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First evidence of association between past environmental exposure to dioxin and DNA methylation of CYP1A1 and IGF2 genes in present day Vietnamese population

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TITLE PAGE

Title: First evidence of association between past environmental exposure to dioxin and DNA methylation of CYP1A1 and IGF2 genes in present day Vietnamese population

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RUNNING TITLE

Dioxin and DNA methylation in Vietnamese population

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COMPETING INTERESTS

The authors declare that they have no competing interests

KEYWORDS

DNA methylation, Vietnamese population, past exposure, dioxin, CYP1A1, IGF2

IN BRIEF

This gene-candidate study on 188 individuals from a Vietnamese population indicates that past environmental exposure to dioxin (AO/TCDD) shapes the DNA methylation profile of regions in CYP1A1 gene and that the place of living of parents in former spray zones influences DNA methylation of CYP1A1 and IGF2 genes in the offspring.

ABSTRACT

During the Vietnam War, the United States military sprayed over 74 million litres of Agent Orange (AO) to destroy forest cover as a counterinsurgency tactic in Vietnam, Laos and Cambodia. The main ingredient was contaminated by 2,3,7,8-tetrachlorodibenzo-paradoxin (TCDD). DNA methylation (DNAm) differences are potential biomarker of environmental toxicants exposure. The aim of this study was to perform a preliminary investigation of the DNAm levels from peripheral blood of the present-day Vietnamese population, including individuals whose parents, according to historical data, were exposed to AO/TCDD during the war. 94 individuals from heavily sprayed areas (cases) and 94 individuals from non-sprayed areas (controls) were studied, and historical data on alleged exposure of parents collected. 94 cases were analyzed considering those whose father/parents participated in the war (N=29) and considering the place of residence of both parents (64 living in sprayed areas versus 30 in non-contaminated areas). DNAm levels in CYP1A1 and IGF2 genes were measured (MALDI-TOF technology). The analyses showed that: 1) one CpG site in the CYP1A1 and one in the IGF2 gene showed significant differences in DNAm levels between cases and controls; 2) the CYP1A1 region resulted to be hypomethylated (in 9 out of 16 sites/units; p-val<0.01) in 29 individuals whose father/parents participated in the war in the spray zones; 3) we showed that the place of residence of both parents influenced methylation levels of the CYP1A1 and IGF2 genes (p-val<0.05). In conclusion this study indicates that past environmental exposure to dioxin (AO/TCDD) shapes the DNAm profile of CYP1A1 and that the place of living for parents in former spray zones influences DNAm of CYP1A1 and IGF2 genes. These results open the way to new applications of DNAm as potential biomarker(s) of past human exposure to dioxin.

INTRODUCTION

Epigenetic modifications - and in particular DNA methylation - are the result of molecular mechanisms involved in chromatin structure and DNA accessibility that dynamically modulate gene expression following environmental stimuli. The influence of population genetic structure and ancestry in determining DNA methylation profiles was studied in different human populations (Fagny et al., 2015; Galanter et al., 2017; Giuliani et al., 2016; Heyn et al., 2013). However a great part of DNA methylation variability in populations is associated with environmental factors, so that DNA methylation represents the interface between gene function and the external environment (Bollati and Baccarelli, 2010). DNA methylation is a source of phenotypic variability important for rapid adaptation in response to dynamic environmental changes (Feinberg and Irizarry, 2009; Giuliani et al., 2015; Klironomos et al., 2013). Environmental stimuli as well as external and internal stress factors influence DNA methylation profiles as demonstrated by many studies on murine models which indicate that transient environmental influences can produce changes in epigenetic profiles associated with life-long phenotypic consequences (Heijmans et al., 2008; Stouder and Paoloni-Giacobino, 2010; Szyf, 2015). However human studies are difficult to perform and are limited by ethical constraints. Some rare opportunities for studying the relevance of the environment in human epigenetic variability are represented by historical events like the Dutch Hunger Winter of 1944-45 (Finer et al., 2014; Heijmans et al., 2008; Tobi et al., 2009).

10.75

In the present study we analysed a small population sample coming from two mountainous districts of Thừa Thiên Huế Province that were repeatedly exposed to tactical herbicide spraying during the Vietnam War where the United States military sprayed over 74 million (Stellman et al., 2003) litres of herbicides to destroy forest cover as a counterinsurgency tactic in South Vietnam, Laos and Cambodia. The main ingredient in the most common of herbicides used, Agent Orange (AO), was contaminated by 2,3,7,8-tetrachlorodibenzo-*paradioxin* (TCDD) an extremely toxic and persistent chemical. Political and military histories of both districts note that communist-led forces operated logistics bases near present-day district towns beginning in the late 1940s. As key tactical zones (*khu chiến thuật*) in the First and Second Indochina Wars, these bases saw thousands of military and support staff residing there for several years, especially after 1964. Each district was a key entry point into South Vietnam for military units from North Vietnam traveling along the Ho Chi Minh Trail (Đảng Ủy Ban Chỉ Huy Quân Sự Huyện A Lưới, n.d.; Hùng Sơn and Lê Khai, Eds, n.d.; Ngô Kha, Ed., n.d.). The two mountainous districts received the majority of spray runs in the province from 1963 to 1970. The NW-SE diagonal orientation of spray runs in A Lưới District correspond to the A Sầu Valley where the majority of the district's inhabitants live. From 1966, extensive aerial bombing and large-scale battles forced most inhabitants to stay within the valley. A smaller SW-NE pattern of spray runs follows the one highway, Highway 49, that connects A Lưới to Huế. In Nam Đông District, the main arc of spray runs from west to north follows another mountain valley where most people were located. The present day district towns correspond to the past locations for the tactical zones (Figure 1).

