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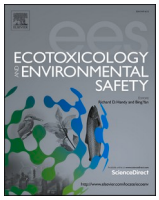

# Chlorinated solvents in groundwater and human DNA methylation: The Italian case of Bussi sul Tirino

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ABSTRACT

Environmental toxicants, including chlorinated solvents present in contaminated water sources, can modify human DNA methylation (DNAm), an epigenetic mechanism that underlies biological plasticity in response to environmental perturbations. Given the relevance of these epigenetic mechanisms, this study examines the DNAm profiles of communities near Bussi sul Tirino (Abruzzo, Italy), an area declared a Site of National Interest (SNI) in 2008 due to extensive environmental contamination, including chlorinated solvents. The aim was to determine whether long-standing groundwater contamination is associated with changes in DNAm variability. Specifically, buccal swabs were collected from 61 volunteers classified into high (HLE) and low (LLE) exposure groups based on proximity to contaminated water sources. Subsequently, bisulfite sequencing (MiSeq, Illumina) was used to assess DNAm in repetitive elements, while genome-wide DNAm and genotyping were performed on a subset of 32 individuals using Illumina MethylationEPIC and HumanOmniExpress 720k BeadChips, respectively. Genome-wide analysis identified differentially methylated positions enriched in genes related to embryonic/cellular development, nervous system development, and immune function. In particular, the strongest association was observed at cg04879348 (GCC2 gene; $p = 6.52 \times 10^{-5}$), previously linked to organochlorine exposure in a group of workers diagnosed with Parkinson's disease. Importantly, the HLE group displayed reduced DNAm variability, potentially reflecting environmental pressure on epigenetic regulation, and a trend toward LINE-1 hypomethylation, suggesting genomic instability. However, no differences were found in epimutation load and epigenetic aging in the oral tissue of this group. In conclusion, this study provides novel evidence that past long-term environmental contamination exposure can shape DNAm profiles in exposed populations, highlighting the relevance of epigenetic markers in environmental health research.

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## 1. Introduction

In 2008, the Bussi sul Tirino area, located in the central Italy (Abruzzo region), was classified as a Site of National Interest (SNI) requiring remediation due to high levels of contamination in the groundwater and soil (Italian Ministerial Decree of May 29, 2008), as also reported on the website of the Italian Ministry of Environment and Energy Security (Guerranti et al., 2017; Vitali et al., 2021; Ministero dell'Ambiente e della Sicurezza Energetica, 2024).

Over the decades, large amounts of industrial waste have been illegally buried and spread in the areas surrounding the plants including in the Tirino river, in Tre Monti dump area, and in other dumps to the North of the chemical complex. This has resulted in the progressive contamination of environmental matrices, with severe pollution reported in soil, river sediments, and groundwater. The area's hydrogeological configuration has further favored the migration of contaminants, especially chlorinated solvents. Over time, these compounds reached the Colle Sant'Angelo well field, the main source of drinking water for the province of Pescara via the Giardino aqueduct (Di Curzio et al., 2018; Filippini et al., 2018; Guerranti et al., 2017; Milan et al., 2019; Vitali et al., 2021; Di Molfetta and Fracassi, 2008; Luchetti et al., 2021; Ministero dell'Ambiente e della Sicurezza Energetica, 2024).

Although systematic long-term monitoring of these substances has not been conducted, the first traces of chlorinated solvents in drinking water were identified in 1992 following an analysis of water from the well field, revealing concentrations from 1 to 10 μg/L for single chlorinated compounds (Filippini et al., 2018). However, historical evidence indicated that contamination was already present in earlier decades. The Colle Sant'Angelo wells, drilled in the early 1980s, were certainly contaminated with chlorinated solvents from the start of their operation, as hazardous industrial wastes from the production of chloromethanes (chlorinated pitches) were buried in the Tre Monti landfill site in the 1970's. At the Tre Monti landfill groundwater samples collected in 2012 and 2017 revealed chlorinated solvent concentrations reaching up to 10,000 μg/L for individual compounds.

In addition to chlorinated solvents, various inspections over the years have confirmed the presence of numerous other contaminants in the different environmental matrices of the Bussi sul Tirino SNI. These include metals, hydrocarbons, BTEX compounds (benzene, toluene, ethylbenzene, and xylenes), boron, polycyclic aromatic hydrocarbons (PAHs) and dioxins. Mercury (Hg) and lead (Pb) were particularly prevalent in the soil and subsoil, while trichloroethylene (TCE) was the main contaminant in drinking water (Table 1S of the Supplementary Tables file).

Despite the wide variety of pollutants, studies on food, environmental matrices, and the local population have yielded contrasting results (Castellani et al., 2023, 2021; Guerranti et al., 2017; Vitali et al., 2021). This uncertainty makes it essential to consider biological mechanisms capable of capturing both current and past exposure. Although current environmental contamination may be minimal or even undetectable in some cases, it is well established that epigenetic mechanisms, such as DNA methylation (DNAm), can retain molecular signatures of past exposures, acting as a biological interface between the environment and human biology (Giuliani et al., 2018; Vineis et al., 2017). Chlorinated solvents, in particular, have been shown to alter DNAm patterns in various experimental models (Cui et al., 2016; Gilbert et al., 2017, 2016; Li et al., 2023). However, most of these studies have been conducted in cell lines or animal models, and data from exposed human populations remain limited (Phillips et al., 2019; Wu et al., 2013; Zhao et al., 2022). Several mechanisms have been proposed to explain how chlorinated solvents may alter DNAm patterns. Experimental and toxicological evidence indicates that compounds such as TCE, perchloroethylene (PCE), and dichloromethane can induce oxidative stress, generating reactive oxygen species potentially affecting epigenetic regulation (Jin et al., 2020). In addition, metabolites of TCE, including dichloroacetic acid (DCA) and trichloroacetic acid (TCA), can reduce the availability of S-adenosylmethionine (SAM), a key methyl donor in one-carbon metabolism, thereby interfering with the DNAm process itself (Tao et al., 2000). Chronic DCA exposure also induces persistent differentially methylated regions (DMRs), indicating long-lasting epigenetic reprogramming (Carswell et al., 2024). These findings support the notion that TCE metabolites can modulate both global and locus-specific DNAm patterns. Taken together, these findings support the hypothesis that exposure to chlorinated solvents may leave persistent epigenetic signatures, underscoring the need to investigate these mechanisms in environmentally exposed human communities.

