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RESEARCH ARTICLE

# Arsenic concentration in topsoil of central Chile is associated with aberrant methylation of P53 gene in human blood cells: a cross-sectional study

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## Abstract

Gene expression can be modified in people who are chronically exposed to high concentrations of heavy metals. The soil surrounding the Ventanas Industrial Complex, located on the coastal zone of Puchuncaví and Quintero townships (Chile), contain heavy metal concentrations (As, Cu, Pb, Zn, among others) that far exceed international standards. The aim of this study was to determine the potential association of the heavy metals in soils, especially arsenic, with the status of methylation of four tumor suppressor genes in permanent residents in those townships. To study the methylation status in genes p53, p16, APC, and RASSF1A, we took blood samples from adults living in areas near the industrial complex for at least 5 years and compared it to blood samples from adults living in areas with normal heavy metal concentrations of soils. Results indicated that inhabitants of an area with high levels of heavy metals in soil have a significantly higher proportion of methylation in the promoter region of the p53 tumor suppressor gene compared with control areas ($p$-value: 0.0035). This is the first study to consider associations between heavy metal exposure in humans and aberrant DNA methylation in Chile. Our results suggest more research to support consistent decision-making on processes of environmental remediation or prevention of exposure.

**Keywords** DNA methylation · Epigenetics · Heavy metal · Soil pollution

## Introduction

In Chile, mining represents a major component of the overall economy, comprising 52% of total exports (OECD 2018). In the 1960s, the Ventanas Industrial Complex (VIC) linked to mining activities was built in the coastal zone of Quintero and Puchuncaví in central Chile. VIC emitted heavy metal–rich particulate matter that was dispersed by the wind and deposited on the soil surrounding it from 1964 until 1992, when environmental regulations were established (Delgado and Serey 2002; Rueda-Holgado et al. 2016; González et al. 2014). As a result, the soil in this area has high concentrations of

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heavy metals (González et al. 2014; Ginocchio 2000), which has been shown to be a risk for humans living in the area (Salmani-Ghabeshi et al., 2016; Berasaluce et al. 2019; Tapia-Gatica et al. 2020). Chronic exposure by ingestion, inhalation, or dermal contact to high amounts of heavy metals in the soil, such as arsenic, chromium, nickel, and cadmium, among others, may lead to detrimental effects on health, including epigenetic changes—modifications of genetic material without modifications of the DNA sequence—such as methylation (Martinez-Zamudio and Ha 2011; Bitto et al. 2014). DNA methylation is a common mechanism of gene silencing that occurs on CpG islands—genomic regions located near or in gene transcription initiation sites (Hou et al. 2011; Moore et al. 2013). Whenever these methylated genes are responsible for coding a tumor suppressor protein, chronic exposure to heavy metals could predispose the carrier to human carcinogenesis. Aberrant methylation and the resulting silencing of a tumor suppressor gene may contribute to tumor initiation and progression (Gauthier et al. 2007; Tanemura et al. 2009; Venza et al. 2016), with the methylations accumulating in response to continuous exposure and persisting even in the absence of the conditions that led to the methylation (Richards 2006; Dolinoy et al. 2007; Anway et al. 2005).

The p53 pathway is upregulated when DNA damage is incurred. This can lead to either cell cycle arrest in order to permit DNA repair or the initiation of sequences leading to apoptosis, thus impeding the replication of damaged cells (Bourdon 2007; Christmann and Kaina 2019). p53 activity is frequently lost in human cancer, either by gene mutation or inactivation (Bourdon 2007; Christmann and Kaina 2019; Sabapathy and Lane 2018). Its mutation has been described as a key prognostic factor in several cancer types, including multiple myeloma, acute myelogenous leukemia, renal cell, and hepatocellular carcinomas (Liu et al. 2012; Knezović Florijan et al. 2019; Abdel-Aziz et al. 2009; Hunter and Sallman 2019).

Other studies have found that p16 has suspected involvement in growth arrest at G1/S, in damaged cells, in cell cycle control, and in the cellular response to genotoxic agents (Lewin, 2001; Sherr and Roberts 1999; Li et al. 2011). Adenomatous polyposis coli (APC) has been shown to be related to phosphorylation and ubiquitination of transcription factors associated with proliferation and RASSF1A in the maintenance of genomic stability and cell motility and invasion (Yang et al. 2006; Song et al. 2004; Vos et al. 2004; Dallol et al. 2005).