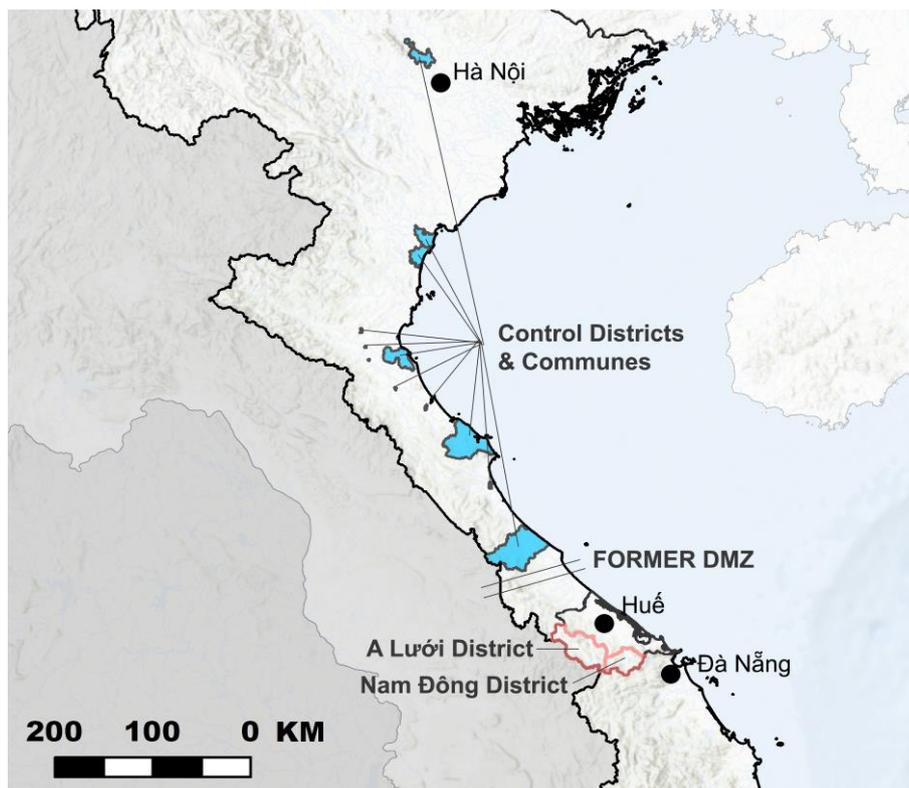


Figure 1. Overview of the geography considered in this study. "DMZ" indicates Vietnamese Demilitarized Zone.

In this context the aim of the present study was to investigate the DNA methylation patterns of two candidate genes, CYP1A1 and IGF2 in order to address the question whether exposure to dioxin in Vietnam is associated with persistent differences in methylation as previously suggested (Manikkam et al., 2012; Skinner et al., 2013) including, for the first time, a very detailed description of historical data (in terms of number of spray runs and spray dates for each district). The rationale beyond the selection of the two genes was the following: CYP1A1 belongs to the cytochrome 450 family, which is involved in the metabolism of various molecules and chemicals within cells (Ko et al., 1996; Mitsui et al., 2014; Okino and Whitlock, 1995; Whitlock et al., 1997). Dioxin is known to be a CYP1A1 inducer (Tsyrllov and Pokrovsky, 1993). In view of the well known effect of dioxin during development we selected also the IGF2 gene which is a maternally imprinted gene and a key factor in human growth and development. DNA methylation of these specific regions of the genome is affected by environmental conditions early in human development and their DNA methylation status changes according to environmental stimuli during life (Pirazzini et al., 2012; Tobi et al., 2011).

DNA methylation of the two genomic regions was measured in 188 Vietnamese individuals, 94 individuals currently living in formerly sprayed areas (Nam Dong and A Luoi District - Thua Thien Hue Province) [CASEs] and 94 individuals living in non-sprayed areas of former North Vietnam from Quang Binh Province to districts and communes near Hà Nội [CTRLs] as reported in Figure 1. Information about parents exposure

during the war (if they participated in the war in the spray zones before 1972 and if they lived in sprayed areas) was collected for each individual.

