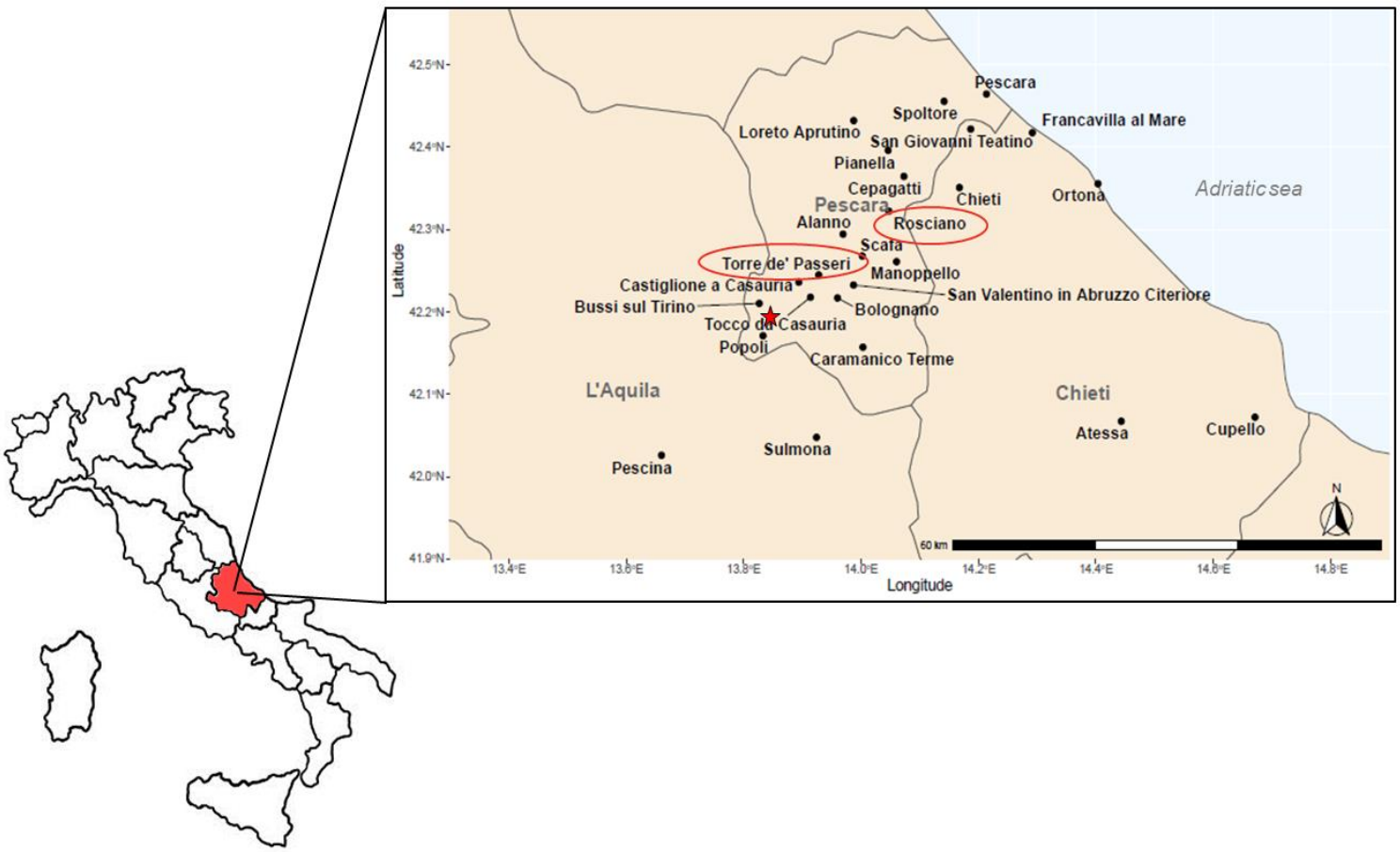

Figure 4. Geographical location of the municipalities included in the SNI of Bussi sul Tirino. The grey lines delimit the provinces of the Abruzzo region, which is highlighted in red on the map of Italy; the red star indicates the location of the industrial pole of Bussi (located near the Colle Sant'Angelo well field); and the two red circles identify the two municipalities where recruitment took place.

3.1.1. Samples classification method

Individuals sampled were divided into two groups using information collected through the questionnaires, including birthplace, residence, and workplace. Specifically, assuming a higher probability of exposure for municipalities located in proximity to the source of contamination and considering a map of the Giardino aqueduct (Figure 5) - as the most likely exposure is ingestion of polluted groundwater - each municipality was assigned a score of 1 if located along the first portion of the main distribution line of the Giardino aqueduct (i.e., from the municipality of Popoli to the municipality of Alanno, excluded), and a score of 0 if located along the second section of the Giardino aqueduct map (from the municipality of Alanno on downstream). This approach is based on the assumption that municipalities farther from the contaminated area and located in zones where the

aqueduct consists of more branches and alternative sources are less susceptible to contamination, as the contaminants in the water are more diluted.

In this way, each individual was assigned three scores: one for the municipality of birth, one for the municipality of residence, and one for the municipality of work. These three scores were then summed to obtain a unique score for each individual, reflecting the overall level of exposure.

Finally, individuals with a score < 2 (N=31) were classified as having a Low Level of Exposure ("LLE" group), while those with a score ≥ 2 (N=30) were classified as having a High Level of Exposure ("HLE" group) to the contaminants present in the Bussi sul Tirino SNI.

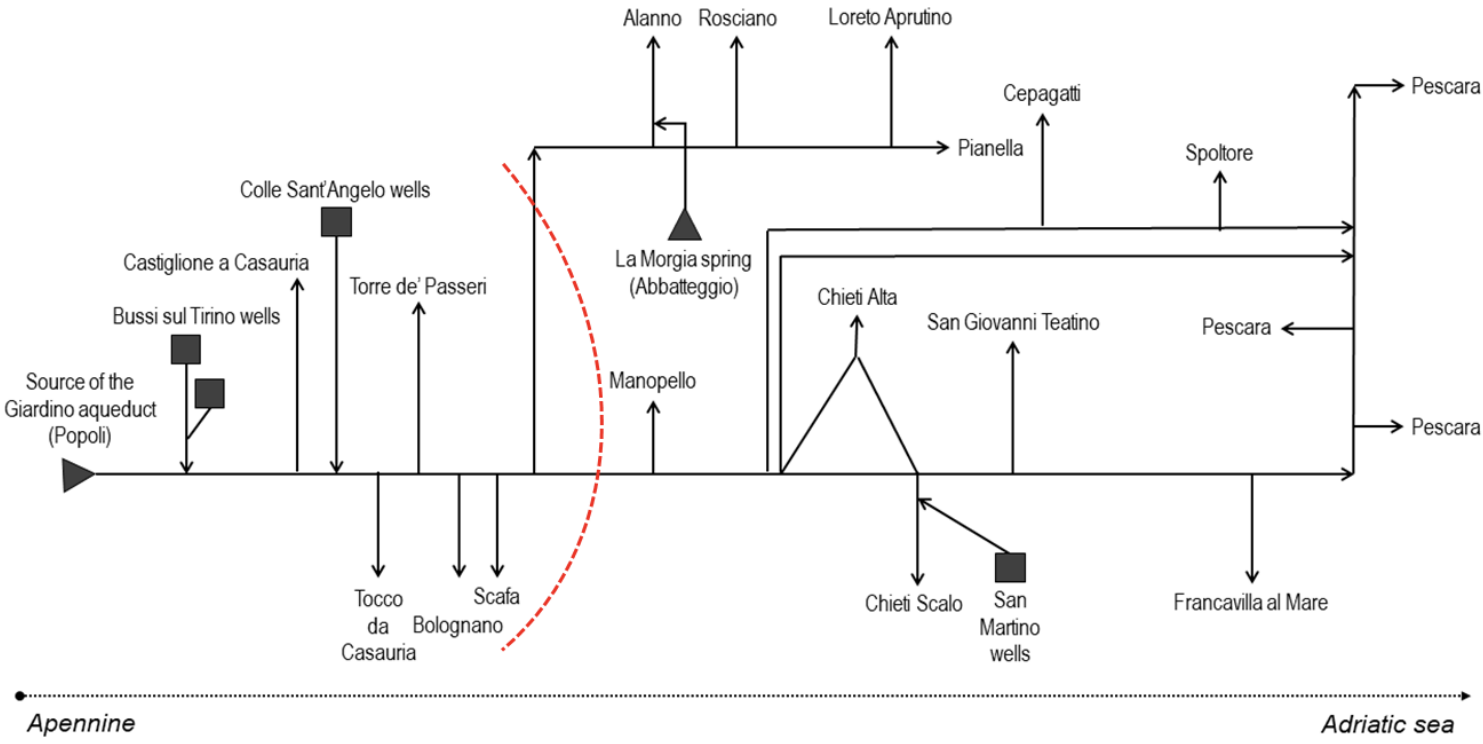

Figure 5. Reconstruction of the Giardino aqueduct map, where arrows indicate the direction of water flow, triangles indicate water sources, squares identify well fields, and the dashed red line indicates the division between HLE and LLE municipalities (adapted from Ing. Livello - A.C.A. S.p.a. in house providing (PE)).

3.2. Epigenome-wide analysis

As outlined in the study's objectives, the DNAm of the sampled individuals was analyzed using two complementary approaches: epigenome-wide analysis and targeted DNAm analysis of candidate genes.

Epigenome-wide analysis was performed on a subset of 33 individuals (14 HLE group and 19 LLE group), consisting of 12 females and 21 males with a mean age of 52.76 years (± 13.76 SD).

DNAm levels were measured using the Illumina MethylationEPIC v1.0 BeadChip, which covers about 850,000 CpG sites across the whole human genome, randomizing samples to minimize potential batch effects.

Raw DNAm data, provided in .idat file format, were processed using RStudio (version 4.2.1) (Posit team, 2023; R Core Team, 2023), with β-values used as the methylation measure. Specifically, quality control and data normalization were performed according to the guidelines by Maksimovic et al. (Maksimovic et al., 2017). Therefore, only CpG sites with a detection p-value < 0.05 in at least 99% of the samples, and individuals with at least 99% of CpG sites showing significant detection p-values, were retained. Additionally, CpG sites exhibiting bimodal or trimodal distributions were excluded using the *dbscan* package, along with those located on sex chromosomes or linked to SNPs.

The *EpiDISH* package (Teschendorff et al., 2017) was used to estimate the proportions of three different cell types (Epithelial Cells (EC), Fibroblasts (Fib), and total Immune Cells (IC)) for each subject. Finally, a t-test was applied to assess whether there were significant differences in the cell type proportions between HLE and LLE groups (p-value < 0.05).

To investigate the close relationship between the human epigenome and genome, the same 33 individuals were also genotyped using the Illumina HumanOmniExpress 720K BeadChip. Quality controls of the raw genotyping data were performed using PLINK 1.9 software (Chang et al., 2015). Specifically, sex assignments were verified by comparing them with those imputed using the X chromosome inbreeding coefficient *(--check-sex/--impute-sex* command-line options). Subsequently, autosomal chromosomes were retained and individuals with a genotyping success rate below 95% *(--mind 0.05*), variants with missing call rates greater than 5% (*--geno 0.05*), and SNPs showing significant deviations from Hardy-Weinberg equilibrium (p-value < $7*10^{-8}$ after Bonferroni correction) (*--hwe*) were excluded. Finally, the proportion of alleles shared Identical By Descent (*--genome*) was calculated, and one sample from each pair of individuals with a kinship coefficient (*PiHat*) greater than 0.25 was filtered out.

3.2.1. Differentially Methylated Positions detection and meQTL analysis

To identify Differentially Methylated Positions (DMPs) between the HLE and LLE groups, the following linear regression model was applied to the DNAm data:

$$Meth = \gamma 0 + \gamma 1\ Group + \gamma 2\ Sex + \gamma 3\ Age$$

where *Meth* indicates the DNAm value of a single CpG site (i.e., the β-value), *γ0* is the intercept, *Group* refers to the classification group of a single individual (HLE group or LLE group), and *Sex* and *Age* are the covariates related to the individual's sex and age.

The linear model was implemented using the *limma* package, followed by correction of model t-statistics for inflation and bias using the *bacon* package. To adjust the p-values for the CpG sites, False Discovery Rate (FDR) analysis was performed using the Benjamini-Hochberg (BH) method.

Significant CpG sites were then ranked according to delta (Δ), which is the difference between the mean β-value of the LLE group and the mean β-value of the HLE group for each CpG site (Beltrami et al., 2017; Nickels et al., 2022; Unruh et al., 2019).

Gene Ontology (GO) enrichment analysis was then performed to determine whether the CpG sites detected were associated with specific biological pathways. Specifically, GO analysis was conducted using the *methylRRA* function (*GSEA* method) of the *methylGSA* package (Ren and Kuan, 2019), which performs enrichment analysis by adjusting multiple p-values for each gene through Robust Rank Aggregation (Kolde et al., 2012).

At this stage, DMRs were also identified using the *combp* function (Pedersen et al., 2012).

Finally, a meQTL analysis was performed by correlating the genetic data (SNPs) of the 33 individuals with the DNAm data of the detected CpG sites to determine whether emerged CpGs were influenced by certain genotypes at specific loci. Specifically, the meQTL analysis was conducted using the following linear regression model: M ~ G + C, where M is a matrix of DNAm values (containing 701,357 CpG sites), G is a matrix of genotype data (676,233 SNPs), and C is a matrix of covariates (specifically sex and age).

Given the small sample size, the initial CpG-SNP association analysis (discovery phase) referenced results from a previously published atlas of genetic effects on DNAm in individuals of European ancestry (Min et al., 2021). The signals identified by Min et al. were subsequently validated in our dataset (replication phase) using the *MatrixEQTL* package in R software (Shabalin, 2012), with a maximum distance of 1 Mb used to define a CpG-SNP pair as local (cis-meQTL). MeQTLs with a nominal p-value < 0.05 were considered significant.