Despite data suggesting an association between heavy metal concentrations in soils in the abovementioned industrial area and effects on human health, no previous studies had provided empirical evidence. Therefore, the objective of this study was to determine the association between the heavy metal concentration in the soil surrounding the VIC and the methylation status in four tumor suppressor genes in permanent residents of the local townships.

## Materials and methods

### Study design

Although the contamination in the soil is polymetallic, background information indicates that among the metals present, only As, Ni, and Cr are proven carcinogens; for the rest of the metals present, there is insufficient evidence on carcinogenicity, or they are proven not to be carcinogenic (ATSDR 2007). Among these metals, according to previous evidence in the area, only As exceeds the international standard levels, specifically, the Italian Standard (20 mg/kg, Ministro Dell' Ambiente 1999). Therefore, based on As mapping, the sampling areas were defined. The Italian Standard was chosen as the reference value because Chile has no quality regulation for soil, and Italy has a geological background (orogeny) and a climate (dry summer, temperate climate) similar to the study area. It is worth mentioning that although a study of background levels of metals in the soil was carried out in the area, which gave a value (UCL 95%) of 12.8 mg/kg for As (PGS Chile 2015), no progress has yet been made in generating quality standards based on them. The values of the Italian standard are consistent with the background values identified.

Further, previous research has demonstrated that As correlates adequately with the remaining heavy metals ($R^2 = 0.5–0.8$) (De Gregori et al. 2003), so its spatial distribution would be representative of the rest of them.

To select the human blood sampling areas, we measured the As concentration in the soil of the study area and, based on the results, generated a continuous map of the concentrations using geostatistical analysis.

Next, we conducted a cross-sectional study (Setia, 2016), using volunteers, to assess the methylation status of promoters from four selected genes, p53, p16, APC, and RASSF1A. The volunteers included inhabitants of areas with high concentrations of heavy metals in the soil and, for a control group, inhabitants of areas with normal concentrations of heavy metals.

### Ethics statement

The research protocol was approved by the ethics committee at the Hospital Dr. Gustavo Fricke Regional Health Service (CEC 46/2012). All research subjects were informed about the study and the sampling protocols, and they provided written consent.

### Area of study

The study took place in the area surrounding the VIC, which comprised two entire townships, Quintero and Puchuncaví, in Chile's Valparaíso region (Fig. 1). The sampling also

included a township south of Quintero and Puchuncaví, Concón. This third township was included because it comprised a large area with normal concentrations of heavy metals in the soil, where control blood samples could be collected.

## Sampling procedures

### Soil samples

The study area was stratified by intersecting the study area boundaries with layers of variables related to potential dissemination of contaminants (theoretical information about altitude, distance from main source, slope and wind direction) (following the methodology of Vasilakos et al. 2007; Gohm et al. 2009; Child et al. 2018). A total of 485 sampling units were defined in order to finally obtain a representative sample of the study area. This procedure was performed using GIS Software application Arc Map (10.7 version, ESRI Inc).

The field sampling campaign had a success rate of 70% (of all sampling units) and generated 340 final topsoil samples 0–15 cm deep and approximately 400 g. For each soil