METHODS

Samples collection and DNA extraction

Peripheral venous blood samples and questionnaires were collected from 188 patients attending the Paraclinical Laboratory, Hospital of Hue University of Medicine and Pharmacy (Hue UMP). All individuals came from either A Luoi District or Nam Dong Districts in Thua Thien Hue Province and various communities north of the former DMZ (Vietnamese Demilitarized Zone). According to maps and historical information, the 188 individuals were divided into 94 cases (*i.e.* individuals who lived in formerly sprayed areas of Nam Dong and A Luoi Districts) and 94 controls (*i.e.* individuals who came from Quang Binh Province). Both cases and controls were interviewed using questionnaires which included information about parents' exposure during the war (living in spraying zones or not, district of residence, and whether they participated in the VN military before 1972). Characteristics of the cohort recruited are described in Table 1 which breaks down the 94 individuals who currently live in former sprayed areas (Nam Dong and A Luoi District - Thua Thien Hue Province), called CASEs, and the 94 individuals who currently live in areas from Quang Binh Province north, called CTRLs.

Group	CASEs		CTRLs	
	N	Average Age	N	Average Age
Male	47	41.3 ± 9.7	47	42.8 ± 14.1
Female	47	38.4 ± 14.4	47	39.2 ± 14.3
Total	94	39.9 ± 12.3	94	41.0 ± 14.3

Table 1. Samples description.

Information about parents' exposure during the war (living or not in sprayed areas and attended or not the war during 1961-1972) were collected for each individual and reported in Table 1S. This shows that father (N=23) or both parents (N=6) of 29 individuals participated in the war in the spray zones before 1972 and were therefore highly likely to have been exposed to dioxin during the war (hence subgroup "CASES_F_P" - cases with Father or Parents exposed) while parents of 65 individuals (who currently live in the former sprayed areas) did not participate in the war in the sprayed area (subgroup "CASES_NO_F_P" - cases with no Father or Parents in the army). Ethical approval for this experiment was obtained from the Ethics Committee of the Hue University of Medicine and Pharmacy (date: May 11th, 2016 to Nguyen Thanh Tin)

Blood samples were collected in EDTA and stored at -80°C at the Department of Microbiology, Hue UMP. DNA extractions were performed by Salting out technique. DNA samples were quantified using the NanoDrop spectrophotometer.

EpiTYPER Assay on MALDI-TOF Platform

From each sample 1,000 ng of DNA was bisulfite-converted using the EZ-96 DNA Methylation Kit (Zymo Research Corporation, Orange, CA) with the following modifications of the manufacture's protocol: bisulfite conversion was performed with thermal conditions that repeatedly varied between 55 °C for 15 min. and 95 °C for 30 seconds for a total of 21 cycles; after the desulfonation and the cleaning steps, bisulfite-treated DNA was eluted in 100 µl of water. Quantitative analysis of methylation status of CpG sites in two candidate genes (CYP1A1 and IGF2) was performed using the EpiTYPER assay (Agena Bioscience Inc., San Diego, CA previously Sequenom Inc.), a MALDI-TOF mass spectrometry-based method. Bisulfite-treated DNA was PCR-amplified and then processed following manufacturer's instructions. DNA methylation levels of 16 CpG sites/units in CYP1A1 genes (chr15:75,019,159-75,019,654, GRCh37/hg19) and 17 CpG sites/units located in IGF2 gene (chr11:2154089-2154542, GRCh37/hg19) located in the DMR2 were measured. The following bisulfite specific primers were used:

IGF2-F: aggaagagagTATAGGGGTGGTTTGTAGGTTAGG

IGF2-R: cagtaatacagactcactataggagaaggcTAAATCAAAAAAACCCEAAAAAAC,

CYP1A1-F aggaagagagTTTGGTATGGTTTGTAGTTGTTTGT

CYP1A1-R cagtaatacagactcactataggagaaggcACCTTCCCTAACCCCTTATTTTA

Raw data are available upon request to the authors.

Statistical Analysis

R software was used to test if bisulphite conversion reaction runs to completion (*package MassArray*) (Thompson et al., 2009). CpG sites with missing values in more than the 20% of the samples were checked, as well as samples with missing values in more than the 20% of CpG sites. No samples were removed following the above mentioned criteria. Statistical analysis was performed on 33 CpG units (16 located in CYP1A1 gene out of 17 and 17 located in IGF2 gene out of 21). ANOVA, pairwise t-test was used to analyze differential methylation. P-values < 0.01 were considered significant. Mean and standard deviation were calculated considering the DNA methylation levels of each CpG site.