3.2.2. Differentially Variable Positions detection

To identify Differentially Variable Positions (DVPs) between the HLE and LLE groups, the following linear model was applied:

$$(Meth - mean(Meth))^2 = \gamma 0 + \gamma 1\ Group + \gamma 2\ Sex + \gamma 3\ Age$$

where *(Meth - mean(Meth))²* represents the squared deviation of the DNAm value from the mean DNAm value for a given CpG site. The linear model was executed using the *varFit* package (Phipson and Oshlack, 2014) and the resulting t-statistics were adjusted for inflation and potential bias using the *bacon* package. To adjust the p-values for the CpG sites, a FDR analysis using the BH method was performed. Finally, the Empirical Cumulative Distribution Function (ECDF) was applied to assess whether the distribution of differentially variable CpGs was significantly different between the HLE and LLE groups.

3.2.3. Epimutations and epigenetic lesions analysis

The beta values from the DNAm data were also used to identify epimutations as extreme outliers within a reference interval derived from all subjects (see Section 1.1). This reference interval was determined by defining the lower and upper boundaries for each probe as Q1 - (3 * IQR) and Q3 + (3 * IQR), respectively. Any beta value for each CpG site in a subject that fell outside this range was classified as SEM. All identified epimutations were then annotated to obtain, for each analyzed subject, a record of the total number of epimutations and their genomic locations. SEMs were further categorized into hypermethylated and hypomethylated sites, and CpGs were classified by their genomic context, including genic versus non-genic, island versus non-island, and grouped by chromosome. Wilcoxon's test and generalized linear models were used in R software to assess differences between the HLE and LLE groups.

To identify regions enriched with SEMs, referred to as epigenetic lesions, a sliding window approach was applied across the entire genome. This method pinpointed SEM-enriched regions, which were then quantified for each gene in every individual. A t-test was then applied to compare HLE and LLE groups. Finally, the genes containing epigenetic lesions were mapped to biological pathways using the Over-Representation Analysis (ORA) method in the WEB-based GEne SeT AnaLysis Toolkit (WebGestalt).

3.2.4. Epigenetic clocks analysis

To further investigate the DNAm data and evaluate the health status of individuals from the Bussi sul Tirino SNI, their biological age was estimated using "epigenetic clocks", which are established biomarkers of biological aging (Horvath and Raj, 2018). To compare the biological aging rates between the HLE and LLE groups, the DNAmAge Calculator (Horvath, 2013) was employed. This tool was provided with an input file containing the DNAm levels for approximately 30,000 CpG sites, along with a file detailing the individuals' information, such as identification number, chronological

age, sex, and the biological tissue analyzed (buccal mucosa cells). The Calculator generated several epigenetic clocks, including five applicable to oral mucosal cells: *AgeAccelerationResidual* (Horvath, 2013), *Intrinsic Epigenetic Age Acceleration* (*IEAA*) (Chen et al., 2016), *Extrinsic Epigenetic Age Acceleration* (*EEAA*) (Chen et al., 2016), *AgeAccelPheno* (Levine et al., 2018), and the *skin & blood clock* (Horvath et al., 2018). The *AgeAccelerationResidual* is an epigenetic marker of age acceleration, representing the residuals from the regression of biological age on chronological age (Horvath, 2013). *IEAA* and *EEAA* assess age acceleration by considering the intrinsic cellular aging process and external environmental influences, respectively (Chen et al., 2016). The *AgeAccelPheno* is an epigenetic aging index that links DNAm changes to aging-related phenotypes (Levine et al., 2018). Lastly, the *skin and blood clock* is based on a penalized regression model that correlates a calibrated version of chronological age with training CpG probes (Horvath et al., 2018).

After calculating the epigenetic clocks, the aging rates between the HLE and LLE groups were compared using a t-test with statistical significance set at p-value < 0.05.

3.3. Candidate gene analysis

The DNAm levels of three REs were measured in all 61 individuals using bisulfite sequencing with the Illumina MiSeq system. Specifically, 12 CpG sites on Alu, 18 CpG sites on LINE-1, and 133 CpG sites on rDNA (comprising 27 CpG sites at the 18S1 target, 13 CpG sites at the 18S2 target, 30 CpG sites at the 28S target, 37 CpG sites at the RiboProm1 target, and 26 CpG sites at the RiboProm2 target) were analyzed as suggested by Marson et al. (2023). The Alu target was located within the body of the repetitive unit, while the LINE-1 target was positioned in the 5' untranslated region (5'-UTR). In rDNA, the 18S1, 18S2, and 28S targets were located at the 5' end of their respective sequences. The RiboProm1 target, on the other hand, was situated upstream of the rDNA promoter, and the RiboProm2 target encompassed the rDNA promoter, including the upstream control element, central promoter region, and transcription start site (Marson et al., 2023) (Figure 6).

To ensure data quality, raw sequencing data were pre-processed following the procedures outlined by Ravaioli et al. (Ravaioli et al., 2023). In particular, *FastQC* (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), *cutadapt* (Martin, 2011), and *PEAR* (J. Zhang et al., 2014) were used for quality control of paired-end reads, adapter trimming, and read assembly, respectively. The assembled reads were then converted to FASTA format using *seqtk* (GitHub - lh3/seqtk: Toolkit for processing sequences in FASTA/Q formats). DNAm profiles were generated using the *AmpliMethProfiler* pipeline (Scala et al., 2016), which assigns a value of 1 to methylated CpG sites, 0 to unmethylated CpG sites, and 2 to positions with nucleotide ambiguity

following bisulfite conversion. Samples with fewer than 500 reads were excluded from analysis. The average DNAm level for each CpG site was then calculated as the percentage of methylated CpG sites relative to the total number of methylated and unmethylated CpG sites (Ravaioli et al., 2023). Finally, a t-test was performed for each CpG site in each RE to identify significant differences in mean DNAm levels between the HLE and LLE groups.

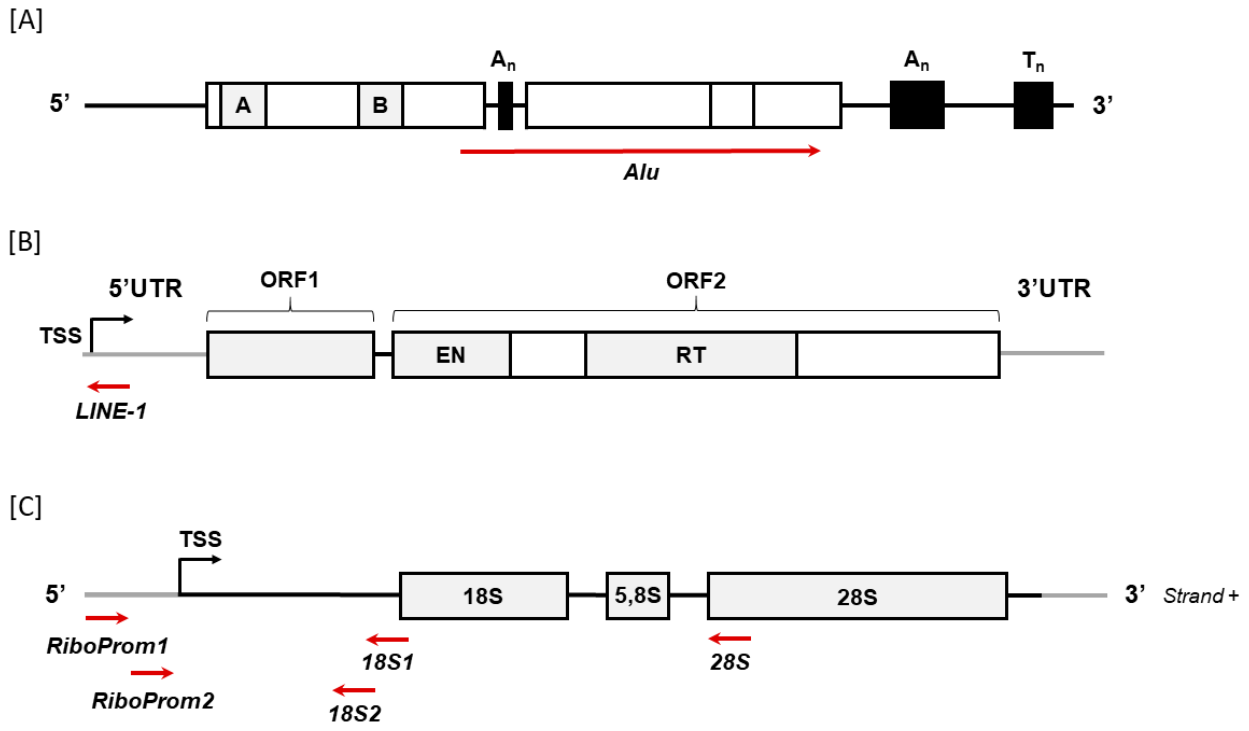

Figure 6. Basic structure of Alu (A), LINE-1 (B), and rDNA (C) repetitive units (adapted from Marson et al., 2023). The locations of the analyzed amplicons (Alu, LINE-1, RiboProm1, RiboProm2, 18S1, 18S2, and 28S) are indicated by red arrows. A and B represent the Box A and the Box B promoters, respectively; An, from left to right, is the adenine-rich middle region separating the two monomers and the terminal poly-A tail; Tn is the T-rich transcription terminator sequence; TSS is the Transcription Start Site; UTR indicates the Untranslated Region; ORF is the Open Reading Frame; EN is the ENdonuclease coding region; and RT represents the Reverse Transcriptase coding region. For further details refer to Marson et al., 2023.

4. RESULTS

4.1. Epigenome-wide analysis results

After performing quality checks on the DNAm data, a total of 701,357 CpG sites and 32 individuals were retained for further analysis. The selected sample had a mean age of 52 years and consisted of 12 females and 20 males. Of these individuals, 14 belonged to the HLE group and 18 to the LLE group.

The estimated proportions of the three cell types (EC, Fib, and IC) showed no statistically significant differences between the HLE and LLE groups (EC p-value = 0.38; Fib p-value = 0.40; IC p-value = 0.38). Therefore, the subsequent models were not adjusted for cell counts.

4.1.1. DMPs and meQTLs

The epigenome-wide analysis performed on the 32 individuals revealed a total of 622 significant DMPs between the two groups (nominal p-value < 0.001), located on 604 genes. However, none of these CpG sites remained significant following FDR correction. Among the 622 DMPs, 161 CpG sites were hypomethylated in the HLE group compared to the LLE group. Of the 622 DMPs, the top five according to delta were: cg04879348 (nominal p-value = $6.52*10^{-5}$) mapping in the GCC2 gene, cg14498475 (nominal p-value = $5.98*10^{-4}$) mapping in the LY6K gene, and cg14433904, cg21397540, and cg04190888 (nominal p-values = $9.86*10^{-4}$, $1.04*10^{-4}$ and $5.02*10^{-4}$, respectively) which map in intergenic regions (Table 2 and Figure 7). Three of these five DMPs were found to be associated with specific traits in the Epigenome-Wide Association Studies Atlas (EWAS Atlas) (Table 2).

Table 2. Summary statistics of the top five DMPs between the HLE and LLE groups (GRCh37/hg19 assembly) and traits found associated with them (EWAS Atlas - Li et al., 2019).

CpG name	Chr	Position	Relation to island	Nominal p-value	Δ LLE_HLE	Gene name	Related trait	Reference
cg04879348	2	109124576	OpenSea	6.52E-05	0.157153839	GCC2	Exposure to organochlorinated compounds during plantation work and risk of Parkinson's disease	(Go et al., 2020)
cg14498475	8	143781814	Island	0.000597624	0.147480664	LY6K	Ancestry and immune gene regulation	(Husquin et al., 2018)
cg14433904	8	78518428	OpenSea	0.000985593	0.092095058	-	-	-
cg21397540	1	247537159	OpenSea	0.000103934	0.091796384	-	-	-
cg04190888	10	49879914	OpenSea	0.000501523	0.090258724	-	Multiple sclerosis, smoking, and kidney disease	(Hannon et al., 2021; Maltby et al., 2018; Smyth et al., 2021)

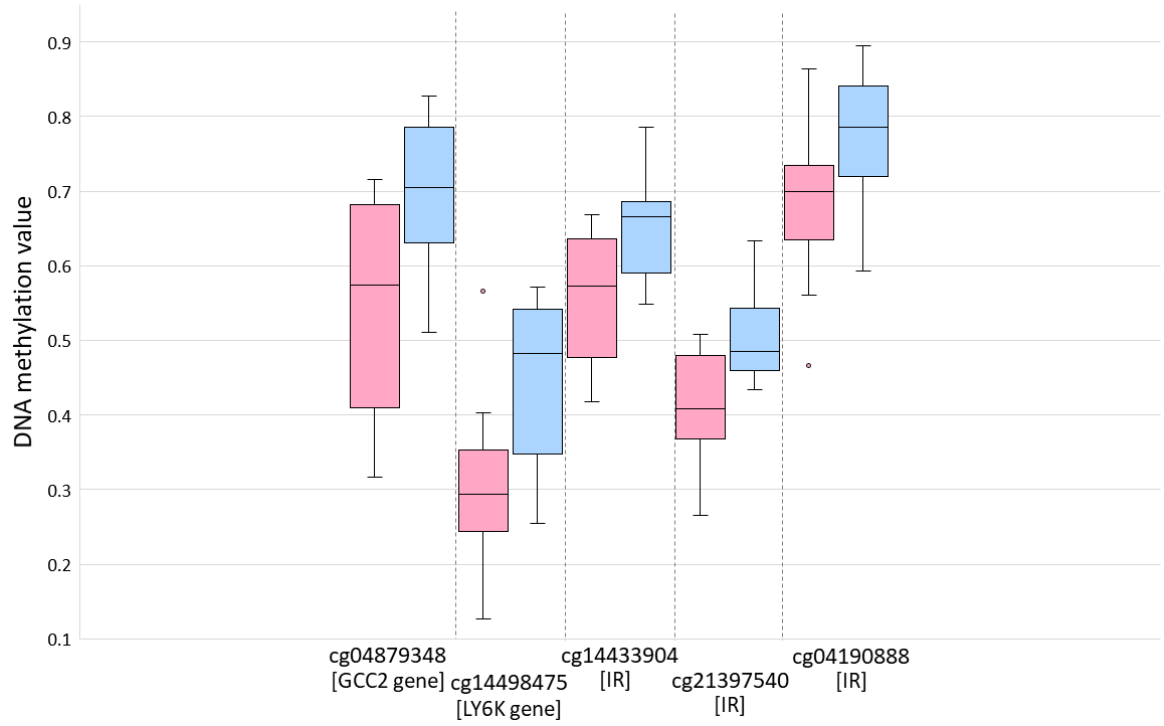

Figure 7. Boxplots of the top five DMPs, ranked by the delta value, comparing the HLE (in pink) and LLE (in blue) groups. For each DMP the gene or intergenic region (IR) is indicated.