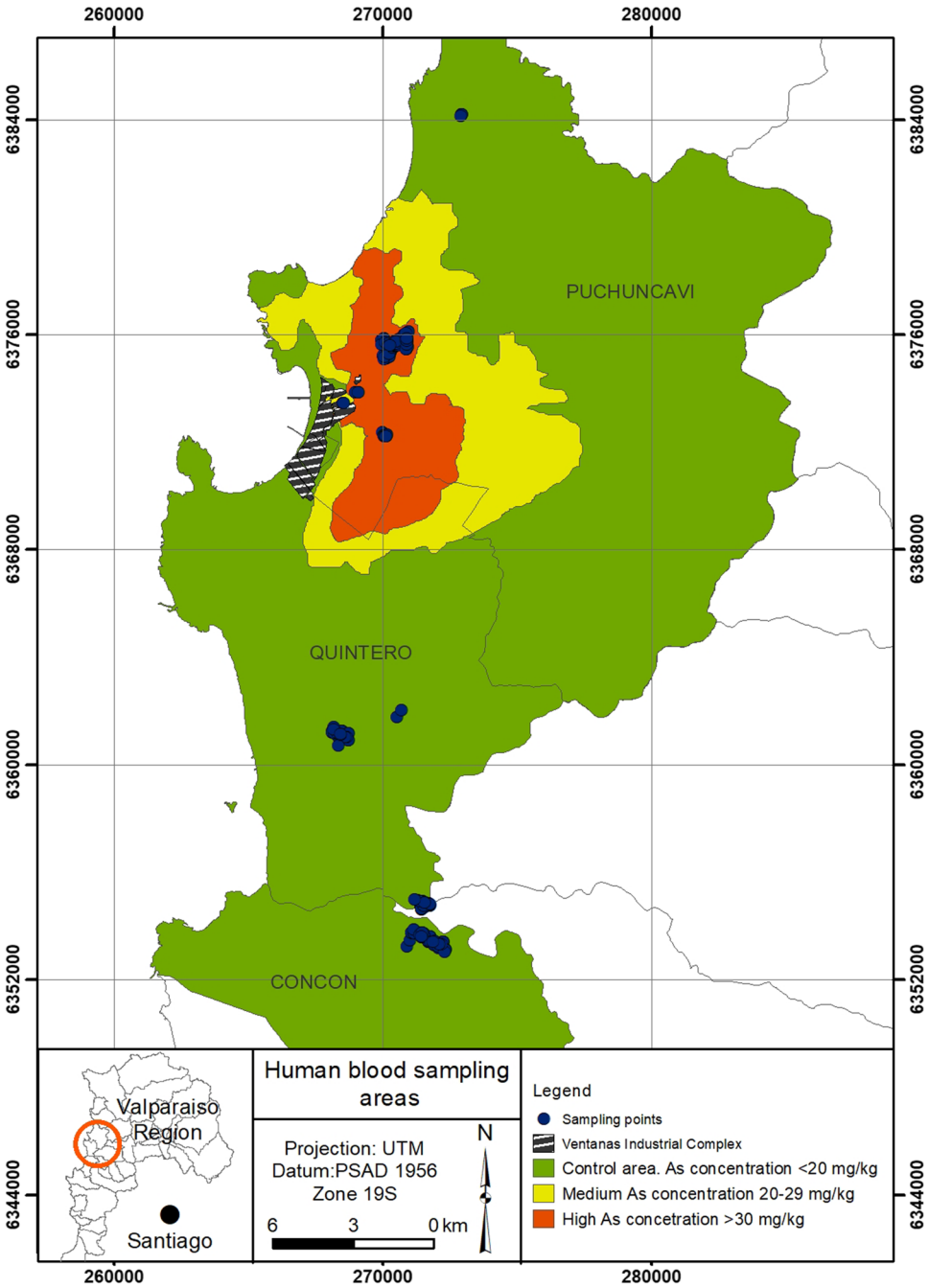


**Fig. 1** Human blood sampling areas and sampling points

sample, geographic coordinates were recorded using GPS equipment (Garmin eTrex Summit).

### Blood samples

Based on the geostatistical analysis of the data, described in detail in "Geostatistical analysis and human blood sampling areas", two types of blood sampling areas were defined: (1) "exposed" (i.e., high concentrations of heavy metals) and (2) "control" (low concentrations of heavy metals) (Fig. 1).

We then calculated the proper sample size for detecting a difference in methylation of 10% with a statistical power of 80% and a 5% significance level. The minimum sample size was 128 participants per group. A total of 280 individuals agreed to participate in the study—140 from the "exposed" area and 140 from the control area. The study participant inclusion criteria were (1) area resident for at least 5 years, (2) 18 years of age or older, and (3) the provision of informed consent. The criterion of 5 years of residence was chosen because the minimum time of exposure that generates epigenetic changes is not fully known. The authors studying epigenetic changes due to exposure to polluted air indicate that more than 1 year is considered long-term exposure (White et al. 2019; Prunicki, et al. 2018).