For the analysis, we first divided the population in cases and controls considering 94 individuals presently residing in sprayed districts and 94 individuals presently residing in historically non-sprayed areas. Then - on the basis of the questionnaire - we subdivided the former 94 individuals into 2 subgroups. The first included 29 cases where at least one parent (23 only father and 6 both parents) participated in the war in the spray zones before 1972 and thus were likely exposed to dioxin during the war, indicated in the result section as "CASES_F_P" (mean age 41.7 ± 5.5 y). The second subgroup is made of 65 individuals who presently live in former sprayed areas but whose parents did not participate in the war in the spray zones, indicated in the result section as CASES_NO_F_P (mean age 39.0 ± 14.3 y). In conclusion we divided the 94 individuals presently residing in sprayed districts in 2 groups according to the historical place of residence of the parents

during the years of the spray missions: 64 parents were living in sprayed areas (PcPL, Parents contaminated Place of Living) and 30 parents were living in non-contaminated areas (called PncPL, Parents non contaminated Place of Living). A detailed description is reported in Table 1S. We reported for each comparison nominal p-values but also p-values after the adjustment for multiple test (using R package *stats* and function *p.adjust* setting "fdr" as method of adjustment, that in this case is Benjamini, Hochberg method). No data on co-pollutant were collected for this study.

PCA were performed using *prcomp* function and *factoextra* package in R software. The score (s) were calculated to identify for each person a level of exposure (and thus toxicity) and it was estimated as follows (according to Supplementary Material 1): $s = N*i/z$, where: N = number of spray runs, i = years each individual lived in the sprayed area, $z = [\text{date of the last spray run}] - [\text{date of the first spray run}]$.

Since DNA methylation is a tissue specific process and data on cell count of the samples collected are not available, we selected from Illumina 450k BeadChips the CpG sites located in the regions here analyzes. We selected cg1785238, cg13570656, cg12101586 (here CYP1A1 CpG 2.3.4), cg22549041 (here CYP1A1 CpG 5), cg11924019 (here CYP1A1 CpG 6), cg18092474 (here CYP1A1 CpG 10), cg26516004 (here CYP1A1 CpG 14), cg22956483 (here IGF2 CpG 5), cg02613624 (here IGF2 CpG 6.7), cg07096953 (here IGF2 CpG 18.19) and cg11717189 (here IGF2 CpG 26) and the correlation between DNA methylation levels and cell count (CD8 T cells, CD4 T cells, Natural Killer, B cells, monocyte and granulocyte) was calculated and reported in Supplementary Material. These analysis was performed using the R package *FlowSorted.Blood.450k* (Jaffe, 2017) that consider 60 samples from a paper from Reinius and colleagues (Reinius et al., 2012), which can be used by the minfi package to estimate cellular composition from whole blood samples. Only cg11717189, cg12101586 and cg11924019 significantly correlate with cell count p-value < 0.01 f-statistic. Results are reported in Table 2S.

RESULTS

DNA methylation levels for the 16 CpGs unit/sites included in the CYP1A1 region and 17 CpGs unit/sites in the island of IGF2 gene were measured. First we compared the 94 individuals presently residing in sprayed districts (CASES) with the 94 individuals who presently live in non-sprayed areas (CTRL) for DNA methylation levels in the CYP1A1 and IGF2 genes. For the region located in the CYP1A1 gene only one CpG site showed a significant p-value (CpG 5, nominal p-values=0.001, FDR adjusted p-value = 0.016) as reported in Figure 2A and for the sites located in the IGF2 gene the CpG 14 showed a nominal significant p-value (nominal p-values=0.009, FDR adjusted p-value=n.s.) as indicated in Figure 2B. We then divided the CASE group into two subgroups. Those individuals whose father/parents participated in the war in sprayed zones before 1972 (29 CASES_F_P) and those who presently live in sprayed districts but whose father/parents did not participate in the war in sprayed areas (65 CASES_NO_F_P).

The observed DNA methylation changes in theory could be due to: 1) persistent contamination of water and soil in sprayed areas or to 2) high exposure to dioxin of parents during the war who might have transmitted to their offspring these methylation changes as hypothesized by other studies in different environmental situations (Skinner et al., 2010; Szyf, 2015). We compared therefore the 29 CASES_F_P to the 65 CASES_NO_F_P. We observed a general hypomethylation of the CYP1A1 region in CASES_F_P. In particular 9 out of 16 differentially methylated units (CpG 2.3.4, CpG 5, CpG 6, CpG 9, CpG 10, CpG 11, CpG 12.13, CpG 17, CpG 18.19) showed FDR adjusted p-values < 0.05 as Figure 2C shows. Moreover, the number of significant CpGs differences is reduced when CTRL and CASES_NO_F_P subgroup were compared (CpGs 18.19 FDR adjusted p-value < 0.05) which indicates a closer epigenetic profile between controls and individuals who presently live in former contaminated areas but whose parents never participated in the war in the spray zones. On the contrary the IGF2 genomic region showed no significantly differentially methylated regions (FDR adjusted p-value < 0.05) between CASES_F_P and CASES_NO_F_P as Figure 2D shows.