The GO analysis performed including genes with identified DMPs revealed 104 significant biological pathways (FDR q-value < 0.05) (Table S2), comprising pathways related to embryonic organ morphogenesis and development, cell development regulation, B cell activation, immune response mediator production, neutrophil-mediated immunity, leukocyte migration, extracellular matrix organization, appendage development, small molecule catabolic processes, and nervous system development (including axonogenesis and axon development) (Figure 8).

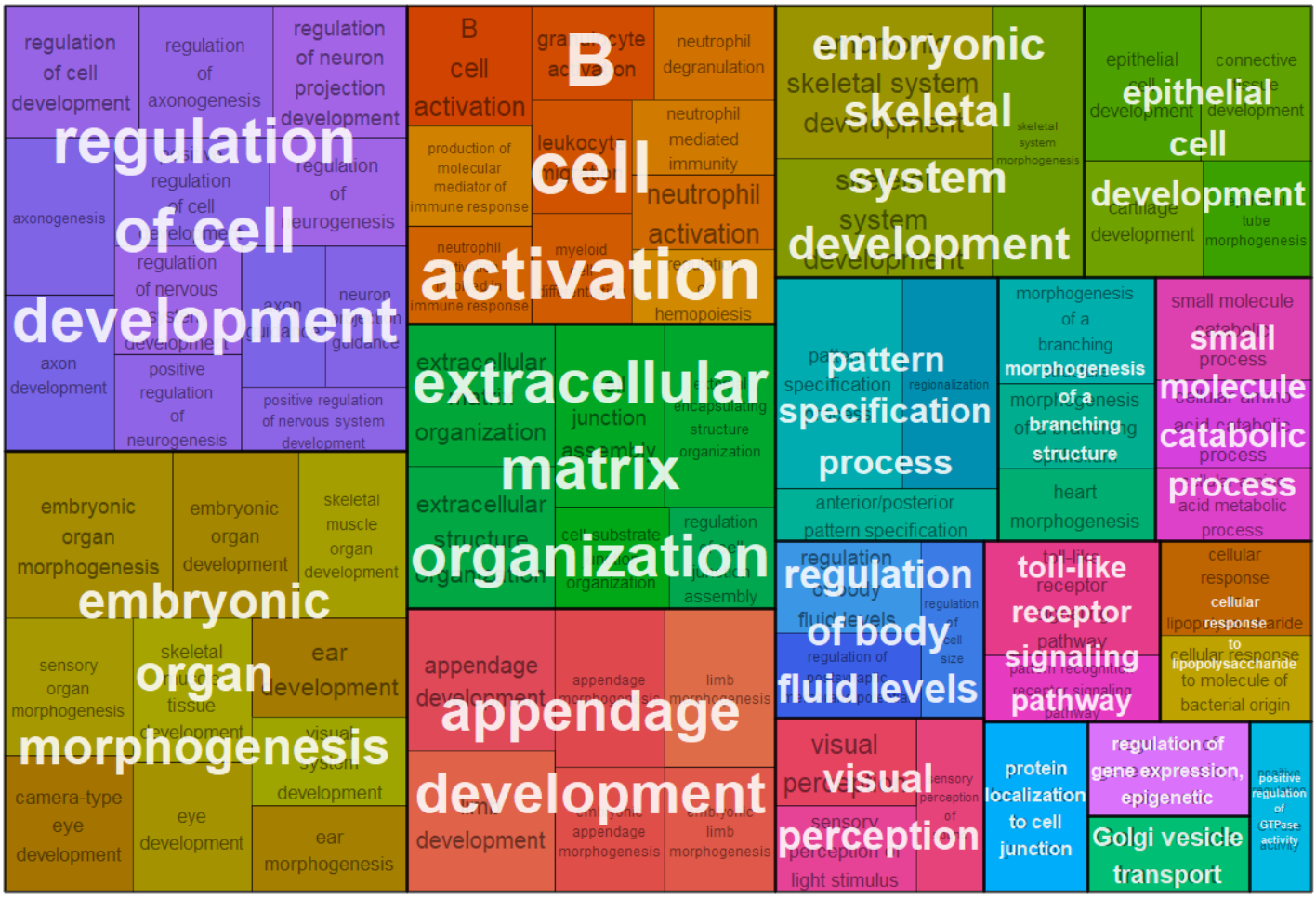

Figure 8. Tree map summarizing statistically significant pathways from the GO analysis (FDR q-value < 0.05), in which each rectangle corresponds to a different functional pathway, the size of each rectangle is proportional to its significance, and the most biologically similar pathways are plotted in the same color.

The study of DMRs, however, showed no statistically significant results.

Lastly, the meQTL analysis was first performed by screening a published atlas on the effects of genetics on DNAm in individuals of European ancestry (Min et al., 2021), in order to reduce the risk of false-positive associations due to the relatively small cohort. Specifically, the atlas identified 169,656 meQTL associations (155,190 cis- and 14,466 trans-meQTL), of which 3,369 (3,147 cis- and 222 trans-meQTL) were replicated also in this study (p-value < 0.05). However, among the 622 DMPs, only cg16393905 was found to be affected by genotypes at the rs11586401 locus (t-statistic = -2.26; p-value = 0.03) (Figure 9).

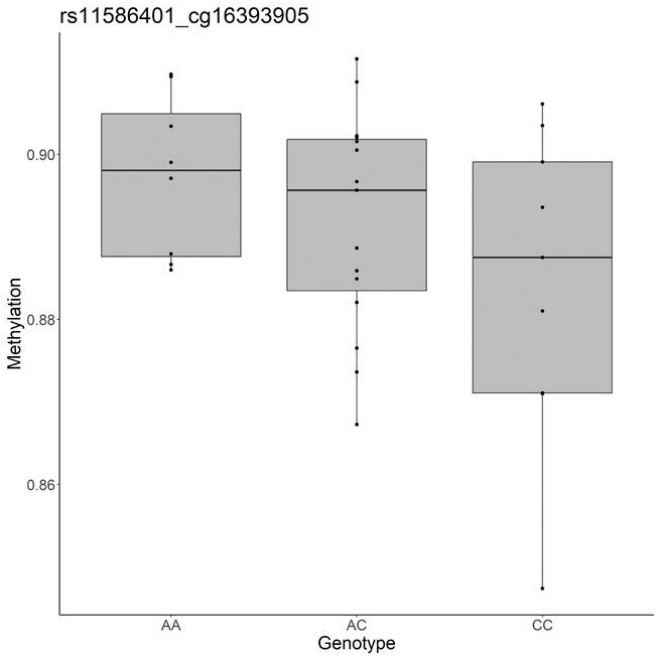

Figure 9. Significant meQTLs identified by Min et al. (2021) that successfully replicated in the present study (p-value < 0.05).

4.1.2. DVPs

Analysis of variance identified 1,138 significant DVPs between the HLE and LLE groups (nominal p-value < 0.001) mapped in 882 genes. However, none of these CpG sites remained significant following FDR correction. Among the 1,138 DVPs, 71.36% showed reduced variability in the HLE group compared to the LLE group. In general, the five most significant DVPs identified were: cg23678634 (nominal p-value = $1.51*10^{-6}$) mapping in the RBM8A, NBPF20, and NBPF10 genes, cg09696644 (nominal p-value = $2.96*10^{-6}$) mapping in the SRGAP2 gene, cg17024333 (nominal p-value = $3.21*10^{-6}$) mapping in the SCTR gene, cg01277615 (nominal p-value = $3.23*10^{-6}$) mapping in the BCAP29 gene, and cg10760539 (p-value = $4.90*10^{-6}$) which maps in an intergenic region (Table 3).

Table 3. Summary statistics of the top five DVPs between the HLE and LLE groups (GRCh37/hg19 assembly).

CpG name	Chr	Position	Relation to island	Nominal p-value	Gene name
cg23678634	chr1	145508314	S_Shore	1.51E-06	RBM8A; NBPF20; NBPF10
cg09696644	chr1	206626583	OpenSea	2.96E-06	SRGAP2
cg17024333	chr2	120233913	OpenSea	3.21E-06	SCTR
cg01277615	chr7	107220080	N_Shore	3.23E-06	BCAP29
cg10760539	chr7	31505908	OpenSea	4.90E-06	-

The ECDF (Figure 10A) showed a significant difference in the distributions of standard deviations of DNAm levels for the DVPs between the HLE and LLE groups (p-value = $2.2*10^{-16}$). Visual inspection of the plotted distributions (Figure 10B) revealed that, on average, the variability of DNAm levels was lower in the HLE group compared to the LLE group.

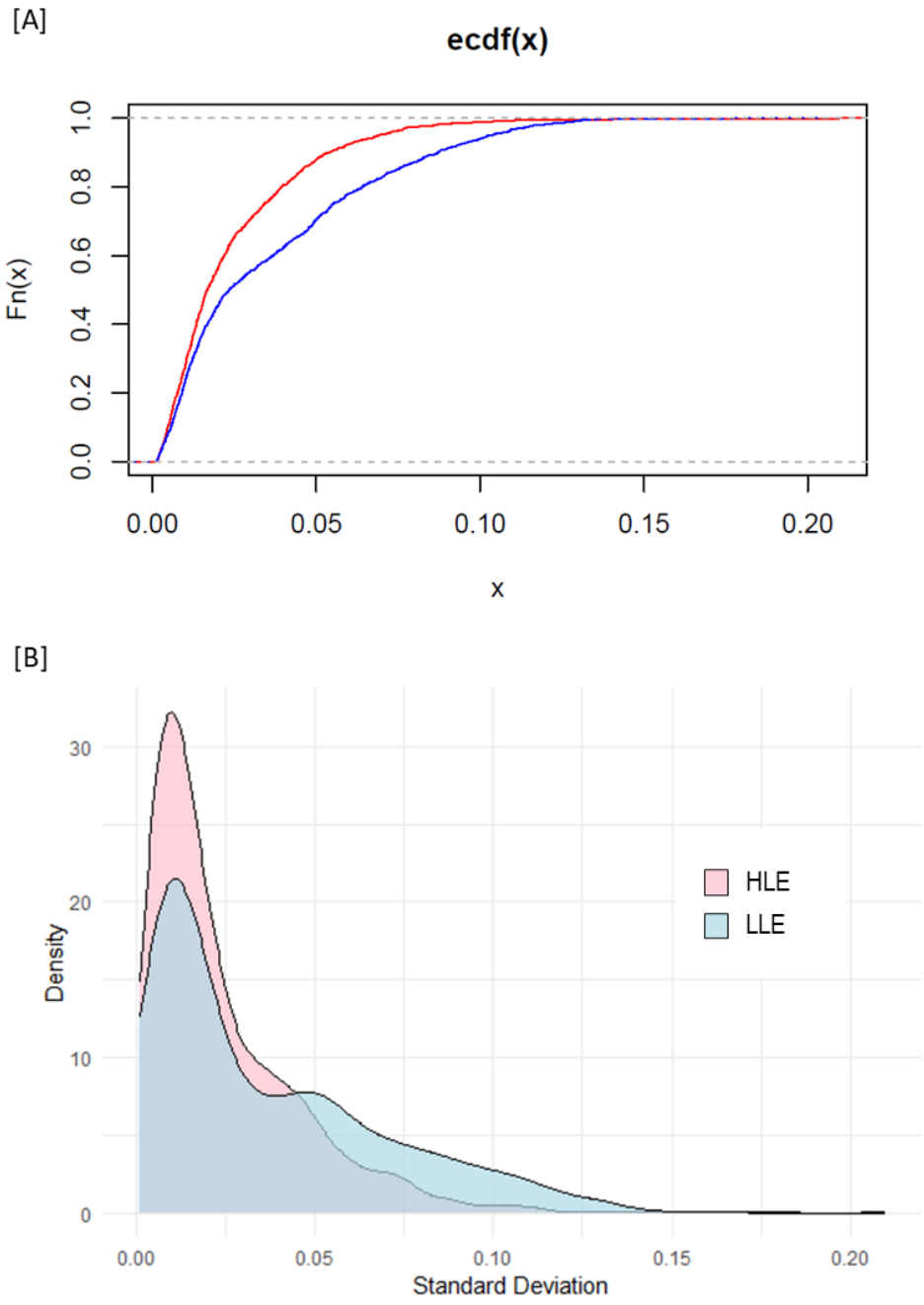

Figure 10. A) Graphical representation of the Empirical Cumulative Distribution Functions (ECDF) test, illustrating a significant difference (p-value = $2.2*10^{-16}$) between the DNAm level distributions of the 1,138 significant CpG sites in the HLE group (in red) and the LLE group (in blue). B) Density plot showing the distribution of the *sd(Meth)* variable associated with DVPs (p-value < 0.001) for the HLE (in pink) and LLE (in blue) groups. The width of the curves indicates that the variability is lower in the HLE group compared to the LLE group.

4.1.3. Epimutations and epigenetic lesions

Analysis of epimutations across the 32 subjects revealed a median of 2282.5 and 2550.5 total epimutations for the LLE group and the HLE group, respectively. Specifically, the median hypomethylated epimutations were 1371.0 and 1484.5, while the median hypermethylated epimutations were 968.5 and 987.0 for the LLE and HLE groups, respectively. Log-transformed comparisons of epimutations between the two groups showed no significant differences (Figure 11).