Random sampling was performed to ensure the homogeneity of the two groups was performed. Any differences between the exposed group and the control group with regard to demographic characteristics were assessed and controlled in the final statistical analysis as needed. The selected participants were visited at their homes and donated 15 mL of peripheral blood for the study. The blood samples were stored in ethylenediaminetetraacetic acid (EDTA) tubes, in a cooler, using dry ice to maintain the temperature at – 70 °C until all samples were collected, and then analyzed (Chacón-Cortes and Griffiths 2014).

## Analysis

### Soil analysis

Soil samples were dried for 48 h, at a temperature of 40 °C, and then sieved through a 2-mm mesh. Then, samples were digested during 12 h in boiling nitric acid, followed by perchloric acid addition. In order to prevent volatilization of As during the digestion process, a Teflon stopper with 30-cm-long glass reflux tube was used (Verlinden 1982). Then, total concentrations of arsenic were assessed using atomic absorption spectrophotometry (AAS) (GBC Scientific Equipment PTY Ltd., Dandenong, Victoria, Australia; model 902) coupled with a hydride vapor generator (model VP100) following the procedure of Tapia-Gatica et al (2020). Quality assurance was performed using duplicate assessment and a certified reference soil sample (GRX-2) obtained from the United States Geological Survey. The detection limit of As was 0.01 mg/kg.

### Geostatistical analysis and human blood sampling areas

Various geostatistical methods to generate a raster format map of the concentration of As in the soil were used. This procedure, the As concentration in soil, and the map of cancer risk assessment to As for children and adults were previously published in Tapia-Gatica et al. 2020.

Then, we defined two different blood sampling areas, one with normal concentration of As in soil and one with a high concentration. To depict these areas, we converted the original soil arsenic concentration raster surface to vector polygons, defining 4 categories of concentration.

The lower category, corresponding to the "control" area (normal soil arsenic concentration), was defined according to the criteria for acceptable concentration in soils of green and residential areas reported in the Italian Standard (20 mg/kg). The remaining 3 categories of soil As concentrations were defined as follows, based on the criteria for similar land surfaces: 21–30 mg/kg and 30–150 mg/kg. The category for the highest concentration (> 30 mg/kg) was selected to represent the exposed area.

In summary, we defined two different blood-sampling areas: (1) "exposed," with a soil As concentration > 30 mg/kg and (2) a control area, with a soil As concentration ≤ 20 mg/kg (Fig. 1).

### DNA analysis

Genomic DNA (gDNA) was isolated from leukocytes using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany), following the manufacturer's protocol (Ghaheri et al. 2016). DNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Genomic DNA integrity of all of our clinical samples using gel electrophoresis was assessed.

To carry out the sodium bisulfite conversion, 1 µg of DNA from each sample was modified with sodium bisulfite (SB). Bisulfite conversion was carried out using the EZ DNA Methylation Gold Kit (ZYMO Research Co., Orange, CA), according to the manufacturer's instructions. The converted DNA was stored at – 70 °C until used. Built-in controls to verify bisulfite conversion were used (Wojdacz et al., 2008).

Human placental genomic DNA (gDNA; Sigma-Aldrich) and Universal Methylated Human DNA Standard (ZYMO Research Co., Orange, CA) were used as fully unmethylated and fully methylated controls respectively (Dimitrakopoulos et at, 2012). Both controls underwent sodium bisulfite conversion, and a series of synthetic controls containing 0%, 1%, 10%, 50%, and 100% methylated DNA were prepared by spiking the fully methylated DNA control into the unmethylated (Wojdacz et al., 2008).

Real-time PCR was performed with the KapaHRM Fast master mix 2 × (Kapa Biosystems; USA). Briefly, 1 µL (~ 100 ng) of SB converted DNA was added in the PCR reaction mix, which consisted of 1 × PCR Buffer (Invitrogen, USA), 0.4 mM for each dNTP (Invitrogen, USA), 0.05 U/µL PlatinumW Taq DNA Polymerase (Invitrogen, USA), 0.25 µg/µL BSA (Sigma, Germany), 1 × LCGreen Plus Dye (Idaho Technology, USA), 0.25 µM each primer (forward and reverse), and $MgCl_2$ (2.5 mM). $dH_2O$ was used to supplement up to 10µL. The primer set (Table 1) was designed using the National Center for Biotechnology Information (NCBI) web tool Primer-BLAST (Basic Local Alignment Search Tool), according to guidelines (Wojdacz et al., 2008). The real-time PCR protocol began with one cycle at 95 °C for 3 min followed by 50 cycles of 95 °C for 10 s and 65 °C for 30 s. Following PCR amplification, a re-annealing cycle consisting of 95 °C for 1 min and a rapid cooling to 70 °C for 1 min was introduced. All PCR reactions were performed in triplicate for each sample.