To our knowledge, no studies have directly examined the relationship between past exposure to chlorinated solvents via contaminated drinking water and DNAm profiles measured in buccal swab samples.

Among epigenetic markers, the DNAm levels of repetitive elements (REs), such as Long Interspersed Nuclear Element-1 (LINE-1) and the short interspersed sequence Alu, have been shown to respond dynamically to environmental contaminants (Barchitta et al., 2018; Lee et al., 2017; Liu et al., 2019; Munnia et al., 2023; White et al., 2016). Altered DNAm levels in these elements have been associated with various health conditions including cancer and infertility, with hypomethylation often linked to disease onset (Antelo et al., 2012; Chaiwongkot et al., 2022; Kitahara et al., 2020; Miao et al., 2014; Ye et al., 2020). However, to date, the impact of exposure to chlorinated solvents on the methylation of repetitive elements has not been investigated in exposed human communities.

In this framework, the present study aims to investigate the impact of pollutants, particularly chlorinated solvents, on DNAm profiles by considering 61 individuals from the Bussi sul Tirino area (SNI) who have experienced varying degrees of exposure to environmental contaminants. DNAm variability was analyzed at the epigenome-wide and candidate gene levels (Fig. 1S of the Supplementary Materials). Concerning the epigenome-wide analysis, we investigated differentially methylated positions and regions (DMPs, DMRs), gene ontology, meQTLs, and differentially variable positions (DVPs), as well as epigenetic aging and stochastic epigenetic mutations (SEMs). SEMs are a powerful tool for evaluating epigenetic drift which is associated with various age-related disorders and the accumulation of DNA damage (Brusati et al., 2023; Gentilini et al., 2015). Although several studies have investigated the effects of environmental pollutants on DNAm, none have examined the epimutation load (Lichtenfels et al., 2018; Plusquin et al., 2017). Regarding the candidate gene analysis, we investigated the effect of contaminants on the DNAm of three different REs: ribosomal DNA (rDNA) tandem repeats, LINE-1, and Alu (Fig. 1S).

## 2. Materials and methods

### 2.1. Sample description

Oral mucosal cells were collected via buccal swabs from a cohort of 61 volunteers, comprising 30 individuals recruited in Rosciano (PE) in October 2019 and 31 individuals recruited in Torre de' Passeri (PE) in February 2020. The population sampled comprised 19 healthy females and 42 healthy males, with an average age of 51.95 years (± 13.26 SD). The mean exposure duration to contaminants for the cohort was 18.68 years (± 4.08 SD), ranging from 1 to 20 years, calculated by considering that the Colle Sant'Angelo well field opened in the early 1980s and a significant level of contaminants was present in the wells until the early 2000s. Indeed, the 21st century saw the introduction of procedures aimed at reducing the concentration of contaminants in water (see Table 2S and Fig. 2S in the Supplementary Materials for details).

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

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and the use of the samples was approved by

the Bioethics Committee of the University of Bologna (approval date: 11/11/2015) (Supplementary File 1).

All participants provided written informed consent and completed a bio-demographic questionnaire, while biological samples were anonymized with alpha-numeric codes to ensure donor privacy, and stored at the Laboratory of Molecular Anthropology at the University of Bologna.

#### 2.1.1. Geographic score classification method

The samples were divided into two groups based on information collected via questionnaires. Specifically, data regarding each individual's place of birth, residence district, and workplace were recorded (Fig. 1A). Assuming a higher probability of exposure for locations closest to the source of contamination (Fig. 1B) and taking into consideration a map of the Giardino aqueduct (Fig. 1C) - as the most likely mode of exposure is the ingestion of polluted groundwater - each municipality was assigned a score of 1 if located near the initial section of the main distribution line of the Giardino aqueduct (from the municipality of Popoli to, but not including, the municipality of Alanno), and a score of 0 if located in the downstream section of the aqueduct map (from Alanno onwards). This classification assumes that municipalities situated farther from the contaminated area and served by more branched and alternative aqueduct systems are more likely to receive water with more diluted levels of contaminants. Accordingly, each individual was assigned three scores: one for the municipality of birth, one for residence, and one for workplace. These three scores were then summed to produce a single "geographic score", representing the cumulative potential exposure for each participant. For example, an individual with a geographic score of 3 was born, lives, and works in municipalities classified as having higher exposure. Finally, the 61 study participants were divided into two groups based on their geographic score: individuals with a score $< 2$ ($N = 31$) were assigned to the low-level exposure ("LLE") group, while those with a score $\geq 2$ ($N = 30$) were assigned to the high-level of exposure ("HLE") group. The division into groups is therefore based on the water supply system, since, although no data are available on contaminant concentrations in the water distribution system during the period of operation of the Colle Sant'Angelo well field, drinking water was certainly contaminated and used directly by the local population, while the duration of exposure to contamination did not differ between the two groups.

### 2.2. Epigenome-wide microarray analysis

A subset of 33 individuals, comprising 14 from the HLE group and 19 from the LLE group, was selected for whole epigenome analysis. The mean age of this subgroup was 52.76 years (± 13.76 SD), with a gender distribution of 12 females and 21 males.

DNAm levels were measured using the Illumina MethylationEPIC v1.0 BeadChip, which evaluates approximately 850,000 CpG sites across the whole human genome. To mitigate potential batch effects, the samples were randomized.

The raw methylation data, returned in.idat file format, were preprocessed using RStudio software (version 4.2.1) (Posit team, 2023; R Core Team, 2023), using β-values as the methylation metric. Quality control and data normalization procedures were carried out following the recommendations of Maksimovic et al. (2017). Consequently, only CpG sites with a detection p-value$< 0.01$ in all the samples and individuals with at least 99 % of CpG sites showing significant detection p-values were retained (Bodelon et al., 2019; Maksimovic et al., 2017; Pidsley et al., 2016; Qi et al., 2021). CpG sites displaying bimodal and trimodal distributions were filtered out using the *dbscan* package, and those located on sex chromosomes were excluded (Hahsler et al., 2019). Additionally, the CpG sites identified by Zhou et al. (2017), which include single nucleotide polymorphisms (SNPs), were masked. Finally, the data were normalized using the preprocessFunnorm function (with bgCorr = T and dyeCorr = T) of the *minfi* package.