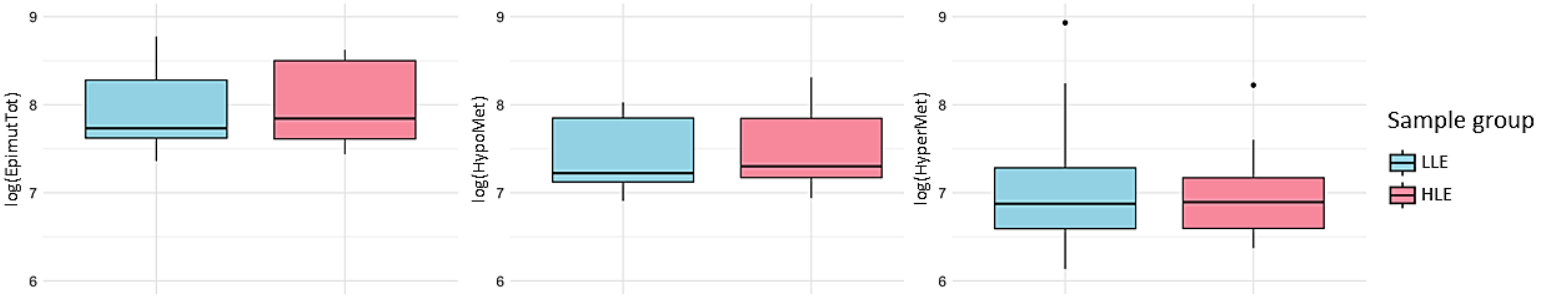

Figure 11. Boxplot showing the logarithm of the total number of epimutations, as well as hypomethylated and hypermethylated epimutations, categorized into HLE (in pink) and LLE (in blue) groups.

Further analysis of epimutations based on CpG annotations—classifying them into genic versus non-genic regions, and island versus non-island regions—did not yield significant results. Additionally, further categorization of these CpG classes into hypo- and hypermethylated epimutations showed no significant differences between the HLE and LLE groups (Tables 4).

Table 4. Median number of hypomethylated and hypermethylated, island and non-island epimutations in both genic and non-genic regions, divided into HLE and LLE groups.

		Median_HLE	**Median_LLE**
Genic region	Island Hyper	544	574
	Island Hypo	410	419
	Non-Island Hyper	197	194
	Non-Island Hypo	604	564
Non genic region	Island Hyper	86.5	87
	Island Hypo	124	112
	Non-Island Hyper	109	104
	Non-Island Hypo	358	334

Similarly, when CpGs were grouped by chromosome, no significant differences were observed between the two groups (Table 5).

Table 5. Median number of epimutations per chromosome divided into HLE and LLE groups.

Chr	Median_HLE	Median_LLE
1	228	226
2	186	176
3	150	126
4	124	120
5	141	138
6	203	200
7	144	133
8	121	118
9	75	68.5
10	132	122
11	150	128
12	129	130
13	66.5	62
14	83.5	77
15	85	81
16	110	104
17	116	97
18	51.5	42
19	118	94.5
20	55.5	52
21	29.5	30.5
22	48.5	42.5

Finally, there were no statistically significant differences in the total number of epigenetic lesions between the two groups, and the biological pathways associated with genes exhibiting epigenetic lesions were also not significant.

4.1.4. Epigenetic clocks

In order to examine the potential connection between contaminant exposure and biological aging in relation to individuals' health, five epigenetic clocks (*AgeAccelerationResidual*, *IEAA*, *EEAA*, *AgeAccelPheno*, and *skin & blood clock*) for all 32 participants were assessed. Differences in epigenetic age between the HLE and LLE groups were then evaluated using a t-test. However, no significant differences (p-value < 0.05) were found between the two groups in the analysis of these epigenetic clocks.

4.2. Candidate gene analysis results

The DNAm levels of three REs (LINE-1, Alu, and rDNA) were measured in all 61 individuals using targeted bisulfite sequencing. Specifically, 12 CpG sites on Alu, 18 CpG sites on LINE-1, and 133 CpG sites on rDNA were studied.

A t-test was then performed for each CpG site to identify significant differences in mean DNAm levels between the HLE and LLE groups. Statistically significant differences (p-value ≤ 0.05) were found in the DNAm levels of one CpG site (referred to as "CpG_62" by Marson et al., 2023) on Alu (nominal p-value = 0.05) and three CpG sites ("CpG_34," "CpG_37," and "CpG_194") on LINE-1 (nominal p-values = 0.04, 0.03, and 0.03, respectively) (Figure 12). However, no rDNA CpG sites showed significant differences between the two groups. In addition, all significant CpG sites were found to be hypomethylated in the HLE group compared to the LLE group.

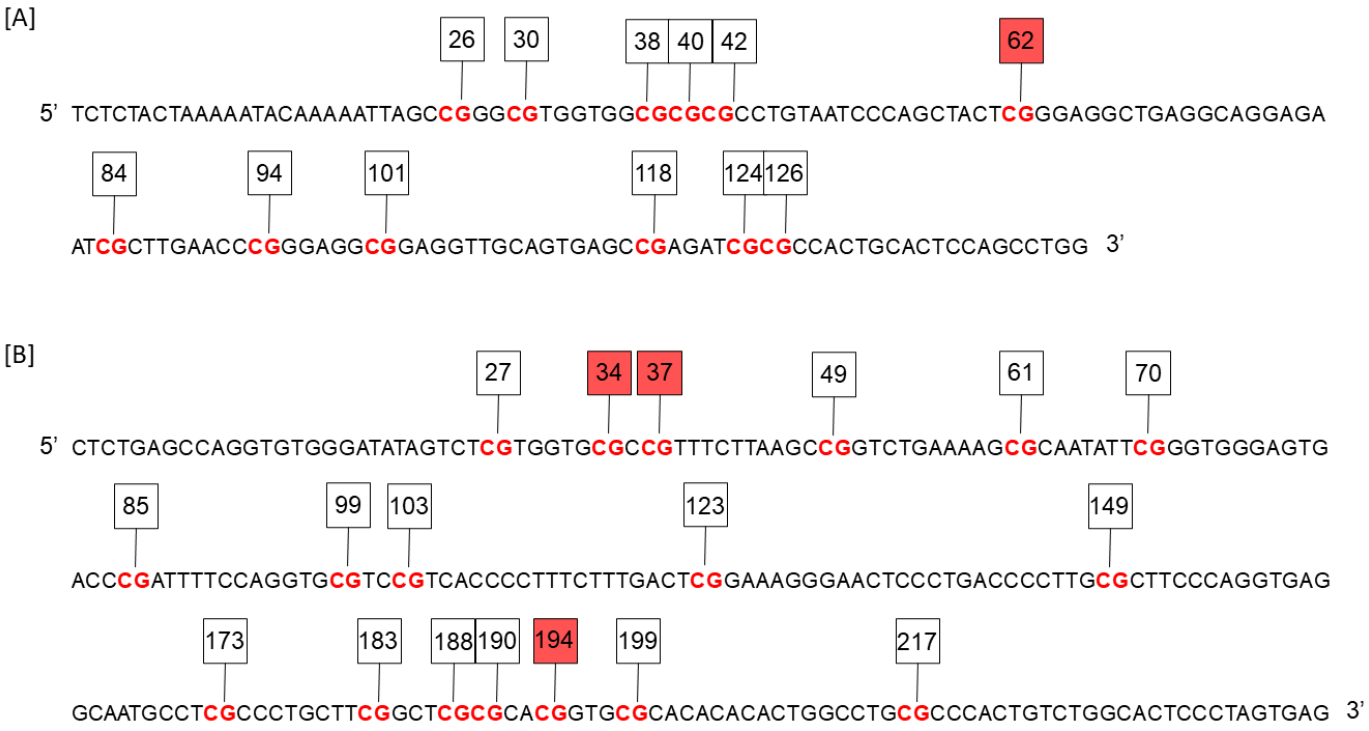

Figure 12. Target sequences for Alu (A) and LINE-1 (B) (GRCh38/hg38) along with the positions of the analyzed CpG sites. The CpG sites showing differential DNAm levels between the HLE and LLE groups are highlighted in red. For more details, please refer to Marson et al. (2023).

Notably, all 18 CpG sites analyzed in LINE-1 were hypomethylated in the HLE group compared to the LLE group (Figure 13).

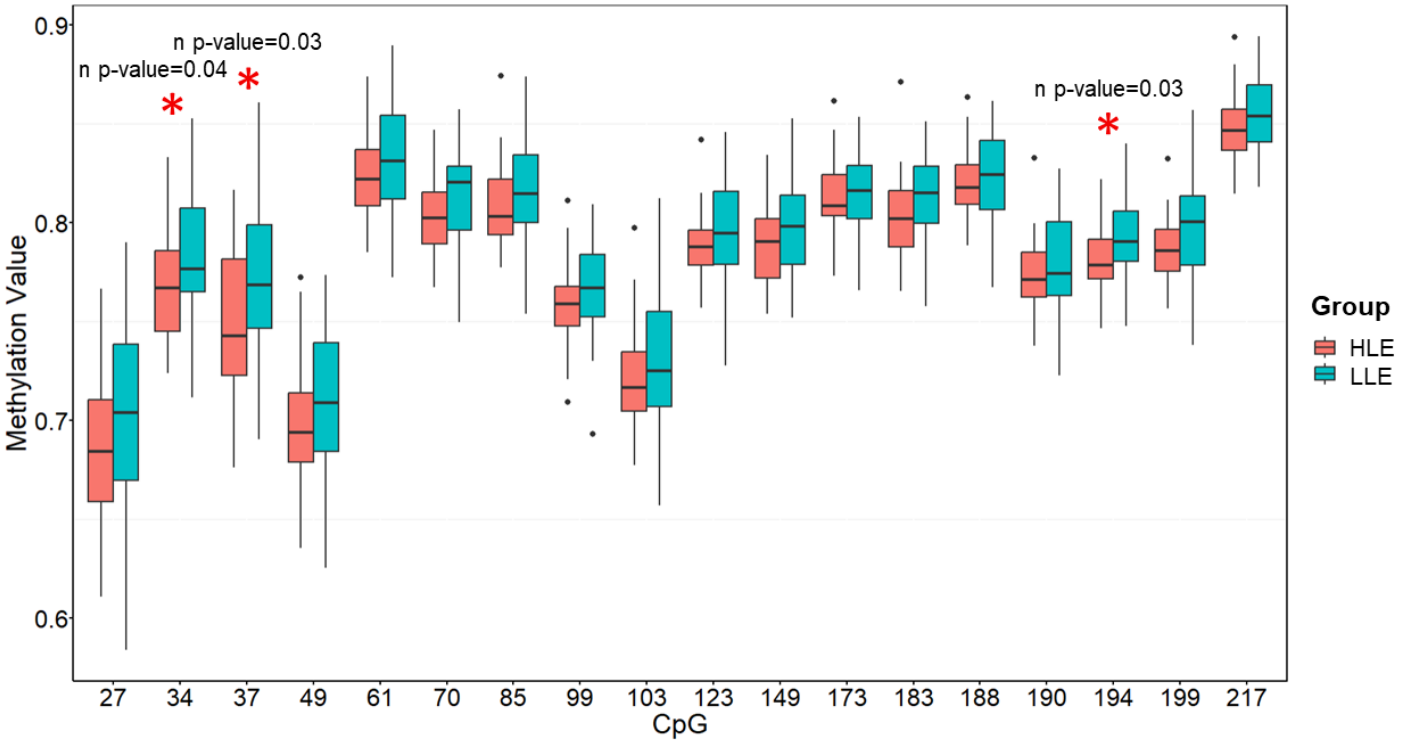

Figure 13. Plot showing the 18 CpG sites measured in LINE-1 for the HLE (pink boxplots) and LLE (blue boxplots) groups. Red asterisks mark the three CpG sites (CpG_34, CpG_37, and CpG_194) that are differentially methylated between the two groups (p-value ≤ 0.05). The boxplot trends reveal a general pattern of LINE-1 hypomethylation in the HLE group compared to the LLE group.

5. DISCUSSION

This dissertation addressed the increasingly relevant issue of environmental contaminants and their impact on human health, focusing in particular on the variability of DNAm, an epigenetic modification strongly influenced by the environment. Specifically, the role of toxic substances as past environmental stresses and current emerging pollutants to which humans have been and are still exposed was explored. The intimate interconnection between genetics and DNAm, which plays a crucial role in human health and with which contaminants can interfere, was also emphasized. In addition, the effects of the most studied contaminants on reproductive health, both male and female, biological aging process, and nervous system disorders through the modification of DNAm profiles, were explored. Finally, the central focus of this thesis was the case study of the SNI of Bussi sul Tirino, a current and highly debated example of prolonged human exposure to environmental contaminants. This study focused in fact on the impact of contaminants illegally discharged into the soil and groundwater of the SNI at Bussi sul Tirino, such as heavy metals and chlorinated solvents, on the DNAm levels of the local population. To this end, buccal mucosal cells were collected from 61 volunteers using buccal swabs, and the study participants were divided into two groups (30 HLE and 31 LLE) according to a classification method that summarized the exposure level of each individual.

DNAm profiles were analyzed using i) epigenome-wide and ii) candidate gene approaches. Epigenome-wide analysis was performed on a subset of 32 individuals (14 HLE and 18 LLE) using DNAm and genetic data generated with the Illumina EPIC BeadChip and HumanOmniExpress BeadChip, respectively. This analysis specifically examined DMPs, DMRs, meQTLs, DVPs, epimutations, epigenetic lesions, and epigenetic clocks, in order to identify possible differences in DNAm levels between the HLE and LLE groups. The candidate gene approach was instead applied to all 61 subjects and used the DNAm data of three REs (LINE-1, Alu, rDNA) generated by bisulfite sequencing (Illumina MiSeq) of specific sites.