Then, High Resolution Melting Analysis was performed. Melting data acquisition began at 69 °C and ended in 95 °C, using a ramp rate of 0.3 °C/s. High Resolution Melting Analysis was also performed in the KapaHRM Fast master mix 2 × (Kapa Biosystems Foster City, CA, USA). Data processing was performed using the EcoStudy 5.0 gene scanning software (Illumina, USA). The settings for data collection were 50 fluorescence acquisition points per degree centigrade resulting on a ramp rate of 0.01 °C/s. Comparing the peaks of the melting curve of an unknown sample with those of the controls gave the semi-quantitative estimation for the methylation level of that sample as reported before (Wojdacz et al. 2008; Wojdacz 2012).

### Statistical analysis

Complementary to the geostatistical analysis, the existence of differences in the concentrations of As, between the control and exposed area, was corroborated. This was done using a Student *t* test for independent samples after data normalization using ln (data with positive skewness) and assuming different variances between groups. A *p*-value of $< 0.05$ was considered to be statistically significant.

We expressed the degree of methylation as the percentage of methylated cytosines (% 5mC). Participants were categorized as free of any methylation versus any degree of methylation in each of the genes we assessed. All reported *p*-values are from two-sided tests. A *p*-value of $< 0.05$ was considered to be statistically significant.

In the inferential analysis, for all continuous variables, we used the Student's *t* test or the Mann–Whitney–Wilcoxon test, depending on the assumptions met by the collected data. When comparing two categorical variables, the chi-square test was used. To control for potential confounders, a multivariate logistic regression model was fitted, controlling for age, smoking status, and years of residence.

Statistical analyses were performed using SPSS Statistic 20 (IBM Corp. 2011).

**Table 1** Primers used in the study

| Forward and reverse primers |
|---|
| p53forward: 5′-GGGTCTGGGCGGGTGAGTGA-3' |
| p53reverse: 5′-GCATCTTGTGACTGGGCTCCTGGG-3' |
| RASSF1A forward: 5′-CTTGAGGCCAGGAGTTTGAG-3′ |
| RASSF1A reverse: 5′- AGTAGAGATGGGGTTCACCA-3′ |
| p16 forward: 5′-CAGGRGGGAGGGTCTTCA-3′ |
| p16 reverse: 5′-TGAGGCAGGAGAATCGCTTG-3′ |
| APC forward: 5′-GGCGTTCCATTTAGTTACAAAGTTG-3′ |
| APC reverse: 5′-GGTTACTGAGGCATTCCATTCT-3′ |

## Results

### Soil contamination of the study area

The results indicated that 17% of the total study area presents As concentrations in the soil that exceed the acceptable limits according to the Italian standard (20 mg/kg). In this area, there are 7 rural towns populated with approximately 2140 inhabitants (INE 2019). The mean As concentration in soils of the control area was $16 \pm 10.9$ mg/kg, while in the exposed area it was $33 \pm 25.2$ mg/kg. The statistical analyses indicate significant differences between both sampling areas ($p = 0.00$).

### Study subjects characterization

The sample of human volunteers consisted of 280 participants—140 from the exposed area and 140 from the control area. The baseline characteristics of both groups were comparable, except age of participants and length of time they had been living in the areas of exposure, which were both higher in the exposed area (Table 2). For age, duration of residence in the area, number of cigarettes smoked per day, the Student's *t* test was used, and for sex and smoking habits, Fisher's exact test was used.