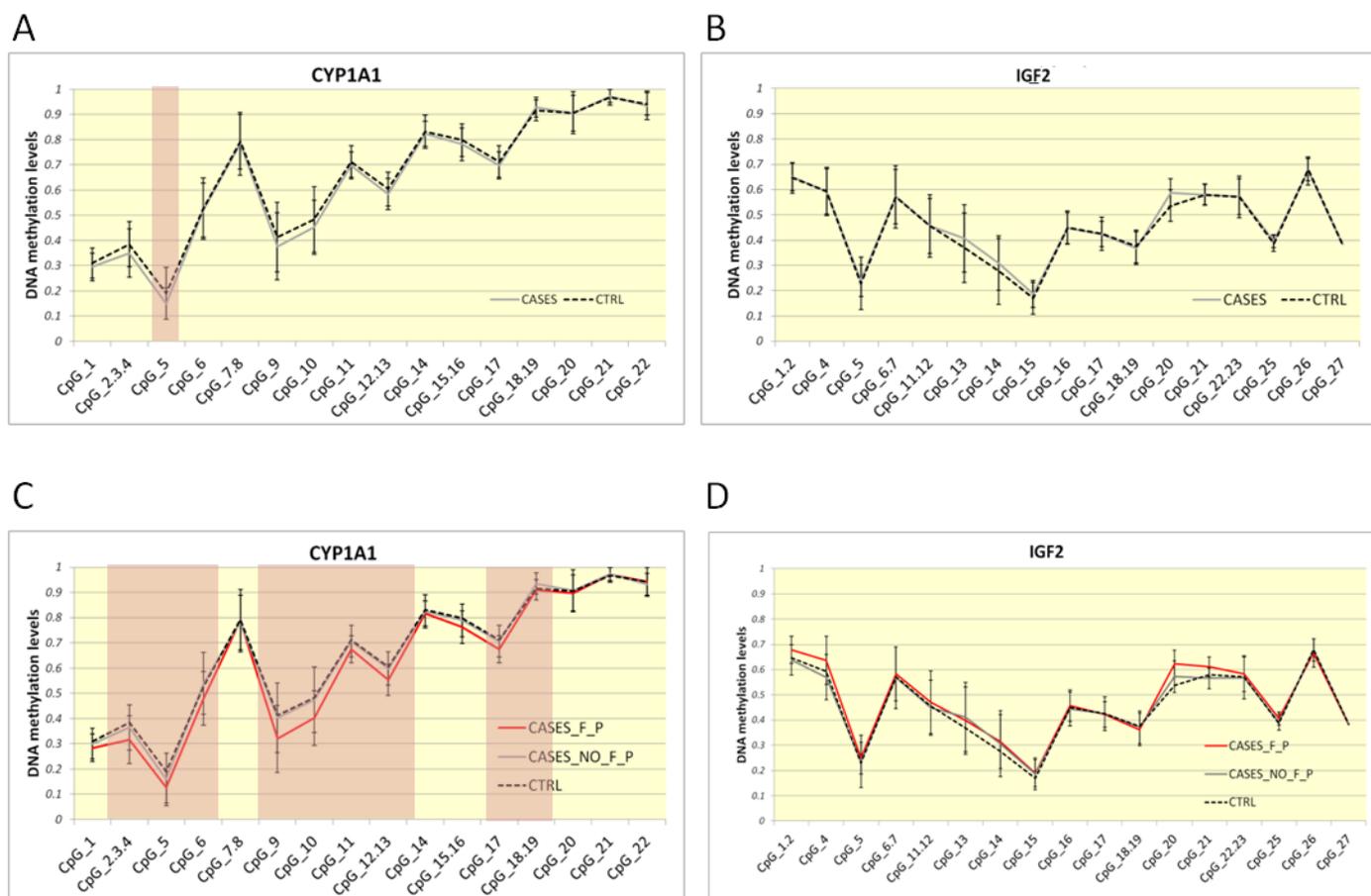


Figure 2. DNA methylation analysis of CYP1A1 and IGF2 gene. DNA methylation levels of individuals presently residing in formerly sprayed districts (CASES: grey line) and individuals who live in areas never sprayed (CTRL: black line) for the regions located in the CYP1A1 (A) and IGF2 (B) genes. In C and D the methylation data in the CYP1A1 and IGF2 genes of CASES were further divided in two subgroups: CASES_F_P (red line) represent the average methylation levels of individuals who lived in regions exposed to dioxin and whose parent(s) participated in the war in the spray zones before 1972; and CASES_NO_F_P (gray line) representing individuals who presently live in formerly sprayed areas but whose parent(s) did not participate in the war in the spray areas; CTRL (black line) refers to individuals who lived in non-sprayed areas and whose parents were never present in the sprayed areas). Red shadows indicates significant FDR adjusted p -values (<0.05) in the pairwise t -test in A and B comparing CASES vs CTRL and in C and D comparing CASES_F_P vs CASES_NO_F_P.