The comparison between the HLE and LLE groups revealed 622 significant DMPs (nominal p-value < 0.001). Interestingly, the most differentiated (Δ) significant site between the two groups (cg04879348) also emerged in a recent study on the impact of organochlorine compounds (Go et al., 2020).

The GO analysis conducted on genes containing DMPs revealed several significant biological pathways (q-value FDR < 0.05), including embryonic, cellular and nervous system development, extracellular matrix organization, small molecule catabolic process, and other immune-related pathways. These findings are in agreement with those of previous studies on human exposure to air

pollution and heavy metals from e-waste, which identified changes in DNAm levels in genes involved in development, nervous system, and immune response (Du et al., 2022; Zeng et al., 2019).

The meQTL analysis, which integrated methylation and genetic data, revealed that most of the DNAm differences observed between the two groups (DMPs) were not attributable to genetic background, suggesting they were likely related to environmental exposure. Specifically, only one CpG site (cg16393905) was found to be influenced by genotypes at a specific locus (rs11586401).

The whole epigenome analysis also showed 1,138 differently variable CpG sites (nominal p-value < 0.001). Of particular interest is the decreasing variability and convergence towards similar DNAm values that were observed in the group of high-exposure individuals (HLE). Similar events were recently described in the study by Tobi et al. (2018), who, using a mathematical model of re-methylation in the early embryo, demonstrated selective processes underlying a reduction in DNAm variance in a human cohort prenatally exposed to environmental stressors such as the Dutch famine (Tobi et al., 2018). On the basis of this study, it is therefore possible to hypothesize that the reduced variability of DNAm levels detected in the HLE group could be the expression of an epigenetic selection event caused by prenatal exposure to pollutants released in the Bussi sul Tirino SNI. In fact, it is possible that embryonic exposure to an altered intrauterine environment, such as that resulting from maternal exposure to environmental contaminants, may compromise the remodeling phase of the embryonic epigenome that occurs after fertilization. This could lead to structural and functional changes in tissues, resulting in pathologies later in life, but also to differential survival of embryos. Indeed, epigenetic selection would operate by removing specific DNAm variants unsuitable for that specific uterine environment from the population, thus changing the distribution of DNAm profiles in survivors (Breton et al., 2017; Germain and Winn, 2024; Tobi et al., 2018). However, it cannot be excluded that the reduction in DNAm variability recorded for the high-exposure group may be part of an adaptive strategy in response to pollutants (Gluckman et al., 2007).

The comparison between the two groups of individuals with different exposure revealed that exposure to contaminants did not seem to affect the number of epimutations and epigenetic lesions. However, it is important to emphasize that this is the first study that investigates a possible relationship between contaminants and epimutation rate, as well as the first that uses DNA samples from oral mucosa cells, as previous studies had applied the epimutation algorithm on DNA samples from blood (Brusati et al., 2023; Gentilini et al., 2022; Spada et al., 2020). Therefore, it will be fundamental in future studies to deepen these analyses, also integrating DNAm data from blood samples, in order to establish with greater certainty whether or not environmental contaminants have an impact on epigenetic mutation rates.

Finally, since previous studies had found an association between exposure to pollutants and the epigenetic aging rate, as seen in Section 1.2.1.2, the population of the Bussi sul Tirino SNI was also studied in terms of biological aging, in order to also capture possible impacts of contaminants on human health. However, the five calculated epigenetic clocks (*AgeAccelerationResidual*, *IEAA*, *EEAA*, *AgeAccelPheno* and *skin & blood clock*) showed no statistically significant differences between the two groups, suggesting that the pollutants discharged in the Bussi sul Tirino area do not appear to influence the biological aging rate of the local population. Although, as mentioned above, some studies have demonstrated an association between toxic substances and epigenetic aging, the results of this study are in line with those of further studies that have found no effect of pollutants on the biological aging rate. Bozack et al. (2022), for example, observed no relationship between the epigenetic aging rate in buccal mucosa cells and exposure to As (Bozack et al., 2022). Similarly, three other studies on exposure to perfluoroalkyl substances (PFAS) (Xu et al., 2020), air pollution (NO2) (Gaskins et al., 2023) and Pb (Herrera-Moreno et al., 2022) did not observe effects on biological aging in blood, granulosa cell and cord blood samples, respectively. However, it is important to remember that there are different epigenetic clocks, and each of them, as well as the type of tissue chosen for analysis, may produce different results. Therefore, for a more in-depth analysis of the health status of individuals in the area of interest, it will be necessary to expand these results by including blood samples, for which a greater variety of epigenetic clocks are available, in order to obtain more precise and clear conclusions.

The Bussi sul Tirino SNI population was also analyzed by applying a candidate gene approach to all 61 samples, which explored the DNAm levels of three REs in the genome (LINE-1, Alu, rDNA) using bisulfite sequencing (Marson et al., 2023).

Comparison between the two groups showed that one CpG site on Alu and three CpG sites on LINE-1 had differential DNAm levels (p-value ≤ 0.05). Particularly, these sites were hypomethylated in the high contaminant exposure group. Notably, for all sites analyzed in LINE-1, a hypomethylation trend was observed in the HLE group. The loss of methylation in this element is also supported by a recent study of exposure to Pb, one of the most prevalent pollutants in the contaminated area of Bussi sul Tirino (Yohannes et al., 2022). Our results thus confirm the susceptibility of LINE-1 DNAm profiles to pollutants, as already reported by several previous studies (Barchitta et al., 2018; Lee et al., 2017; X. Liu et al., 2019). In addition, LINE-1 hypomethylation has also been shown to be related to genomic instability associated with several types of cancer (Baba et al., 2024; Kitahara et al., 2020; Schulz et al., 2002) and infertility (Wang et al., 2020). Therefore, it is possible to speculate that loss

of methylation in this RE may contribute to the onset of certain disease states in individuals exposed to environmental contaminants.

In this regard, the epidemiological survey conducted in the Bussi sul Tirino area in the context of the SENTIERI project (SENTIERI 2023) reported an increase in deaths due to bladder cancer and non-Hodgkin's lymphoma related to the presence of the illegal dump in Bussi. Excess female mortality also emerged for different cancers associated with the presence of the power plant, chemicals, and landfills. In addition, hospitalization rates for colorectal cancer, lung cancer, respiratory diseases, circulatory system diseases, digestive diseases, and urinary diseases increased for both sexes (Zona et al., 2023). Interestingly, urinary system diseases, such as nephritis and renal failure, have also been found at other Italian sites contaminated with solvents and heavy metals (Benedetti et al., 2021).

Although the results obtained are significant and in line with other studies, the present work has some limitations that deserve to be discussed. Firstly, the sample size is relatively small, which may have affected the statistical power of the analyses. Another limitation is that some of the statistical analyses performed did not pass the correction for multiple testing, which may affect the robustness of the results. Finally, it is important to consider that all samples used for analysis were exposed to environmental contaminants, probably reducing the observed differences. Therefore, future studies could benefit from a group of individuals from a contamination-free area.

However, this pilot study provides important preliminary data that could guide future, broader and more in-depth studies, thus improving the understanding of the phenomenon analyzed and laying the foundations for possible prevention strategies in health care and human environmental impact assessment.

6. CONCLUSION

This PhD thesis reflects on the multiple effects of environmental contaminants on human biology by reviewing the current scientific literature, especially regarding the potential of contaminants to shape human DNAm profiles, with consequent effects on human health in terms of reproductive health, epigenetic aging, and neurological diseases. Specifically, this dissertation focuses on the study of an Italian contaminated site, with the aim of contributing with new data to the understanding of the impact of contaminants on human biology and health.

Overall, the results obtained in this study, although preliminary, contribute to clarifying the effects of environmental pollutants present in the area of Bussi sul Tirino SNI on human health and natural variability, offering potential insights for future health prevention and environmental impact assessment strategies. In particular, this study highlights the influence of contaminants in shaping the DNAm profiles of the local population, showing especially a reduced variability in DNAm levels and a convergence towards similar methylation values in high-exposure subjects, suggesting a possible adaptation to the polluted environment or a prenatal epigenetic selection event. High exposure subjects also showed a general loss of methylation in RE LINE-1, which could be the driver of genomic instability associated with the onset of different pathological conditions. However, the contaminants do not appear to impact biological aging or epimutation rate in the study population. Finally, this study suggests the need for further analysis to deepen the impact of environmental contaminants on the welfare of exposed populations.

I. APPENDIX: Groundwater tracing via DNA-labeled nanoparticles

I.1. Introduction

The tracking and reconstruction of contamination distribution dynamics is a growing area of fundamental importance in various fields. In the context of studying the effects of contaminants on populations, it is essential for an accurate reconstruction of the exposure dynamics of individuals, thus enabling a more precise characterization of samples.

Among the various methods currently used for groundwater tracing, such as ionic solutions and fluorescent dyes, the use of DNA-based tracers is recently emerging. The DNA molecule in fact has numerous advantages in tracing groundwater flow compared to classical tracers, such as specificity (i.e. it minimizes the effects of background noise given its different nature), compatibility with the environment (Mora et al., 2015), ultra-sensitivity in terms of analysis, and could also be useful for a multi-tracer tomographic evaluation (Liao et al., 2018; Sharma et al., 2012). Tracking tests using dissolved DNA in groundwater have shown positive results in various hydrological compartments: sewer pollution sources (Georgakakos et al., 2019; Pang et al., 2017), surface water (Foppen et al., 2013, 2011), and glacial systems (Dahlke et al., 2015). However, the widespread use of these tracers in full-field conditions is still evolving due to some technical challenges caused by the nature of the material. Indeed, DNA tends to degrade easily, particularly in an aquatic environment it has an average lifetime of less than 10 hours at 20°C (Mikutis et al., 2018), and can undergo possible adsorption processes. It is precisely to meet these needs that the field of applied hydrogeology has given rise to nanotechnology. Indeed, nanoparticles can be used to facilitate organic molecules such as DNA, which can be encapsulated and protected from chemical and physical stresses within their silica shell (McNew et al., 2018; Mikutis et al., 2018; Wang and He, 2010). Moreover, the slight negative charge created on the surface of the nanoparticles, due to an attenuation of surface effects along the pore walls, improves the mobility of these tracers (Zhuang et al., 2009).

Despite the great improvements that this technology has brought, there are still some technical difficulties, such as gravity deposition, pore size exclusion effects (which must be at least 1.5 times larger than the nanoparticles) due to the colloidal nature of the DNA tracers, and the possibility of preferential migration along streams with higher velocities (McKay et al., 2000; Sirivithayapakorn and Keller, 2003). Furthermore, the high cost of this technology compared to classical tracers and the need for interdisciplinary structures and specialized staff for a proper interconnection of the field of hydrogeology with that of molecular biology must be considered.

I.2. Aim of the study

The purpose of this study was to investigate the use of DNA nanoparticles as tracers for full-field monitoring of groundwater flow dynamics, evaluating their efficacy and transport modes, and overcoming the limitations of conventional tracers in complex hydrologic environments. Specifically, the ultimate goal of this research was to develop a tracer protocol useful for studying the dynamics of contamination distribution through groundwater flows in order to more specifically characterize the degree of pollutant exposure of sampled individuals.

I.3. Materials and Methods

In order to develop an experimental protocol for tracking and characterizing groundwater flow, a collaboration was undertaken with a group of hydrogeologists from the University of Bologna, which provided access to knowledge and technology specific to the hydrogeology field.

The tracer test was conducted in a fractured sedimentary aquifer at a site called “Arpolli”, located in the northern Apennines of Italy, approximately 40km south-west of the metropolitan city of Bologna, in order to experimentally verify the degree of hydrogeological connectivity within this aquifer. To the authors' knowledge, this study is the first to use tracer nanoparticles in an aquifer of this type under full-field conditions. In particular, the aquifer of interest is characterized by a karst-like enlargement of primary or secondary discontinuities and represents a strategic reservoir for public water supply. The spring-boxes at the site have the function of facilitating the drainage of groundwater from the aquifer to a nearby reservoir. The company responsible for the water supply (Gruppo Hera S.p.A.), for study and monitoring purposes, drilled two wells (called B1 and B2) near the spring-boxes, at a distance of 10m from each other.

DNA-labeled nanoparticles used to perform tracking had been manufactured and quality-controlled by the Swiss Federal Institute of Technology (Zürich, CH) according to Paunescu et al. (2013). The DNA tracers, called “LPf32”, were composed of a short synthetic DNA sequence encapsulated in a small silica nanoparticle. The nanoparticles had an average size < 140mm and in each of them <0.1wt% DNA was loaded with the following sequence (65 bp): TACTCGCTGCTGTGACTGACG ACTCGACTCATAGTGAATAGTGACCGGTAGACCTGCTGAACCTA.

0.5ml (0.5 mg) of DNA tracer eluted in 9.5ml of water was injected in the B1 well. Subsequently, 10mL water samples were collected at three different recovery points downstream of the injection point: S2 and S3, that are two spring-boxes, and B2, that are a borehole. Regarding recovery points S2 and S3, samples were collected on average every 10 minutes directly from the end of the tube

conveying groundwater to the local aqueduct reservoir, while for point B2, samples were collected every 15 minutes from inside the water column using a bailer.

Once collected, the samples were transferred to the Molecular Anthropology laboratory of the Department of Biological, Geological and Environmental Sciences (BiGeA) of the University of Bologna, and stored at +4°C.

Subsequently, the nanoparticles contained in the 10ml site water samples were concentrated by transferring 1ml of sample into a 1.5ml tube, centrifuging at maximum speed for 3 minutes at room temperature and discarding the supernatant. This procedure was repeated 10 times in order to transfer the entire contents of the sample.

Afterwards, Buffered Oxide Etch (BOE) solution was prepared inside a polyethylene container by dissolving 0.23 g of ammonium difluoride (NH_4FHF) and 0.19 g of ammonium fluoride (NH_4F) in 10ml of Milli-Q water (pH 4), in order to dissolve the silica envelope in which the DNA was encapsulated. Specifically, 45µL of BOE solution was added to each concentrated sample, resulting in the release of the DNA in aqueous solution.