The *EpiDISH* package (Teschendorff et al., 2017) was employed to estimate the proportions of three cell types (epithelial cells (Epi), fibroblasts (Fib), and total immune cells (IC)) for each participant. Subsequently, a *t*-test was used to identify possible significant differences in the proportions of cell types between HLE and LLE individuals (p-value $< 0.05$).

#### 2.2.1. Differentially methylated CpG sites detection and meQTL analysis

We applied the following linear regression model (1) to our data:

$$\text{Meth} = \gamma 0 + \gamma 1\ \text{Group} + \gamma 2\ \text{Sex} + \gamma 3\ \text{Age} \quad (1)$$

where *Meth* is the methylation value referred to a single CpG site (i.e., the β-value) and *γ0* is the intercept. *Group,* that is the variable under investigation, is categorical (binary), indicating whether each individual belongs to the HLE or LLE group. In the model, this variable was coded as a binary indicator (0 = LLE, 1 = HLE), so that $\gamma_1$ represents the difference in mean methylation levels between the two groups. *Sex* (0 = male, 1 = female) and *Age* (continuous) were included as covariates to control for potential effects of sex and age on DNAm levels.

The linear model was implemented using the *limma* package (Ritchie et al., 2015), with subsequent correction of model t-statistics to remove inflation and bias via the *bacon* package (Van Iterson et al., 2017). False Discovery Rate (FDR) analysis was performed using the Benjamini-Hochberg (BH) method to adjust the p-values for the CpG sites. Significant CpG sites were then ranked by delta (Δ), which is the difference between the mean β-values of the LLE and HLE groups for each CpG site (Beltrami et al., 2017; Nickels et al., 2022; Unruh et al., 2019).

To characterize the genomic distribution of differentially methylated positions (DMPs), each CpG site was annotated according to its genomic context (e.g., TSS200, TSS1500, 5′UTR, 1stExon, Body, 3′UTR, Intergenic) and its relation to CpG islands (e.g., Island, Shore, Shelf, OpenSea), as defined in the Illumina EPIC manifest and following the approach described in the *recountmethylation* package (Maden et al., 2022, 2021). To assess whether promoter-associated CpGs were overrepresented among the DMPs, we performed an empirical enrichment analysis. Specifically, we repeatedly drew 10,000 random sets of CpGs from the pool of probes that passed all quality control filters, each containing the same number of sites as the DMP set, and computed for each iteration the proportion of promoter-associated probes. The empirical *P*-value was estimated as the fraction of iterations with the simulated proportion greater than the observed proportion.

Gene Ontology (GO) enrichment analysis was conducted using the methylRRA function (GSEA method) from the *methylGSA* package (Ren and Kuan, 2019), which applies Robust Rank Aggregation to adjust gene-level p-values (Kolde et al., 2012).

Afterwards, Differentially Methylated Regions (DMRs) were identified using the *combp* function (Pedersen et al., 2012).

Finally, a methylation Quantitative Trait Loci (meQTL) analysis was performed via linear regression ($M \sim G + C$), where M represents a matrix with DNAm values (701,357 CpG sites), G is a matrix with genotype data (676,233 SNPs), and C is a matrix with covariates (sex and age). In order to perform this analysis, the same set of 32 samples were also genotyped for approximately 720,000 SNPs using the Illumina HumanOmniExpress BeadChip. Quality checks of the raw genotyping data were performed according to Anderson et al. (2010) using PLINK 1.9 software (Chang et al., 2015). Due to the limited sample size, the discovery phase of the CpG-SNP association analysis relied on a published atlas detailing genetic effects on DNAm in individuals of European ancestry (Min et al., 2021). Signals reported by Min and colleagues were then tested in our dataset (replication phase) and meQTLs with nominal p-value $< 0.05$ were considered significant. Analysis was conducted using the *MatrixEQTL* package in R (Shabalin, 2012), with cis-meQTL defined as CpG-SNP pairs within 1 Mb.