The Pearson correlation between DNA methylation levels and individual ages for each CpG site was performed to test the hypothesis that the older individuals have been in contact with the pollutant for the longest time and therefore show more pronounced alterations of their methylation profiles. However all the CpGs located in CYP1A1 and IGF2 genes showed no correlation (direct or inverse) between DNA methylation and age.

We extended our analysis using historical data of spray runs to consider whether intensity of past spraying might have had any effect. First, we collected historical data for building accurate maps, indicating the paths, dates, and calculated spray areas for each spraying run and then separated for each commune (as reported by Figure 3 and in more details in supplementary materials Figures 1S).

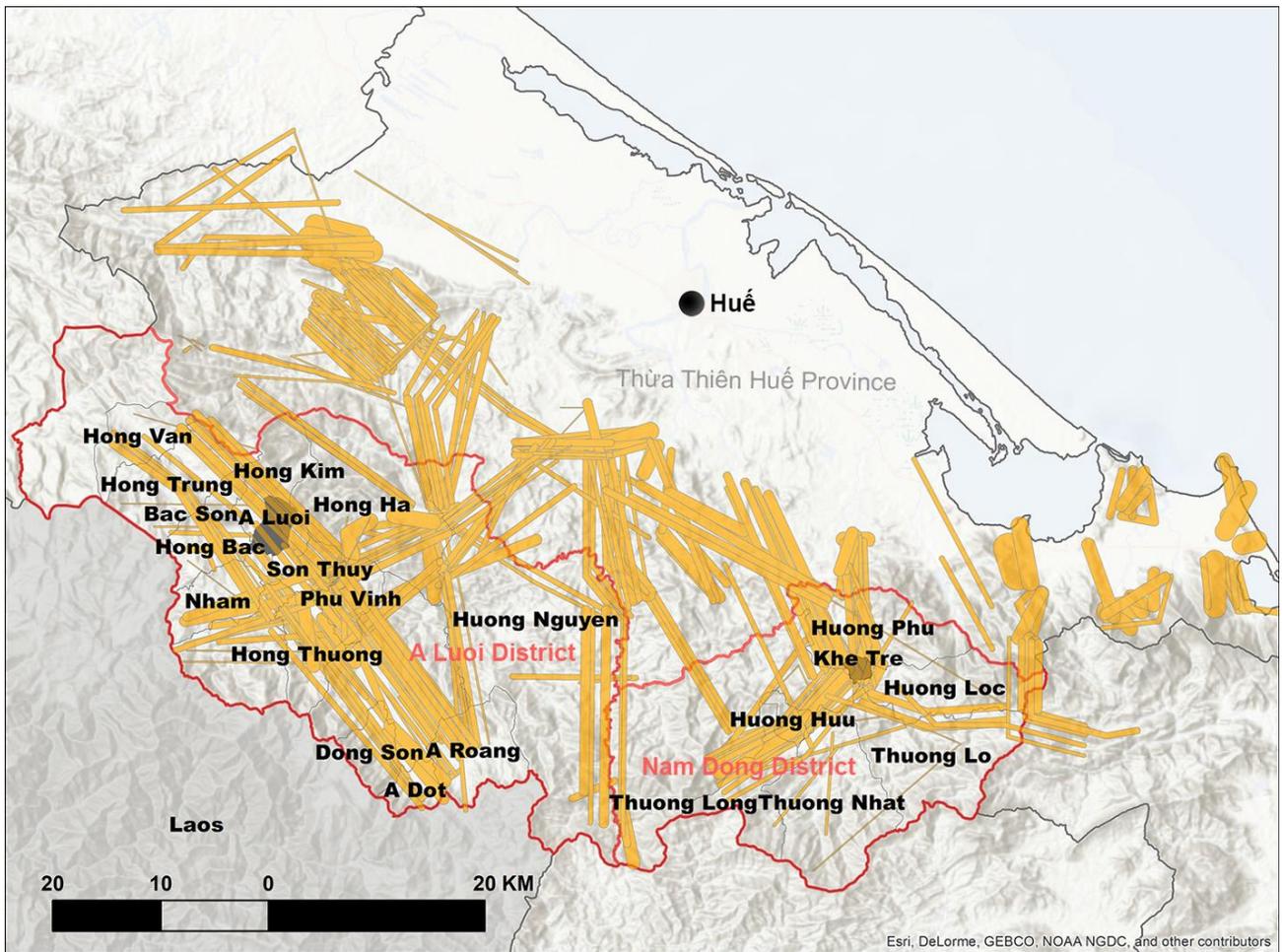


Figure 3. Location of A Luoi and Nam Đông Towns (former tactical zones) and Spray Runs. Map by D. Biggs using spray data from HERBS Tape: Defoliation Missions in South Vietnam, 1965-1971, Data by Province, 1985, Special Collections, USDA National Agricultural Library.