### Gene methylation

Of the four genes assessed in this study, p53 was associated with a higher proportion of methylation in its promoter region in inhabitants of areas with high concentrations of heavy metals in soil compared to control areas (Table 3). Results showed a statistically significant difference for p53 methylation in the exposed area (16.4) compared to the

**Table 2** Baseline characteristics of the study participants

| Characteristics | Control area $n=140$ | Exposed area $n=140$ | $p$-value |
|---|---|---|---|
| Age (years old) | 46.83 ± 13.5 | 53.67 ± 15.2 | 0.0001* |
| Sex (% males) | 58 M (41%) | 62 M (44%) | 0.717 |
| Smoking habits | 68 smokers (56%) | 64 smokers (53%) | 0.796 |
| Duration of residence in area (years) | 27 ± 15.7 | 36.9 ± 20.1 | 0.001* |
| Cigarette consumption (per day) | 4.9 ± 5.4 | 6.3 ± 6 | 0.10 |

*Values for statistical differences are based on the Student's $t$ test or Fisher's exact test depending on the assumptions met by the collected data

**Table 3** Gene methylation compared by area of exposure, expressed in proportion of CpG islands methylated

| Gene | Control area $n=140$ | Exposed area $n=140$ | $p$-value |
|---|---|---|---|
| p53 | 9.36 ± 17.5 | 16.4 ± 22 | 0.0035* |
| p16 | 47.2 ± 41.5 | 43.3 ± 38.9 | 0.41 |
| APC | 25.2 ± 22.6 | 25.6 ± 21.5 | 0.89 |
| RASSF1A | 18.3 ± 18.7 | 19 ± 19.2 | 0.75 |

*Values for statistical differences are based on the two-tailed Student's $t$ test

**Table 4** Proportion of participants with no methylation in their promoter region

| Gene | Control area $n=140$ | Exposed area $n=140$ | $p$-value |
|---|---|---|---|
| p53 | 82 (58.6%) | 53 (37.9%) | 0.0001* |
| p16 | 19 (13.6%) | 26 (18.6%) | 0.255 |
| APC | 23 (16.4%) | 21 (15%) | 0.743 |
| RASSF1A | 35 (25%) | 37 (26.4%) | 0.784 |

*Values for statistical difference are based on Fisher's exact test

control area (9.36), with a $p$-value of 0.0035. There was no significant association between heavy metal concentration in soil and the p16, APC, and RASSF1A tumor-suppressor genes.

The methylation status of the participants was classified into two groups: (1) participants with no methylation in their promoter region and (2) participants with any degree of methylation in their promoter region. The crude odds ratio (OR) of presenting any degree of p53 methylation in the exposed area was 1.94 (95% confidence interval (CI): 1.20–3.10) (Table 4).

To reduce the influence of confounders such as age and smoking habits, a multivariate model was used (Table 5). The adjusted OR for this association was 2.16 (95% CI: 1.28–3.65, $p=0.012$), after adjusting by confounders (duration of residence, smoking habits, age).

Living in an exposed area increased the chance of presenting p53 methylation, with an OR adjusted for age and smoking of 2.16 (95% CI: 1.28–3.65, $p=0.012$), adjusting for a history of current or previous smoking habits and age.

**Table 5** Multiple logistic regressions for p53 gene (methylation versus non-methylation)

| Exposure variables | Beta coefficient (95% CI) | OR (95% CI) | $p$-value |
|---|---|---|---|
| Duration of residence in exposed area | 0.77 (0.24, 1.3) | 2.16 (1.28, 3.65) | 0.004* |
| Smoking habits | 0.05 (– 0.46, 0,57) | 1.05 (0.62, 1.77) | 0.839 |
| > 65 years old | – 0.17 (– 0,90, 0.55) | 0.84 (0.41, 1.74) | 0.637 |
| Constant (alpha) | – 0.23 (– 0,7, 0.24) | 0.79 (0.50, 1.27) | 0.339 |

*CI* confidence interval, *OR* odds ratio

## Discussion

Our study demonstrates that permanent inhabitants of an area with high levels of As and other heavy metals in soil show a significantly higher proportion of methylated CpG islands in the promoter region of p53 tumor suppressor gene compared with control areas. This is the first study to consider associations between heavy metal exposure in humans and DNA methylation in the Quintero and Puchuncaví townships in Chile.