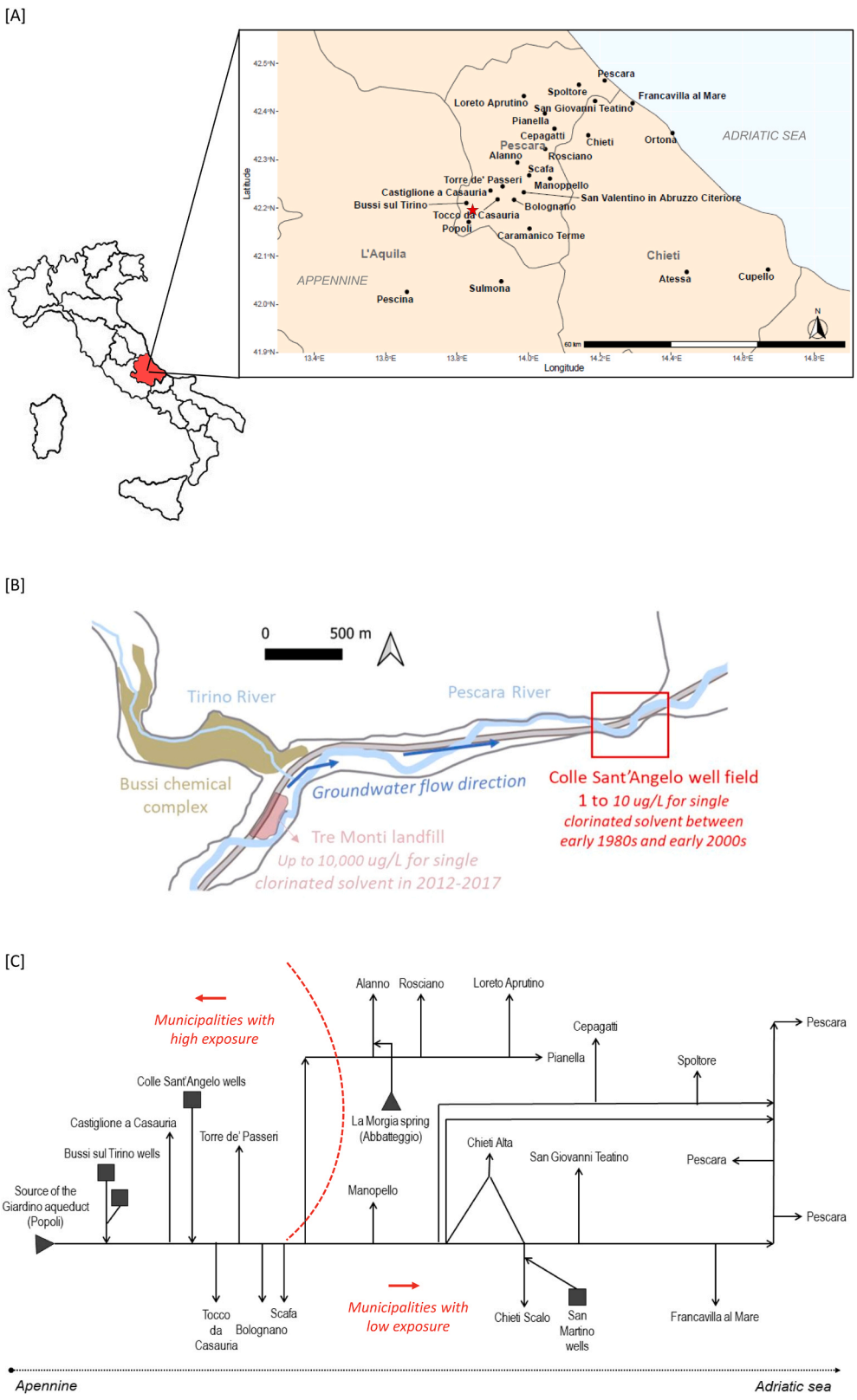


**Fig. 1.** Contamination distribution in the Bussi sul Tirino SNI. A) Geographical location of the Bussi sul Tirino SNI and the municipalities related to the individuals studied. The red star indicates the position of the Bussi industrial pole, located near the Colle Sant'Angelo well field. The gray lines delimit the provinces of the Abruzzo region (Italy). B) Map of the contaminated area of Bussi sul Tirino and groundwater analysis points. The first traces of chlorinated solvents in drinking water were identified in 1992 (1–10 ng/L per compound), while subsequent water analyses (in 2012 and 2017) detected chlorinated solvents (up to 10,000 μg/L per compound) in the Tre Monti landfill. C) Map of the Giardino aqueduct, 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)).

#### 2.2.2. Differentially variable CpG sites

We applied the following linear model (2) to the DNAm data:

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

where *(Meth-mean(Meth))²* represents the squared deviation of the methylation value from a mean methylation value for a single CpG site.

The linear model was implemented using the *varFit* package (Phipson and Oshlack, 2014), and the resulting model t-statistics were corrected to remove inflation and potential bias via the *bacon* package (Van Iterson et al., 2017). To adjust the p-values for the CpG sites, an FDR analysis was performed with the BH method. Subsequently, the Empirical Cumulative Distribution Function (ECDF) test was applied.

#### 2.2.3. Epimutations and epigenetic lesions

Epimutations were identified as extreme outliers in DNAm beta values relative to a reference interval defined across all subjects. For each probe, the interval was set as Q1-(3 ×IQR) to Q3 + (3 ×IQR), where Q1 and Q3 are the first and third quartiles, respectively, and IQR is the interquartile interval (Gentilini et al., 2015). Beta values beyond this range were classified as Stochastic Epigenetic Mutations (SEMs). Finally, all epimutations were annotated to obtain a matrix with their count and their genomic position. SEMs were further classified as hypermethylated or hypomethylated, and CpG sites were grouped by genic and non-genic, island and non-island, and by chromosome. Both Wilcoxon's test and sex- and age-adjusted generalized linear models were implemented in RStudio to test for differences between the HLE and LLE groups.

SEMs-enriched regions were identified as epigenetic lesions and obtained using a sliding window algorithm that covered the entire genome. This process provided a matrix of the number of epigenetic lesions for each gene for each individual to compare HLE and LLE individuals through a *t*-test. Lists of genes containing epigenetic lesions were then translated into biological pathways using WEB-based GEne SeT AnaLysis Toolkit (WebGestalt) (Elizarraras et al., 2024) with the Over-Rapresentation Analysis (ORA) method.

#### 2.2.4. Epigenetic clocks analysis

We estimated the biological aging of individuals in the selected subgroup using "epigenetic clocks" (Horvath and Raj, 2018). Specifically, we used the online DNAmAge Calculator (Horvath, 2013) to compute five epigenetic clocks suitable for oral mucosal cells, respectively the: *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 *skin & blood clock* (Horvath et al., 2018). Subsequently, we analyzed potential differences in epigenetic aging rates between HLE and LLE individuals using a sex-adjusted linear model with the *lm* function of R software.

### 2.3. Targeted bisulfite sequencing

For all 61 individuals, the DNAm level of three REs was measured by the bisulfite sequencing technique using the Illumina MiSeq system. Specifically, 12 CpG sites on Alu, 18 CpG sites on LINE-1, and 133 CpG sites on rDNA (27 CpG sites at 18S1 target, 13 CpG sites at 18S2 target, 30 CpG sites at 28S target, 37 CpG sites at RiboProm1 target, and 26 CpG sites at RiboProm2 target) were analyzed, as suggested by the experimental design of Marson et al. (2023) (more details in Supplementary File 2). The Alu target was located in the body of the repeated unit, the LINE-1 target was located in the 5' untranslated region (5'-UTR) of the repetitive element, 18S1, 18S2 and 28S targets were located at the 5′ end of the respective sequence in the rDNA, RiboProm1 target was located upstream of the rDNA promoter, and RiboProm2 target was located in the rDNA promoter including the upstream control element, the central part of the promoter, and the transcription start site (Marson et al., 2023) (Fig. 3S).