We looked at DNA methylation levels for individuals according to their near-present place of residence (*i.e.* A Luoi Town, Ha Tinh Province, Nam Dong District, Nghe An and Quang Binh Provinces). We did not observe significant differences in DNA methylation profiles based on present-day place of residence. Then we considered differences between more or less heavily sprayed communes in the two CASE districts, checking the number of spray runs per commune and years of spray missions in order to estimate the duration of potential dioxin exposures (= date last spray mission - date first spray mission) (data reported in supplementary Material 1) and no association with DNA methylation levels were demonstrated. Then considering the number of years (in days) each individual has lived in the historically contaminated area, we calculated a score ($s = N^{\circ} \text{ runs} * \text{exp in the contaminated area (in days)} / \text{duration of the exposure}$) as described in materials and methods. We correlated this score (s) to DNA methylation level of each CpG site/unit but no significant correlation was detected even considering single parameters (duration of exposure, number of spray runs, and number of years, in days). This means that in individuals presently

living in the contaminated area we did not observe any significant association with DNA methylation profiles of the CYP1A1 and IGF2 regions. A limitation of this analysis is that the level of dioxin for each run is not included in the score here calculated.

We finally divided the 94 CASES according to the historical place of residence of the parents during the years of the spray missions: 64 living in sprayed areas (PcPL, Parents contaminated Place of Living) and 30 living in non-contaminated areas (called PncPL, Parents non contaminated Place of Living). We observed statistically significant differences in CpG 5, CpG 6, CpG 9, CpG 18.19 located in CYP1A1 gene and in CpG 1.2, CpG 4, CpG 5, CpG 20, CpG 21 located in IGF2 gene (nominal p-values 0.009, 0.009, 0.009, 0.001, 0.04, 0.02, 0.008, 0.01, 0.03 respectively). FDR adjusted p-values were significant (<0.05) only for the region located in CYP1A1 (0.038, 0.038, 0.038, 0.02 respectively), while DNA methylation variation considering the same comparison in the region located in IGF2 are nominally significant but they are not significant after adjustment with FDR method. Figure 4 and Figure 5 showed the differences between the two groups (PcPL and PncPL) by using principal component analysis.

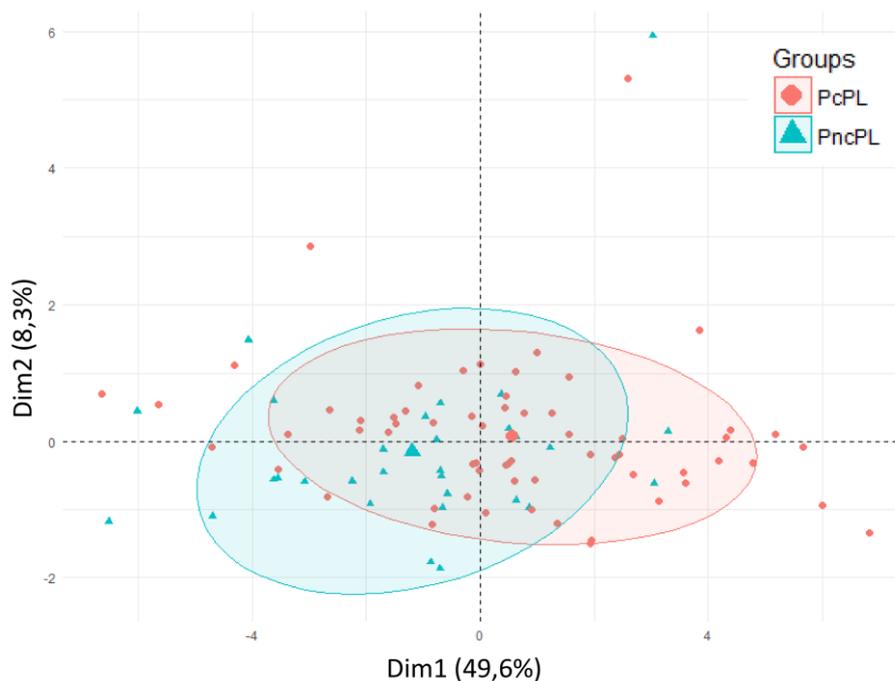


Figure 4. Principal Component analysis for methylation data of CYP1A1 region. Individuals were divided according to the place of living of their parents. In blue 30 PncPL (Parents from non contaminated Place of Living) and in red 64 PcPL (Parents from contaminated Place of Living) are reported. The proportion of the variance is reported in x-axis and y-axis.