Changes caused by heavy metals have been reviewed by different authors (Fragou et al., 2011, Cheng et al. 2012, Ho et al. 2012; Bailey and Fry 2014; Cardenas et al. 2017; Vaiserman and Lushchak 2021). Our results support existing evidence that human exposure to heavy metals can cause epigenetic changes in important tumor-suppressor genes, such as hypermethylation of promoter CpG islands of the p53 gene (Jones and Laird 1999). Previous studies have reported a dose–response relationship with hypermethylation—a mechanism that has been proposed as an explanation for hotspot mutations in the p53 gene. Mutations in the p53 gene are present in 50–60% of human cancers, and

this promotes genetic instability as well as the acquisition of additional mutations, as p53 plays a central role in the response to DNA-damaging agents, such as carcinogens (Chanda et al. 2006, Baugh et al. 2018).

When all p53 missense mutations in the DNA-binding domain are examined, there is a correlation between the frequency of p53 mutant alleles and the altered structure of the mutant protein (Baugh et al. 2018; Mitra et al. 2020 and references therein).

The evidence for significant correlations between this gene methylation and human exposure to heavy metals in the studied areas suggests that more attention should be focused on assessing the public health impact of this exposure in both the Quintero and Puchuncaví townships in Chile. It is particularly relevant given the fact that methylation changes can occur with in utero exposure, persist over time, and continue to accumulate with frequent exposure to heavy metals, even in the absence of the conditions that originally favored their development (Richards 2006; Dolinoy et al. 2007; Anway et al. 2005; Intarasunanont et al. 2012; Guo et al. 2018; Bjørklund et al. 2018).

Regarding this last point, it should be taken into consideration that since the appearance of the first environmental regulations in 1991, the rate of metal deposition in soils has decreased significantly (González et al. 2014; Ministerio de Minería, 1992). However, despite the evolution of environmental regulations and corporate responsibility, no measures have been taken to remedy the environmental impact of heavy metals already accumulated for almost 40 years of unregulated emissions (Arellano 2017). Furthermore, the metals contained in the soils of the study area have very little mobility within the soil profile concentrating in the first 15 cm. (Neaman et al. 2009; De Gregori et al. 2004). These characteristics added to the drought that has been affecting the region for more than 10 years are unfavorable for the inhabitants that continue to be exposed to metals. Considering these facts and the results of this research, we strongly recommend the implementation of an environmental remediation program focused on reducing as much as possible the human exposure to soils with high levels of heavy metals.

One weakness of this study is the observational nature of the collected data, which makes it difficult to reliably establish the mechanism and source of contamination and limits interpretation of our main finding to association rather than causation. Although it is clear that heavy metals were emitted by the smelter in the VIC (Parra et al. 2014), the presence of other compounds, such as $SO_2$, from other companies, such as the thermoelectric plants, could have enhanced the availability of the metals by acidifying the soil, while also causing loss of sensitive vegetation and thus facilitating erosive processes, which have become a serious problem because they facilitate the resuspension of the contaminated dust (Ginocchio et al. 2004; Muena et al. 2010).

The main strengths of this study include the interdisciplinary nature of the work, from sampling soil and human subjects at the same time. We used a large sample size and a multiple logistic regression to adjust the established association for confounders.

Although various media outlets and scientific publications have reported the potential health harms to humans in the region from heavy metal concentrations in the soil (e.g., Salmani-Ghabeshi et al. 2016, Berasaluce et al. 2019, Tapia-Gatica et al. 2020), this study is the first to find evidence of an association between human health and soil contamination in the area surrounding the VIC.

We believe that future studies should take into account the importance of different environmental media and pathways of human exposure to heavy metals, different vulnerable groups, and the potential association with morbidities, especially malignancies.

## Conclusion

The results of this study suggest that the environmental pollution in the townships of Quintero and Puchuncaví in Chile has induced epigenetic changes in the genome of its inhabitants. According to these results, gene p53 and its aberrant methylation is associated with duration of residence in an area with higher heavy metal concentration in soil.

**Author contribution** Conceptualization: EM, SM, IG.
Project administration: EM, IG.
Methodology: FM, EM, FC, SM, MB, CR.
Data curation: CR, EM, IG, MP.
Writing Original draft: EM, MB, IG, JPC.
Review and editing: All authors.

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**Data availability** The datasets generated and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

## Declarations

**Ethics approval and consent to participate** The research protocol was approved by the ethics committee at the Hospital Dr. Gustavo Fricke Regional Health Service (CEC 46/2012). All research subjects were informed about the study and the sampling protocols and each one provided written consent.

**Consent for publication** Not applicable.

**Conflict of interest** The authors declare no competing interests.

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