Quality checks of the raw sequencing data were performed following Ravaioli et al. (2023) (Supplementary File 2). An age- and sex-adjusted linear model was implemented using the *lm* function for each CpG site of each RE in order to identify significant differences in DNAm levels between HLE and LLE individuals. The p-values were adjusted for multiple testing by FDR correction.

To investigate overall patterns of variation in methylation levels within REs, we focused on those showing multiple differentially methylated CpG sites. For these elements, a correlation matrix was computed among CpG sites using the *cor* function (method = "Pearson") after verifying the normality of methylation values with the Shapiro–Wilk test. A principal component analysis (PCA) was then performed with the *prcomp* function in R to reduce dimensionality and summarize variability across sites. After excluding outliers (mean ± 2 SD), PC1 scores were subsequently compared between the two groups using a *t*-test.

## 3. Results

### 3.1. Contamination distribution

Based on a detailed territorial analysis (Figs. 1A and 1B) and an examination of the Giardino aqueduct map (Fig. 1C) — showing water distribution routes and alternative intakes — we reconstructed the probable dynamics of contamination spread. Using this reconstruction and the geographic score method, the 61 participants were divided into two exposure groups: HLE (N = 30) and LLE (N = 31).

### 3.2. Epigenome-wide analysis

After quality checks on DNAm data, a total of 701,357 CpG sites and 32 individuals (12 females and 20 males, with a mean age of 52 years), including 14 HLE and 18 LLE, were retained for further analysis.

The estimated proportions of the three cell types (Epi, Fib, and IC) showed no statistically significant differences between HLE and LLE individuals (p-values: 0.38 (Epi), 0.40 (Fib), and 0.38 (IC)) (Figure 4S of the Supplementary Figures). Consequently, the subsequent models were not adjusted for cell counts.

#### 3.2.1. Differentially methylated positions (DMPs) and MeQTLs

A linear model identified 622 DMPs (Figure 5S) across 604 genes (nominal p-value < 0.001) between HLE and LLE groups (more information in Table 3S and Table 4S for the top five DMPs). However, no CpG sites remained significant following FDR correction. Among the 622 DMPs, 161 CpG sites were hypomethylated in the HLE group compared to LLE group. Of the 622 DMPs, the five most differentiating between HLE and LLE individuals (Δ LLE_HLE) are listed in Table 1 (Figure 6S). Three out of these five most significant DMPs were found to be associated with specific traits from the literature of Epigenome-Wide Association Studies (EWAS Atlas) (Table 1). Notably, most DMPs were located within promoter-associated regions and CpG islands (Supplementary Figures 7S, 8S, 9S). To assess whether this pattern reflected a non-random distribution, we performed an enrichment analysis, which revealed that 48.7 % of the DMPs were located in promoter-associated regions compared with an expected 34.1 % based on the empirical distribution obtained from 10,000 random resamplings (see Material and Methods for details), indicating a significant overrepresentation of promoter-associated sites (empirical P < 0.001) (Supplementary Figure 10S).

A GO analysis, performed by considering all the genes that included the DMPs identified above, revealed 104 significant biological pathways (FDR q-value < 0.05). These pathways specifically encompass: embryonic organ morphogenesis and development, regulation of cell development, B cell activation, production of molecular mediator of immune response, neutrophil mediated immunity, leukocyte migration, extracellular matrix organization, appendage development, small molecule

**Table 1**
Summary statistics of the five most different DMPs between HLE and LLE individuals (GRCh37/hg19 assembly) and traits found associated with them (EWAS Atlas (Li et al., 2019)). The CpG sites are ordered according to the delta value (Δ), which is the difference between the mean β-values of the LLE and HLE groups for each CpG site. IR is an abbreviation for intergenic region.

| CpG name | Chr: position | Mean (±SD) HLE | Mean (±SD) LLE | Nominal p-value | Δ | Gene name | Related trait |
|---|---|---|---|---|---|---|---|
| cg04879348 | chr2:109124576 | 0.54 (±0.15) | 0.71 (±0.10) | $6.52 \times 10^{-5}$ | 0.16 | GCC2 | Exposure to organochlorinated compounds and risk of Parkinson's disease (Go et al., 2020) |
| cg14498475 | chr8:143781814 | 0.30 (±0.11) | 0.45 (±0.11) | $5.98 \times 10^{-4}$ | 0.15 | LY6K | Ancestry and immune gene regulation (Husquin et al., 2018) |
| cg14433904 | chr8:78518428 | 0.56 (±0.08) | 0.66 (±0.06) | $9.86 \times 10^{-4}$ | 0.09 | IR | - |
| cg21397540 | chr1:247537159 | 0.41 (±0.07) | 0.50 (±0.06) | $1.04 \times 10^{-4}$ | 0.09 | IR | - |
| cg04190888 | chr10:49879914 | 0.68 (±0.10) | 0.77 (±0.09) | $5.02 \times 10^{-4}$ | 0.09 | IR | Multiple sclerosis, smoking, and kidney disease (Hannon et al., 2021; Maltby et al., 2018; Smyth et al., 2021) |

catabolic process, regulation of nervous system development, axonogenesis, and axon development (details in Table 5S).

No statistically significant results were instead observed when the analysis was carried out on DMRs.

To assess whether the 622 DMPs reflected epigenetic variation or genetic influence, a meQTL analysis was conducted. To minimize false positives in our small cohort, we first screened a published atlas of genetic effects on DNAm in Europeans (Min et al., 2021). Specifically, Min et al. (2021) identified 169,656 meQTL associations, consisting of 155,190 cis-meQTLs and 14,466 trans-meQTLs. From these, 3369 meQTLs (3147 cis- and 222 trans-meQTLs) replicated also in our analysis (p-value $< 0.05$). Notably, among the 622 DMPs, only cg16393905 was associated with genetic variation at rs11586401, where each additional C allele corresponded to a 0.746 % decrease in DNAm levels (Figure 11S).

### 3.2.2. *Differentially variable positions (DVPs)*

A linear model was applied to identify DVPs between the two groups. The analysis identified 1138 CpG sites mapping in 882 genes with significantly different variance between HLE and LLE individuals (nominal p-value $< 0.001$) (more information in Table 6S). However, no CpG sites remained significant following FDR correction. 71.36 % of the 1138 identified DVPs showed reduced variability in the HLE group compared with the LLE group. The top five DVPs are listed in Table 2.