Finally, the samples were diluted 1:100 in water in order to reduce the tendency of the BOE solution to limit detection by quantitative PCR (qPCR) (Mikutis et al., 2018; Paunescu et al., 2013). The dissolved DNA was in fact used directly for the qPCR reaction without any purification.

The quantification of the DNA present in each sample was performed by qPCR (StepOnePlus Real-Time PCR, Applied Biosystems) in triplicate as follows: 10µL Power SYBR Green PCR Master Mix (Applied Biosystems), 1µL forward primer (10µM), 1µL reverse primer (10µM), 3µL Milli-Q water, and 5µL DNA template. The primers used had the following sequence: TACTCGCTGCTGTGACTGAC (forward primer) and TAGGTTCAGCAGGTCTACCG (reverse primer). The thermal cycle used was: 95°C for 10 minutes (pre-activation), 95°C for 15 seconds, 54°C for 30 seconds, and 72°C for 30 seconds (40 cycles) (Paunescu et al., 2013). As part of each experiment, three negative controls containing water instead of DNA were included.

Concurrently with the preparation of the samples, seven 1:10 serial dilutions (D1-D7) of the tracer at a known concentration (mother) were also prepared (Figure a1; Table a1), in order to construct a standard curve for each experiment from which the sample data could be interpolated and the concentration derived. Specifically, the mother (0,33 ng/µl) was prepared by centrifuging 50µl nanoparticles at maximum speed for 3 minutes at room temperature, removing the supernatant, and adding 150µl of BOE solution until a clear solution was obtained.

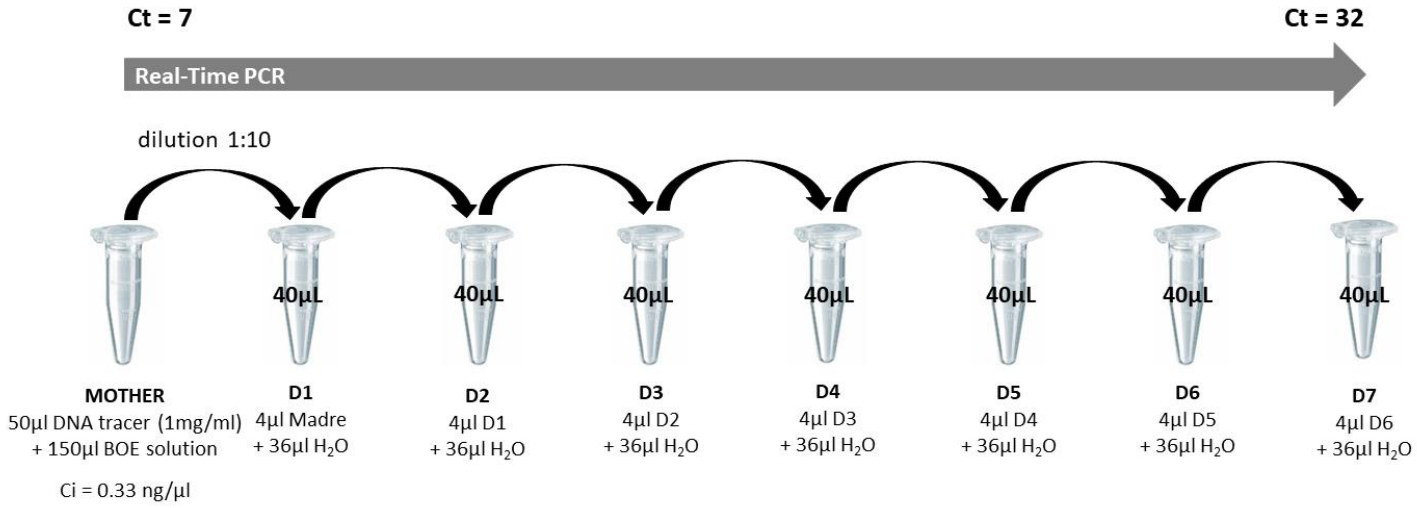

Figure a1. Schematic procedure for preparing the seven 1:10 serial dilutions (D1-D7) used to construct the standard curve.

Table a1. Example of qPCR plate layout showing the triplicate samples, the three negatives, and some serial dilutions (D4, D5, D6, D7) useful for constructing the standard curve.

	1	2	3	4	5	6	7	8	9	10	11	12
A	55	55	55	56	56	56	57	57	57	58	58	58
B	59	59	59	60	60	60	61	61	61	62	62	62
C	63	63	63	64	64	64	65	65	65	66	66	66
D	67	67	67	68	68	68	69	69	69	70	70	70
E	71	71	71	72	72	72	73	73	73	74	74	74
F	75	75	75	76	76	76	77	77	77	78	78	78
G	79	79	79	80	80	80	81	81	81	neg	neg	neg
H	D4	D4	D4	D5	D5	D5	D6	D6	D6	D7	D7	D7

Among other information, the output file returned by qPCR shows for each sample the Ct value (threshold cycle) of each triplicate – an indication of the number of amplification cycles required for the fluorescent signal emitted by SYBR Green to be detected – and the standard deviation (SD) of the triplicate Ct values. The following quality control was then performed on the raw qPCR data: when SD < 0.5, the arithmetic mean of the three triplicate Ct values (Ct mean) was calculated, when SD > 0.5, the sample was re-analyzed. However, if one replicate pair out of the three had an SD < 0.5, the mean Ct value was calculated based on the mean of the Ct values from that replicate pair. After deriving the Ct mean value of each sample, further filtering was done using the Ct mean value of the negative control, which in each experiment defined the minimum detection limit. Therefore, all samples with a Ct mean value higher than the detection threshold were also considered negative.

As mentioned above, in order to derive the DNA concentration value from the Ct mean value of the samples, a regression line starting from the Ct mean values of the serial dilutions was constructed for each experiment (Table a2; Figure a2).

Table a2. Example of table summarizing the serial dilution data of an experiment, in which the Ct mean and concentration value in ng/µl and log10(g/l) are given for each dilution, and in which the intercept and beta value of the regression line obtained are indicated (Figure a3).

Sample Name	Cт Mean	Concentration (ng/µl)	Concentration log10(g/l)	Intercept	Beta
D2	5.5345	0.0033	-2.48148606	-1.7762	-3.03
D3	9.4066	0.00033	-3.48148606		
D4	11.6062	0.000033	-4.48148606		
D5	14.3997	0.0000033	-5.48148606		
D6	17.8225	0.00000033	-6.48148606		
D7	21.1362	0.000000033	-7.48148606		

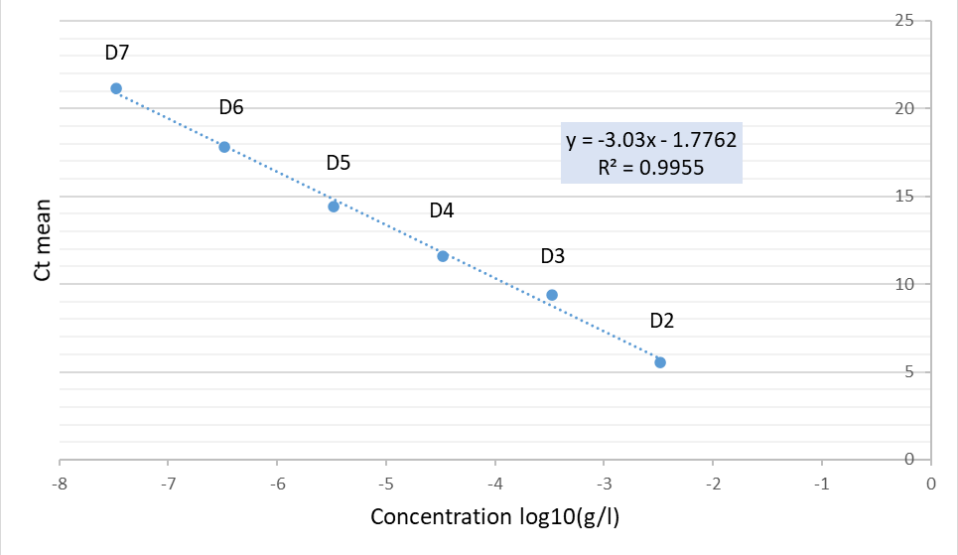

Figure a2. Example of regression line constructed from the Ct mean values of the serial dilutions and their known concentrations reported in Table a4.

The Ct mean of each sample was then entered into the equation of the obtained line in order to derive the sample concentration value.

Similarly, it was also possible to derive the concentration value of the negative controls and thus establish the method's limit of detection.

Once the concentration values for each sample were obtained, it was possible for the hydrogeologists to construct the tracer breakthrough curve (BTC) for each sampling point. In particular, these curves

show the time trend of the DNA tracer concentration (ng/l), providing information on the distribution and flow patterns of groundwater.

I.4. Results and Discussion

Considering the most conservative negative control concentration value among all experiments, the method's limit of detection was found to be $2.64*10^{-11}$ g/l, corresponding to an estimated total number of 37 particles.

BTCs were observed at all three sampling points (S2, S3, B2) (Figure a3). The first tracer detection occurred between ≤ 10 minutes, at points S2 and B2, and 15-30 minutes, at point S3, after injection, therefore very early. A first concentration peak of the tracer was recorded at S2 (5.44 ng/l) and B2 (1.31 ng/l), with a difference of approximately 30 minutes after injection. In particular, the S2 BTC showed the highest DNA concentration value and its main peak was followed 80 minutes later by a secondary peak (1.84 ng/l). In contrast, the BTC of S3 was noisier than the others, showing scattered values above and below the detection limit, with a maximum concentration of 0.19 ng/l 60 minutes after injection. Additionally, the sharp shapes of the first arrival peaks have suggested a low dispersivity of the tracer. The elapsed time from the BTCs to the first detection limit was approximately 3 hours for point S2 and 4 hours for point B2.

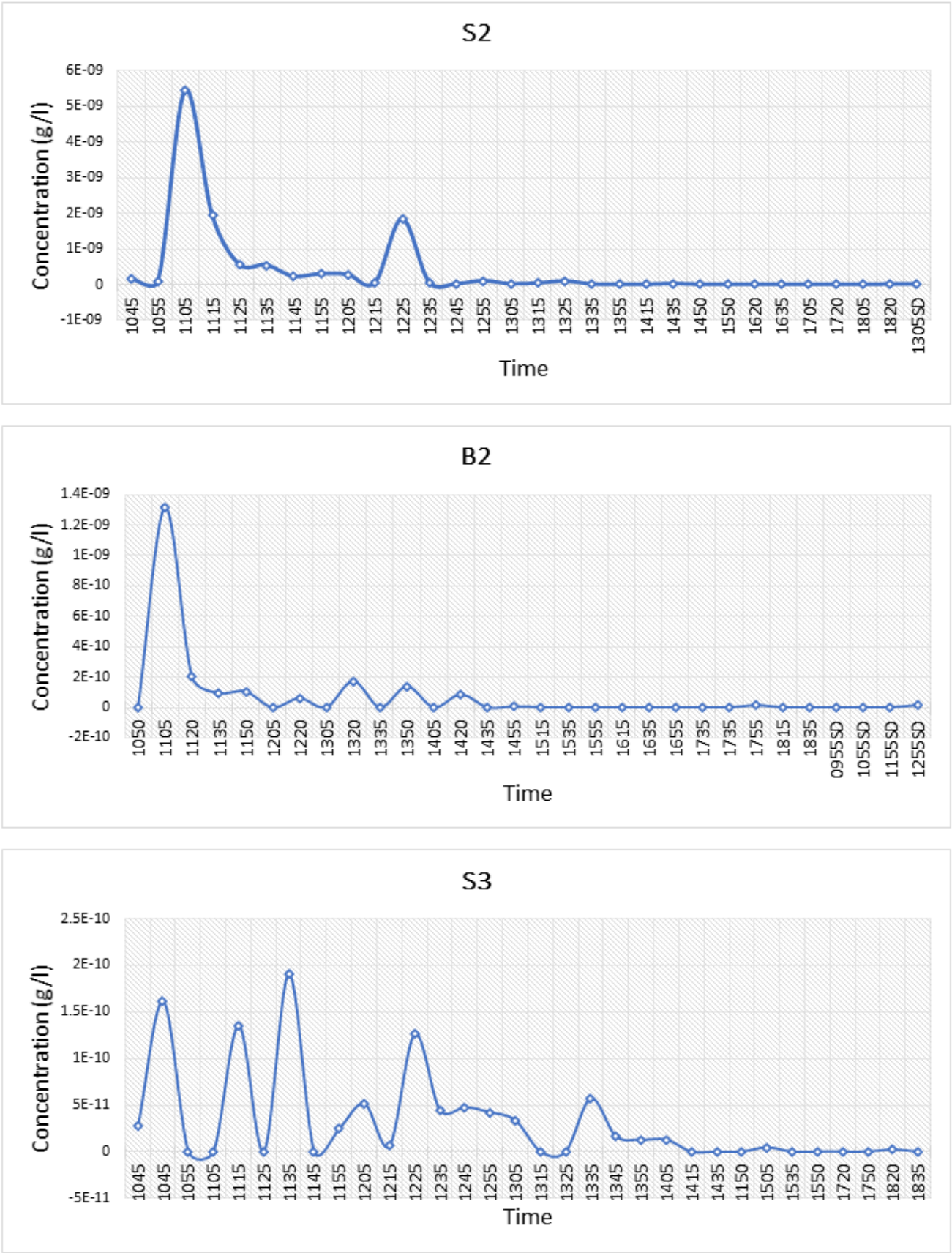

Figure a3. BTCs for sampling points S2, B2 and S3, where the DNA tracer concentration (g/l) is plotted against time.