ECDF (Figure 12S) demonstrated that the distributions of standard deviations of DNAm levels for the DVPs differs significantly between HLE and LLE individuals (p-value $= 2.2 \times 10^{-16}$). The visual inspection of the plotted distributions particularly revealed that the variability of DNAm levels was, on average, lower in the HLE group compared to the LLE group (Fig. 2).

### 3.2.3. *Epimutations and epigenetic lesions*

Analysis of epimutations showed a median of 2282.5 total epimutations for LLE individuals and 2550.5 for HLE individuals. Specifically, the median number of hypomethylated epimutations was 1371.0 and 1484.5, while the median number of hypermethylated epimutations was 968.5 and 987.0 for LLE and HLE groups, respectively. No significant differences were observed when comparing the log-transformed number of epimutations between the two groups (Figure 13S).

A comparison of the number of epimutations between the HLE and LLE groups was conducted by annotating and classifying all the CpGs as either genic or non-genic, and then as wither island or non-island. This

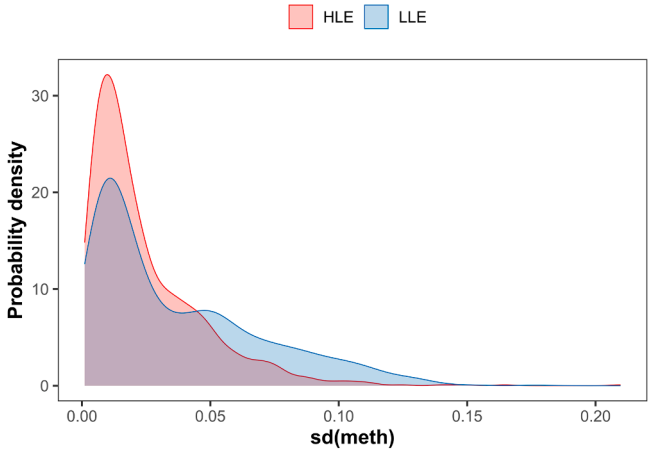


**Fig. 2.** Reduced variability in the high-exposure group. Density plot showing the distribution of the *sd(Meth)* variable related to DVPs (p-value $< 0.001$) for HLE (in pink) and LLE (in blue) individuals. The widths of the curves show that the variability is lower in the HLE group than in the LLE group.

did not result in any significant findings. Similarly, there was no significant difference in the number of epimutations between HLE and LLE when CpGs were grouped by chromosome (Table 7S). Lastly, further subdivision of all these CpG classes into hypo- and hypermethylated epimutations showed no significant differences between the two groups (Tables 8S and 9S).

Moreover, there were no statistically significant differences in the number of total epigenetic lesions between HLE and LLE individuals (p-value $< 0.05$), and the biological pathways related to genes with epigenetic lesions were also not significant.

### 3.2.4. *Epigenetic clocks analysis*

To investigate a possible relationship between contaminant exposure and the health status of individuals, especially in terms of biological aging, we calculated five epigenetic clocks (*AgeAccelerationResidual*, *IEAA*, *EEAA*, *AgeAccelPheno*, and *skin & blood clock*) for the 32 individuals and searched for differences in epigenetic age acceleration between HLE group and LLE group through a sex-adjusted linear model. However, the epigenetic clock analysis revealed no statistically significant differences (p-value $< 0.05$) between the two groups – even at the

**Table 2**
Summary statistics of the top five DVPs between HLE and LLE individuals (GRCh37/hg19 assembly). IR is an abbreviation for intergenic region.

| CpG name | Chr: position | Relation to island | Nominal p-value | Gene name |
|---|---|---|---|---|
| cg23678634 | chr1:145508314 | S_Shore | $1.51 \times 10^{-6}$ | RBM8A; NBPF20; NBPF10 |
| cg09696644 | chr1:206626583 | OpenSea | $2.96 \times 10^{-6}$ | SRGAP2 |
| cg17024333 | chr2:120233913 | OpenSea | $3.21 \times 10^{-6}$ | SCTR |
| cg01277615 | chr7:107220080 | N_Shore | $3.23 \times 10^{-6}$ | BCAP29 |
| cg10760539 | chr7:31505908 | OpenSea | $4.90 \times 10^{-6}$ | IR |

nominal level.

### 3.3. DNAm analysis of REs

For all 61 individuals, the DNAm level of three REs (Alu, LINE-1, rDNA) was measured using targeted bisulfite sequencing. Specifically, 12 CpG sites on Alu, 18 CpG sites on LINE-1, and 133 CpG sites on rDNA were analyzed. In order to identify significant differences in the mean DNAm levels between the two groups, an age- and sex-adjusted linear model was performed for each CpG site. Significant differences (p-value $< 0.05$) between the two groups were observed in the DNAm levels of three CpG sites (named "CpG_34", "CpG_37", and "CpG_194" by Marson et al., 2023) of LINE-1 (nominal p-values = 0.03, 0.01, and 0.02 respectively) (Figure 14S), while no CpG sites of Alu and rDNA showed statistically significant differences between the two groups.

Since LINE-1 was the only element with multiple differentially methylated CpG sites, we further explored methylation variability within this element. All 18 CpG sites of LINE-1 satisfied the assumption of normality according to the Shapiro–Wilk test, and a correlation matrix revealed strong covariance among these sites (Pearson's $R \geq 0.7$). A PCA was then performed to summarize methylation variation across all sites, with PC1 explaining 80 % of the total variance and subsequently used to compare the two groups. After excluding outliers (defined as mean ± 2 SD), PC1 scores were found to be significantly lower in HLE than in LLE individuals (*t*-test, p-value = 0.03). Furthermore, all the CpG sites analyzed in LINE-1 (N CpGs = 18) were hypomethylated in the HLE group compared with the LLE group, suggesting a general trend toward loss of methylation in the group with a high level of exposure to contaminants (Fig. 3).

## 4. Discussion

Scientific interest in the potential epigenetic effects of environmental contaminants, particularly chlorinated solvents, is steadily increasing (Carswell et al., 2024; Cui et al., 2016; Gilbert et al., 2017, 2016; Jin et al., 2020; Li et al., 2023; Tao et al., 2000), while evidence regarding their direct impacts on humans remains limited (Phillips et al., 2019; Wu et al., 2013; Zhao et al., 2022). Within this framework, the present study contributes to the current knowledge by examining the effect of industrial contaminants released into the soil and groundwater of the Bussi sul Tirino SNI on the DNAm profiles of local residents (N = 61), who were sampled via buccal swabs and classified into high (HLE, N = 30) and low (LLE, N = 31) exposure groups.

A subset of 32 individuals (14 HLE and 18 LLE) was analysed at the epigenome-wide level using data generated by the Illumina EPIC BeadChip, with a specific focus on the identification of DMPs, DMRs, meQTLs, DVPs, epimutations, epigenetic lesions, and biological aging rates (epigenetic clocks).

To complement epigenome-wide results, we measured the DNAm level of three REs (Alu, LINE-1, and rDNA) in all 61 individuals using targeted bisulfite sequencing (Marson et al., 2023). REs are present in multiple copies across the genome and are often used as markers of global methylation, which in turn can reflect health status (Yang, 2004).

The first key finding of this study is that epigenome-wide analyses reveal pathways related to cellular development, neurosystem development and immune response. Specifically, the epigenome-wide analysis revealed 622 significant DMPs, emerged at the nominal level (p-value $< 0.001$). Although these signals did not survive correction for multiple testing, we observed an enrichment of DMPs in promoter associated regions, suggesting that the methylation changes identified in this study may preferentially affect regulatory elements involved in gene transcription. The downstream gene ontology (GO) enrichment analysis based on these loci revealed biologically relevant pathways, including those related to embryonic development, cell development regulation, extracellular matrix organization, nervous system development, small molecule catabolism, and other immune-related pathways. Notably, several of these pathways have been previously implicated in the biological effects of chlorinated solvent exposure, as described in the existing literature, although direct literature on the epigenetic effects of industrial chlorinated solvents remains limited. In particular, maternal exposure to chlorinated solvents has been linked to disrupted cellular development, including altered proliferation and differentiation during fetal growth (Brender et al., 2014). These effects may be mediated by epigenetic changes, consistent with our findings on DNAm alterations in exposed individuals.

Regarding immune-related GO terms identified in our study, both murine models and human studies have demonstrated DNAm changes in key immune-related genes following chlorinated solvent exposure

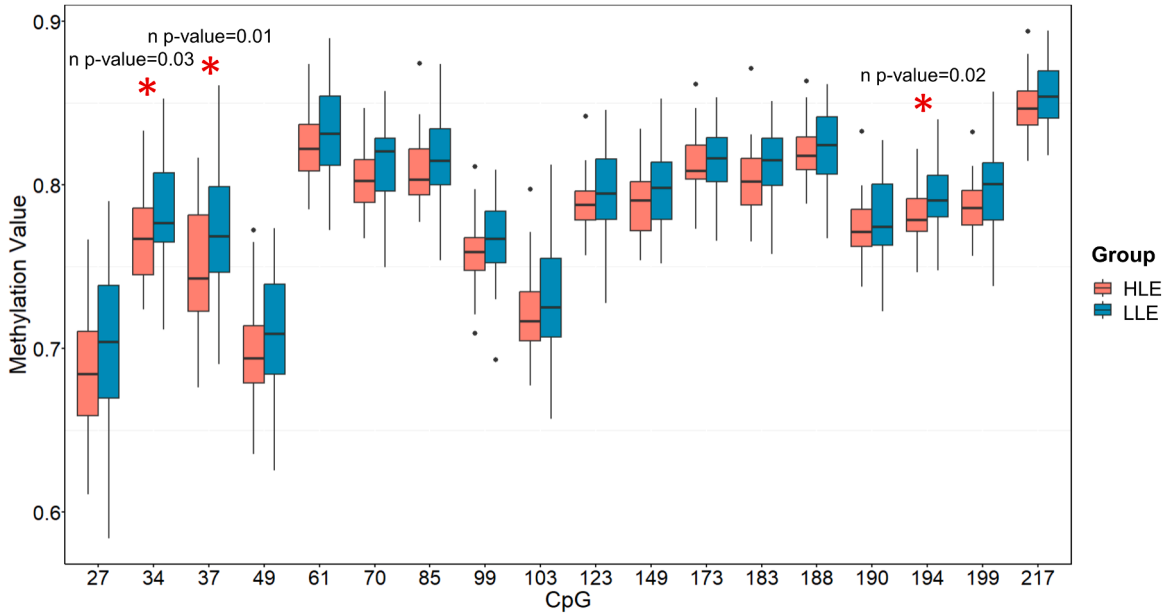


**Fig. 3.** LINE-1 hypomethylation. Plot of the 18 CpG sites measured in LINE-1 in the HLE (pink boxplots) and LLE (blue boxplots) groups. Red asterisks indicate the three differentially methylated CpG sites (CpG_34, CpG_37, and CpG_194) between the two groups (n p-value $< 0.05$). From the boxplots trend, the general tendency of LINE-1 hypomethylation in HLE compared with LLE individuals can be observed.

(Gilbert et al., 2017, 2016; Phillips et al., 2019), supporting a potential role of these compounds in immune dysfunctions or autoimmune disorders. Moreover, recent studies have increasingly highlighted the potential neurodevelopmental risks associated with exposure to chlorinated solvents. For example, research on zebrafish has demonstrated that developmental exposure to TCE leads to morphological and behavioral alterations indicative of neuroxicity during embryogenesis (Horzmann et al., 2020). Additionally, neurotoxicity and a Parkinson's-like disease is also observed in adult workers with occupational exposure to TCE (Gash et al., 2008; Kilburn, 2002). Importantly, the CpG site cg04879348, which showed the largest methylation difference between HLE and LLE groups in our study, has also been associated with exposure to organochlorinated compounds in a previous genome-wide epigenetic study of exposed Japanese immigrant plantation workers diagnosed with Parkinson's disease (Go et al., 2020). Furthermore, early life exposure to chlorinated solvents via contaminated drinking water has been linked to a higher likelihood of developing substance use disorders later in life, suggesting long-term neurobiological impacts (Aschengrau et al., 2020). Collectively these findings reinforce the biological plausibility of chlorinated solvents influencing neurodevelopment and highlight the importance of continued research in this area.