Finally, the groundwater velocity estimated through nanoparticle BTCs, compared with that of other tracers such as salt, showed that DNA-based tracers move faster than other soluble tracers. This suggests that their migration follows larger pathways associated with higher flow velocities, making them a selective tracer more suitable for tracking preferential flow paths. Furthermore, the low concentration of recovered nanoparticles is a further indication of the preferential transport of DNA tracers along highly conductive channels. As mentioned above, in fact, nanoparticles may suffer from fracture exclusion, where flow velocities tend to be low, due to their size or gravitational sedimentation especially in aquifers with irregular or tortuous ducts. Furthermore, the BTC of sampling point S3 showed that as the distance between the injection point and the sampling point increases, the probability of the nanoparticles reaching their destination decreases.

Although preliminary, these results confirm DNA nanoparticles as optimal tracers for high-permeability pathways and contribute to increasing knowledge on the estimation of actual groundwater velocities and flow paths. Moreover, these data could prove useful in the future to more precisely identify the distribution dynamics of contaminants in groundwater, thus providing more detailed information to characterize individuals more accurately according to their level of exposure.

II. APPENDIX: Genetic variability, DNA methylation, and environmental toxicants

II.1. Introduction

All living organisms in the course of their evolutionary history on our planet have interacted with a constantly changing environment to which they have had to adapt. Some substances initially considered toxic have become resources for some organisms over time. Oxygen, heavy metals, UV rays and various chemical compounds are just some of the substances that have shaped and led to the organisms present on planet Earth today (Brady et al., 2017). As mentioned in Section 1.2, humans throughout their evolutionary history have also been exposed to various harmful external agents for which they had to develop survival strategies, leading over time to the human evolution and biodiversity we observe today.

For example, innate immunity receptors are immune system proteins that recognize certain features of pathogens or signals of cellular damage. These proteins are very important in the organism's primary defense, as they activate rapid signaling pathways that lead to the production of proinflammatory cytokines and activation of the immune system. There are different types of innate immunity receptors, including the Scavenger Receptors (SRs), which are mainly expressed on macrophages and dendritic cells, and involved in phagocytosis and degradation of exogenous particles and molecules, contributing to tissue cleansing and regulation of the immune response. Among the class A SRs is the MAcrophage Receptor with COllagenous domain (MARCO), which binds bacteria and environmental particles, such as crystalline **silica**, leading to alveolar macrophage apoptosis and release of proinflammatory cytokines (Hamilton et al., 2006; Thakur et al., 2008). A recent study compared hominoid genomes and reported two positively selected amino acid substitutions in MARCO. The first is located at position 282 within the MARCO collagenous domain and led from serine present in great apes, Neanderthals, and Denisova to phenylalanine found exclusively in *Homo sapiens*. This position in *Homo sapiens* is polymorphic (rs6761637) and shows a higher prevalence of the derived allele in modern human populations (global frequency of the minor ancestral allele = 16.8%). In contrast, the second substitution is located at position 452 within the C-terminal Scavenger Receptor Cysteine Rich (SRCR) domain of MARCO and led from histidine in primates to glutamine in Neanderthals, Denisova, and *Homo sapiens*. Both substitutions increase MARCO efficiency by enhancing receptor-ligand association and phagocytosis (Novakowski et al., 2018). According to some researchers, it is possible that the exposure of human ancestors to pathogens and the increased amount of silica dust and pollen due to the transition from forest to savanna in East Africa contributed to the genetic selection of more efficient SRs in order to better defend the organism from inhaled environmental toxins (Trumble and Finch, 2019).

As cited in Section 1.2, another exposome that humans have faced throughout their evolutionary history are the products of domestic fire, namely toxic fumes and noxious substances produced by burning food, which are known to affect human health and fertility (Aarts et al., 2016). **PAHs**, for example, are carcinogens generated by the incomplete combustion of organic substances such as wood that can be inhaled through smoke (Leachi et al., 2020). Before the discovery of fire, humans were only exposed to PAHs during sporadic forest fires, therefore the systematic inhalation of these substances proved to be a new environmental challenge for the human organism (Trumble and Finch, 2019). It has been observed that inhalation of PAHs in humans causes activation of the Aryl hydrocarbon Receptor (AhR) (Liu et al., 2018), which in turn moves into the cell nucleus to form a heterodimer with the Aryl hydrocarbon Nuclear Translocator (ARNT protein). The AhR/ARNT complex binds to a specific DNA sequence of target gene promoters called the Xenobiotic Response Element (XRE), activating the transcription of several genes, including cyp1a1, cyp1a2, and cyp1b1, which encode for enzymes of the CYtochrome P450 1 family and are important for detoxification from drugs, carcinogens, and environmental toxins (Degrelle et al., 2022; Trumble and Finch, 2019). Comparison of Neanderthal and *Homo sapiens* revealed a substitution at position 381 of the AhR. *Homo sapiens* in fact has valine while Neanderthal has alanine (Aarts et al., 2016; Hubbard et al., 2016). The ancestral variant present in Neanderthal has higher affinity for some PAHs, higher DNA binding capacity, and higher transcriptional activity of cyp1a1 and cyp1b1 genes than the derived variant present in *Homo sapiens*. However, CYP1A1 and CYP1B1 enzymes regulating the metabolism of PAHs can lead to the production of toxic intermediates. The AhR of *Homo sapiens*, on the other hand, has an altered ligand binding site, which is therefore less activated by PAHs resulting in lower production of toxic intermediates, suggesting possible adaptation to combustion products (Hubbard et al., 2016).

Nowadays, a striking and highly visible example of genetic human adaptation to environmental stresses is that of adaptation to extreme environments. **Oxygen-deficient** environments, such as those at high altitudes, are harmful environments for most human populations. However, there are populations such as Tibetans who over time have developed genetic variations that affect their response to hypoxia, energy metabolism, and lung function, enabling them to adapt to high altitudes (Shi et al., 2023).

Another example of genetic adaptation to harmful environments is that of South American indigenous communities that have adapted to environments rich in **As**, a naturally occurring toxic chemical found within mineral deposits, thermal springs, rivers, and volcanic rocks (Tapia et al., 2019). The As-contaminated drinking water to which these populations have been exposed for thousands of years

over time has led to increased frequencies of protective variants of the as3mt gene, which encodes for the enzyme As 3 Methyltransferase (AS3MT), which is essential for As metabolism and excretion in humans (De Loma et al., 2022; Schlebusch et al., 2015, 2013).

Finally, the heterozygous GSTP1 genotype, involved in the detoxification of benzene, appears to be more prevalent in gas station attendants exposed to **BTEX** (Silvestre et al., 2020).

However, the evidence of human genetic adaptations to new pollutants is very limited, as the spread of these substances has only occurred recently compared to human evolutionary history. For this reason, the study of DNAm is extremely important, as it represents a molecular mechanism that is subject to variation in response to environmental factors that is also closely linked to genetic background (Sections 1.1 and 1.2). DNAm and genome interaction can indeed be used to investigate potential ongoing environment adaptations.

II.2. Aim of the study

The purpose of this pilot study was to investigate European genetic variability in relation to exposure to air pollution, as well as one of the currently most widespread and studied environmental contaminants, by studying the interconnection between the DNAm and the human genome, in order to identify possible differences between populations.

II.3. Materials and Methods

As previously mentioned, one of the most studied contaminants is air pollution. In fact, between 2017 and 2024 alone, a total of 335 studies linking DNAm and air pollution were collected on PubMed (National Library of Medicine). As cited in paragraph 1.2.1.1, air pollution is a mixture of pollutants, such as PM10, PM2.5, exhaust gases, O3, heavy metals, and organic compounds, which, collectively or individually, in several studies have been shown to alter DNAm levels with possible adverse effects on human health.

Initially, therefore, a literature review was conducted in search of CpG sites whose methylation levels tended to vary in response to air pollution exposure in European populations (Abraham et al., 2018; Mostafavi et al., 2018; Plusquin et al., 2017; Rabinovitch et al., 2021). Subsequently, further research was undertaken in order to identify whether the methylation status of these CpG sites was influenced, and thus associated, by certain genotypes at specific loci (SNPs), thus forming meQTLs. This research was carried out by consulting a previously published atlas (Min et al., 2021).

Once the variants associated with CpG sites sensitive to air pollution had been identified, an analysis of allele frequencies in five European populations (British, Finnish, Iberian, Tuscan, and Utah residents with Northern and Western European ancestry (CEU)) was carried out in order to identify possible differences between different populations. This analysis was implemented using the PLINK 1.9 software (Chang et al., 2015) and the genetic data available in the database constructed in the context of the 1000 Genomes project (The 1000 Genomes Project Consortium et al., 2015). Then, for each population, the number of haploid individuals carrying the minor allele and the major allele was derived. Finally, Fisher's test was applied between pairs of populations to identify possible differences in frequency (p-value < 0.05).

II.4. Results and Discussion

The literature analysis revealed many differentially methylated CpG sites following exposure to various components of air pollution, however, among these, only 19 CpGs were found to be influenced by genetic background in Europeans (Table a3).

Table a3. CpG sites showing differential methylation levels after exposure to air pollution components, and associated with specific SNPs (meQTLs) in European populations.

Toxicant	CpG	Chr	Gene	SNP	Allele 1	Allele 2
NO_2	cg17580614	17	ADORA2B	rs10221188	G	A
NO_2	cg07563400	17	ADORA2B	rs4792682	T	C
PM_{10}	cg04112100	2	KYNU	rs351696	A	G
PM_{10}	cg23075260	8	ADCK5	rs2280836	G	A
NO_2	cg20491726	2	-	rs56321608	A	G
PM_{10}	cg03215416	8	PSD3	rs2638662	A	G
diesel exhaust	cg05361273	2	GPR17	rs2248754	T	C
$PM_{2.5}$	cg02556634	4	PPP2R2C	rs71599895	T	C
$PM_{2.5}$	cg02508204	11	-	rs12420350	C	A
$PM_{2.5}$	cg19850855	5	FAM172A	rs13361613	T	C
$PM_{2.5}$	cg23468453	10	ASCC1	rs1271352	C	A
$PM_{2.5}$	cg26559703	10	APBB1IP	rs1775227	G	T
$PM_{2.5}$	cg03408122	22	SBF1	rs13054572	T	C
NO_2	cg16205861	12	-	rs7968810	A	G
NO_2	cg13918628	9	CD72	rs58419281	C	T
PM_{10}	cg03513315	22	PES1	rs9608938	G	A
$PM_{2.5}$	cg04319606	2	C2orf70	rs58644455	A	G
$PM_{2.5}$	cg09568355	2	-	rs62130902	C	G
$PM_{2.5}$	cg23890774	19	-	rs2141959	C	G

Subsequently, allele frequency analysis performed on the 19 variants returned 26 statistically significant population pairwise comparisons, among which the most significant (p-value ≤ 0.002) were: Tuscan vs Finnish (p-value = 0.0003) and British vs Finnish (p-value = 0.0009) for variant

rs12420350; Tuscan vs Iberian (p-value = 0.0017) for variant rs13054572; Tuscan vs CEUs (p-value = 0.0025) and Iberian vs CEUs (p-value = 0.0004) for variant rs1775227; and Finns vs Iberians (p-value = $7.71*10^{-5}$) for variant rs7968810 (Figure a4).

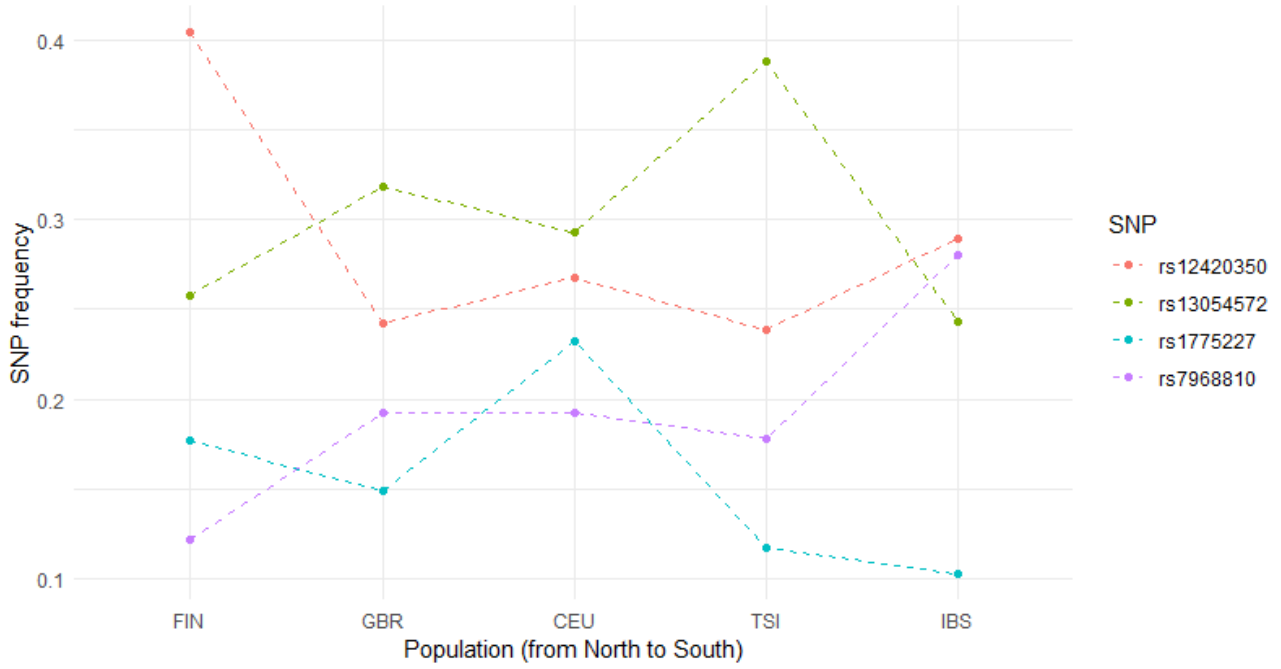

Figure a4. Frequencies of the four most significant SNPs (p-value ≤ 0.002) in the five European populations ordered from north to south: Finnish (FIN), British (GBR), CEU, Tuscan (TSI), and Iberian (IBS).