The second key finding of this study is the reduced DNAm variability in the HLE group. This finding is consistent with Tobi et al. (2018), who, using mathematical models of early embryonic re-methylation, described selective processes underlying a reduction in DNAm variance in a human cohort prenatally exposed to stressors, such as the Dutch famine (Tobi et al., 2018). This phenomenon suggests that only embryos better adapted to adverse intrauterine conditions may survive, shaping the methylome of the surviving population (Breton et al., 2017; Germain and Winn, 2024; Silvestre et al., 2020). Based on these assumptions, it is possible to hypothesize that the decreased variability observed in the HLE group may be due to a prenatal epigenetic selection event resulting from maternal exposure to environmental chemicals. Alternatively, the reduction in variability may represent an adaptive response to exposure to pollutant during life (Gluckman et al., 2007). Further data are needed to distinguish between these scenarios.

The third major finding of this study is the absence of evidence of epimutations or epigenetic aging acceleration in buccal cells.

Epimutations are stochastic alterations in the epigenome that are associated with aging and environmental stress. They serve as indicators of an individual's level of epigenetic entropy, providing insight into their biological aging and overall health status. In this study, we showed for the first time that exposure to contaminants does not appear to impact the number of epimutations and epigenetic lesions in the analyzed communities. However, this finding may reflect tissue specificity, as previous studies on different diseases applied the epimutation algorithm to blood cells (Brusati et al., 2023; Gentilini et al., 2022; Spada et al., 2020), whereas we analyzed buccal mucosal epithelial cells. Therefore, further research should assess whether similar results can also be observed in blood samples, where the higher cellular heterogeneity may increase the likelihood of detecting epimutations.

Epigenome-wide methylation data was also used to explore whether exposure to environmental pollutants affected biological aging in the analyzed population groups, as previously investigated in other studies involving various toxicants, such as organochlorine pesticide (Lind et al., 2018), air pollution (Blechter et al., 2023; White et al., 2019), benzene, and trichloroethylene (Van Der Laan et al., 2022). However, no statistically significant associations were observed between exposure level and biological aging, as estimated by five different epigenetic clocks (*AgeAccelerationResidual*, *IEAA*, *EEAA*, *AgeAccelPheno*, *skin & blood clock*). These findings suggest that pollutants present in the Bussi sul Tirino SNI do not appear to influence the acceleration or deceleration of aging in the exposed individuals, consistent with other previous studies (Bozack et al., 2022; Gaskins et al., 2023; Herrera-Moreno et al., 2022; Xu et al., 2020). Nevertheless, it is important to consider that different epigenetic clocks may yield divergent results and that the type of tissue analyzed can also influence the results obtained.

The fourth major finding of this study is the tendency toward LINE-1 hypomethylation in the HLE group. Specifically, the PCA revealed significant differences (p-value $< 0.05$) in LINE-1 DNAm levels between the two groups, and a general trend towards hypomethylation was observed for all 18 CpG sites in the HLE group, as also found in previous studies on other contaminants (Yohannes et al., 2022). It has been demonstrated that LINE-1 hypomethylation correlates with genomic instability in several tumor types (Baba et al., 2024; Kitahara et al., 2020; Schulz et al., 2002), and that it may also interfere with fertility (Wang et al., 2020). This is particularly relevant in light of the 2023 SENTIERI epidemiological survey conducted in the Bussi sul Tirino area, which reported an excess of deaths from bladder malignancies and non-Hodgkin lymphomas associated with the presence of abusive waste disposal sites during the period 2013–2017 (Zona et al., 2023). In addition, it documented elevated mortality rates among women for all malignancies, breast cancer and stomach cancer, which were associated with the presence of the power plant, landfills and chemicals, respectively. Furthermore, it reported an increase in hospitalizations for colorectal and lung cancers, as well as for respiratory, circulatory, digestive, and urinary diseases for both sexes (Zona et al., 2023). The diseases related to the urinary system found in the Bussi sul Tirino area, such as nephritis and kidney failure, have also been found at other Italian sites contaminated by solvents and heavy metals (Benedetti et al., 2021).

While offering important new insights, this study has some limitations that are worth considering. Firstly, although chlorinated solvents are the predominant contaminants in the aqueduct water — which represents the most likely contamination pathway and the one for which the most data are available — it cannot be excluded that the observed effects may also be attributable to other contaminants. Some of GO terms identified are in fact consistent with exposure to other pollutants such as the one from heavy metals described by Zeng et al. (2019). Further studies will be needed to explore the combined effects of multiple pollutants, building on the results presented in this study. Secondly, the small number of samples analyzed could represent a limitation for the statistical power of all the analyses performed in the present study. To overcome this limitation, future studies should consider larger cohorts to increase statistical power. Thirdly, the collection of additional tissues, such as blood samples, would allow investigation of tissue-specific DNAm patterns, offering a broader view of how this type of exposure may be differently recorded across biological systems. In addition, including individuals completely outside the contaminated area could be useful to better differentiate the two cohorts, likely resulting in more pronounced DNAm differences.

## 5. Conclusion and policy recommendation

This study provides new evidence that past long-term exposure to environmental contaminants, such as chlorinated solvents in the Bussi sul Tirino area, can alter epigenetic profiles, particularly DNAm and LINE-1 methylation. Although these changes are not yet linked to specific clinical outcomes, they suggest potential biological effects from past chronic exposure. Given the growing evidence connecting pollutants to epigenetic alterations and health risks, we recommend prioritizing regular and transparent monitoring of water quality, supporting biomonitoring programs that include epigenetic markers, and adopting precautionary measures—such as improving water infrastructure and providing alternative safe water sources—to minimize ongoing exposure while long-term data are collected. Moreover, for such industrial sites with a very long history and exposure potential, it is important to emphasize the need to conduct an epidemiological study across the broader region to assess health effects and to provide an assessment paradigm for similar sites in other areas.

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## CRediT authorship contribution statement

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2025.119548.

## Data availability

Data will be made available on request.

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