Although very preliminary, these data contribute to expanding the knowledge of the role of genetic variability in the detoxification of contaminants, highlighting the presence of specific variants associated to CpG sites that are sensitive to air pollution components. Future studies will be necessary to further investigate these findings, including CpG sites located on gene promoters involved in detoxification.

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8. SUPPLEMENTARY MATERIAL

Demographic information of study participants

Table S1. Table summarizing data of study participants: sample identifier, classification group (HLE = high level of exposure; LLE = low level of exposure), sampling site (ROS=Rosciano; TDP=Torre de' Passeri), year of birth, age at the time of sampling (occurred in Rosciano in 2019 and in Torre de' Passeri in 2020), and exposure time (calculated considering that the Colle Sant'Angelo well field was opened in the early 1980s and a high level of contaminants was present in the wells until the early 2000s). The outlier sample emerged during epigenome-wide analysis is highlighted in gray.

Sample ID	Group	Sampling site	Year of Birth	Age at time of sampling (2019-2020)	Exposition time (yrs)
AL002	LLE	ROS	1960	59	20
AL004	HLE	TDP	1965	55	20
AL007	LLE	ROS	1980	39	20
AL008	LLE	TDP	1984	36	16
AL010	HLE	TDP	1960	60	20
AL012	LLE	ROS	1963	56	20
AL014	HLE	TDP	1949	71	20
AL016	LLE	ROS	1974	45	20
AL017	LLE	ROS	1978	41	20
AL018	HLE	TDP	1981	39	19
AL020	HLE	TDP	1958	62	20
AL022	LLE	ROS	1974	45	20
AL024	HLE	TDP	1973	47	20
AL026	HLE	ROS	1961	58	20
AL027	LLE	ROS	1951	68	20
AL028	HLE	TDP	1957	63	20
AL029	HLE	TDP	1976	44	20
AL030	HLE	TDP	1997	23	3
AL032	HLE	ROS	1945	74	20
AL034	HLE	TDP	1965	55	20
AL036	LLE	ROS	1967	52	20
AL037	LLE	ROS	1996	23	4
AL038	HLE	TDP	1969	51	20
AL040	LLE	ROS	1959	60	20
AL042	LLE	ROS	1977	42	20
AL044	HLE	TDP	1976	44	20
AL046	LLE	ROS	1972	47	20
AL047	LLE	TDP	1955	65	20
AL048	HLE	TDP	1978	42	20
AL050	LLE	ROS	1986	33	14

AL052	LLE	ROS	1950	69	20
AL054	HLE	TDP	1980	40	20
AL056	LLE	ROS	1946	73	20
AL057	HLE	TDP	1968	52	20
AL058	HLE	TDP	1965	55	20
AL060	LLE	ROS	1964	55	20
AL062	HLE	TDP	1964	56	20
AL064	HLE	TDP	1972	48	20
AL066	LLE	ROS	1977	42	20
AL068	LLE	TDP	1988	32	12
AL070	LLE	ROS	1971	48	20
AL071	LLE	ROS	1959	60	20
AL072	HLE	TDP	1978	42	20
AL074	HLE	TDP	1971	49	20
AL076	LLE	ROS	1979	40	20
AL078	HLE	TDP	1963	57	20
AL080	LLE	ROS	1999	20	1
AL081	HLE	ROS	1943	76	20
AL082	HLE	TDP	1989	31	11
AL084	HLE	TDP	1959	61	20
AL085	LLE	ROS	1965	54	20
AL086	LLE	ROS	1950	69	20
AL088	HLE	TDP	1959	61	20
AL090	LLE	ROS	1956	63	20
AL091	LLE	ROA	1969	50	20
AL092	HLE	TDP	1952	68	20
AL094	HLE	TDP	1949	71	20
AL095	LLE	ROS	1963	56	20
AL096	LLE	ROS	1955	64	20
AL098	HLE	TDP	1980	40	20
AL100	LLE	ROS	1951	68	20

Table S2. Output file of Gene Ontology analysis reporting significant biological pathways (FDR q-value < 0.05).

ID	Description	Size	enrichmentScore	NES	pvalue	padj
GO:0048706	embryonic skeletal system development	114	0.738449525	1.252	4.51E-08	7.23E-05
GO:0048562	embryonic organ morphogenesis	269	0.667012668	1.151	3.96E-07	3.17E-04
GO:0007389	pattern specification process	401	0.642834503	1.115	7.71E-07	4.12E-04
GO:0001501	skeletal system development	450	0.636083534	1.105	1.46E-06	5.86E-04
GO:0048705	skeletal system morphogenesis	205	0.676945337	1.162	2.22E-06	0.00071
GO:0048736	appendage development	160	0.679718512	1.162	1.26E-05	0.00233
GO:0060173	limb development	160	0.679718512	1.162	1.26E-05	0.00233
GO:0048568	embryonic organ development	398	0.635597024	1.102	1.34E-05	0.00233
GO:0030198	extracellular matrix organization	372	0.634908383	1.1	1.58E-05	0.00233
GO:0030695	GTPase regulator activity	415	0.632583352	1.098	1.61E-05	0.00233
GO:0060589	nucleoside-triphosphatase regulator activity	457	0.628090542	1.091	1.66E-05	0.00233
GO:0043062	extracellular structure organization	373	0.635310272	1.101	1.75E-05	0.00233
GO:0002224	toll-like receptor signaling pathway	143	0.68512039	1.168	2.69E-05	0.00312
GO:0003002	regionalization	314	0.637993865	1.103	3.00E-05	0.00312
GO:0034329	cell junction assembly	397	0.631601203	1.096	3.06E-05	0.00312
GO:0045229	external encapsulating structure organization	375	0.634873165	1.1	3.12E-05	0.00312
GO:0002064	epithelial cell development	191	0.658432136	1.129	5.70E-05	0.00537
GO:0060538	skeletal muscle organ development	147	0.671983666	1.146	6.04E-05	0.00537
GO:0001216	DNA-binding transcription activator activity	427	0.625342756	1.086	7.61E-05	0.00616
GO:0001228	DNA-binding transcription activator activity, RNA polymerase II-specific	423	0.625376739	1.085	7.95E-05	0.00616
GO:0060284	regulation of cell development	458	0.622917841	1.082	8.07E-05	0.00616
GO:0050770	regulation of axonogenesis	140	0.674896023	1.15	8.63E-05	0.00622
GO:0010975	regulation of neuron projection development	398	0.628592373	1.09	9.08E-05	0.00622
GO:0090596	sensory organ morphogenesis	236	0.646592635	1.113	9.31E-05	0.00622
GO:0015629	actin cytoskeleton	469	0.62040667	1.078	9.98E-05	0.00633
GO:0043010	camera-type eye development	301	0.631608119	1.091	0.00011	0.00633
GO:1902414	protein localization to cell junction	100	0.704015753	1.19	0.00011	0.00633
GO:0035107	appendage morphogenesis	128	0.682610579	1.161	0.00012	0.00658
GO:0035108	limb morphogenesis	128	0.682610579	1.161	0.00012	0.00658
GO:0007409	axonogenesis	434	0.62321724	1.082	0.00013	0.00672
GO:0007519	skeletal muscle tissue development	139	0.67386712	1.148	0.00013	0.00698
GO:0061448	connective tissue development	222	0.646318612	1.112	0.00014	0.0071
GO:0061564	axon development	475	0.618887093	1.076	0.00015	0.0071
GO:0010720	positive regulation of cell development	274	0.639669822	1.104	0.00016	0.00754
GO:0001763	morphogenesis of a branching structure	182	0.655983876	1.124	0.00018	0.00805
GO:0047485	protein N-terminus binding	101	0.687472482	1.162	0.00018	0.00823
GO:0005096	GTPase activator activity	239	0.641717462	1.105	0.0002	0.00859
GO:0017171	serine hydrolase activity	172	0.655757659	1.123	0.00022	0.00933
GO:0044282	small molecule catabolic process	411	0.621302556	1.078	0.00023	0.00946
GO:0001654	eye development	347	0.625034711	1.082	0.00029	0.0116
GO:0005774	vacuolar membrane	388	0.621454142	1.078	0.00031	0.01191

GO:0043583	ear development	194	0.652113823	1.118	0.00031	0.01191
GO:0005085	guanyl-nucleotide exchange factor activity	182	0.652437998	1.118	0.00033	0.01232
GO:0042113	B cell activation	231	0.639297375	1.1	0.00036	0.01278
GO:0050767	regulation of neurogenesis	330	0.629422086	1.089	0.00036	0.01278
GO:0040029	regulation of gene expression, epigenetic	129	0.670088039	1.14	0.00037	0.01278
GO:0005667	transcription regulator complex	382	0.617942936	1.071	0.00048	0.01636
GO:0071222	cellular response to lipopolysaccharide	186	0.646552229	1.108	0.00049	0.01636
GO:0051216	cartilage development	169	0.653198151	1.118	0.0006	0.01906
GO:0051960	regulation of nervous system development	400	0.617218171	1.071	0.0006	0.01906
GO:0030326	embryonic limb morphogenesis	108	0.674712073	1.143	0.00062	0.01906
GO:0035113	embryonic appendage morphogenesis	108	0.674712073	1.143	0.00062	0.01906
GO:0009063	cellular amino acid catabolic process	101	0.677393061	1.145	0.00064	0.01925
GO:0150063	visual system development	351	0.620110721	1.073	0.00066	0.01971
GO:0042471	ear morphogenesis	106	0.678206193	1.148	0.00071	0.02022
GO:0050878	regulation of body fluid levels	463	0.614629712	1.068	0.00072	0.02022
GO:0061138	morphogenesis of a branching epithelium	169	0.65240518	1.116	0.00072	0.02022
GO:0071219	cellular response to molecule of bacterial origin	194	0.646055414	1.108	0.0008	0.02215
GO:0008236	serine-type peptidase activity	170	0.653195192	1.118	0.00082	0.02227
GO:0060562	epithelial tube morphogenesis	301	0.621661447	1.074	0.00097	0.02582
GO:0007601	visual perception	199	0.639810364	1.098	0.00106	0.02782
GO:0048193	Golgi vesicle transport	342	0.618547265	1.07	0.00108	0.02784
GO:0002440	production of molecular mediator of immune response	175	0.645070711	1.105	0.0011	0.02787
GO:0002283	neutrophil activation involved in immune response	459	0.610884098	1.061	0.00113	0.02836
GO:0060078	regulation of postsynaptic membrane potential	127	0.662970209	1.128	0.00115	0.02837
GO:0050953	sensory perception of light stimulus	202	0.639776532	1.098	0.00121	0.02886
GO:0005765	lysosomal membrane	337	0.621519843	1.075	0.00122	0.02886
GO:0098852	lytic vacuole membrane	337	0.621519843	1.075	0.00122	0.02886
GO:0050769	positive regulation of neurogenesis	205	0.641098668	1.101	0.00126	0.02929
GO:0030135	coated vesicle	275	0.626350707	1.081	0.00133	0.03056
GO:0031983	vesicle lumen	305	0.619422907	1.07	0.00143	0.03221
GO:0007605	sensory perception of sound	139	0.655194767	1.116	0.0015	0.03325
GO:0007411	axon guidance	266	0.628848433	1.085	0.00152	0.03325
GO:0019838	growth factor binding	128	0.661160934	1.125	0.00154	0.03325
GO:0002221	pattern recognition receptor signaling pathway	188	0.642521026	1.101	0.00156	0.03325
GO:0009952	anterior/posterior pattern specification	192	0.640160741	1.098	0.00165	0.03475
GO:0030667	secretory granule membrane	287	0.623064838	1.076	0.00168	0.03507
GO:0097485	neuron projection guidance	267	0.628838257	1.085	0.00176	0.03596
GO:0150115	cell-substrate junction organization	104	0.667966999	1.13	0.00178	0.03596
GO:0006520	cellular amino acid metabolic process	289	0.623279726	1.076	0.00179	0.03596
GO:0005911	cell-cell junction	449	0.609974828	1.059	0.00191	0.03707
GO:0043312	neutrophil degranulation	456	0.610754873	1.061	0.00191	0.03707
GO:0022803	passive transmembrane transporter activity	434	0.611232457	1.061	0.00194	0.03707

GO:0036230	granulocyte activation	475	0.608579049	1.058	0.00194	0.03707
GO:0043235	receptor complex	380	0.615011477	1.066	0.00198	0.03733
GO:0005938	cell cortex	272	0.625294684	1.079	0.00205	0.03808
GO:0043292	contractile fiber	220	0.631707108	1.086	0.00209	0.03808
GO:0050900	leukocyte migration	418	0.612940019	1.064	0.00209	0.03808
GO:0003007	heart morphogenesis	230	0.626948327	1.079	0.00213	0.03832
GO:0099240	intrinsic component of synaptic membrane	132	0.656272533	1.117	0.00216	0.03855
GO:0008361	regulation of cell size	161	0.641358631	1.096	0.00228	0.03965
GO:0030099	myeloid cell differentiation	370	0.613348291	1.063	0.00228	0.03965
GO:0042734	presynaptic membrane	130	0.652034129	1.109	0.00253	0.04368
GO:1901888	regulation of cell junction assembly	181	0.639214495	1.095	0.00261	0.04448
GO:0051962	positive regulation of nervous system development	248	0.627592777	1.081	0.00272	0.04589
GO:0002446	neutrophil mediated immunity	470	0.608529074	1.058	0.00276	0.04603
GO:0043547	positive regulation of GTPase activity	363	0.614316261	1.064	0.00279	0.04616
GO:0015267	channel activity	433	0.611243188	1.061	0.00283	0.0463
GO:0042119	neutrophil activation	470	0.608197499	1.057	0.0029	0.04635
GO:0030016	myofibril	213	0.631764905	1.086	0.00298	0.04635
GO:0042470	melanosome	104	0.662202322	1.12	0.00298	0.04635
GO:0048770	pigment granule	104	0.662202322	1.12	0.00298	0.04635
GO:1903706	regulation of hemopoiesis	365	0.614646818	1.065	0.00298	0.04635
GO:0034774	secretory granule lumen	299	0.618118155	1.068	0.00309	0.04762

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