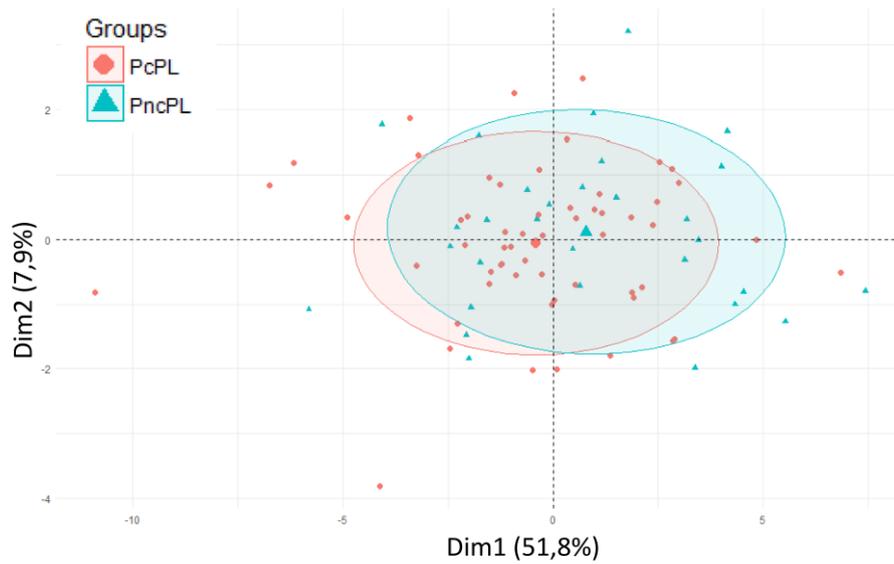


Figure 5. Principal Component analysis for methylation data of IGF2 region. Individuals were divided according to the place of living of their parents. In blue 30 PncPL (Parents non contaminated Place of Living) and in red 64 PcPL (Parents contaminated Place of Living) are indicated. The proportion of the variance is reported in x-axis and y-axis.

DISCUSSION

DNA methylation represents one of the most promising molecular tool to investigate the complex relation between genes and environment and it has been suggested that this epigenetic mechanism is a source of phenotypic plasticity in the changing environment (Feinberg and Irizarry, 2009; Giuliani et al., 2015; Klironomos et al., 2013) by producing variability in the genomes of identical individuals, such as monozygotic twins (Fraga et al., 2005; Pirazzini et al., 2012). Recent studies showed that different human populations present natural variability in DNA methylation profiles and that these are due in part to genetic ancestry and in part to environmental adaptive processes (Fagny et al., 2015; Galanter et al., 2017; Giuliani et al., 2016; Heyn et al., 2013). However, the term "environment" includes many external stimuli and the influence of environmental pollutants on DNA methylation profiles represents a relevant issue in biology and medicine (Bollati and Baccarelli, 2010; Feil and Fraga, 2012; Vineis et al., 2017) with implications for social and anthropological sciences as well.

We hypothesized an effect of Agent Orange (AO) and dioxin on the epigenetic profiles of the present-day population living in areas of Vietnam that were sprayed with these compounds during the last war and we analysed also subset of individuals whose parents were living in sprayed areas at the time of the war or who participated in the war in the spray zones before 1972.

The aim of the present study was therefore twofold: 1) to evaluate the association between an historically contaminated environment (soil, water) and DNA methylation profiles of the Vietnamese population today; 2) to investigate the effect of past exposure of parents on the epigenetic profiles of the offspring.

Following an overview of the recent scientific literature we decided to measure whole blood DNA methylation profiles in two genomic regions represented by the CYP1A1 and IGF2 genes. The region selected in CYP1A1 (chr15:75,019,159-75,019,654) include a GpG island and part of a CpG shore and it is located upstream the CYP1A1 gene nearby the transcription factors binding site. The region selected in IGF2 (chr11:2154089-2154542) is part of a CpG island and shore but it is located in the gene body. DNA methylation of CYP1A1 was associated with exposure to dioxin and external pollutants in different studies (Mitsui et al., 2014; Okino and Whitlock, 1995). DNA methylation of IGF2 was associated with environmental chemical exposure (Hou et al., 2012), while a previous study reported that exposure of mouse preimplantation embryos to dioxin alters the methylation status of imprinted genes H19 and IGF2 (Wu et al., 2004).

Our first results showed that only one CpG site in CYP1A1 (CpG 5) and one in IGF2 (CpG14) are differentially methylated (and IGF2, CpG 14 is not significant after multiple test adjustment) in individuals who presently reside in districts sprayed with dioxin during the Vietnam War when compared with individuals who lived in regions of Vietnam never exposed to AO according to historical data. Although we do not have dioxin exposure estimation for each individual or data on dioxin clearance or urine concentration, these data indicate that living in areas formerly contaminated during the war minimally affects

the methylation profile of the analyzed genes in present day populations. Probably the time past since the end of Agent Orange spray missions (1970) and the half-life of dioxin reduce the amount of toxicant present in the environment (air, water and soil) as indicated in Table 2, thus not producing measurable modifications in DNA methylation profiles of the genes analyzed.

HALF-LIFE	ENVIRONMENT
	Air
200 hours	Estimated OH radical oxidation of fraction in vapor phase
22.3-223 hours	Estimated photo-oxidation by hydroxyl radicals
288 hours	Estimated with respect to gas-phase reaction with OH radical in troposphere
<1 hours	Photolysis of fraction in vapor phase
	Water
600 days	Model surface water environment
32 days	Calculated volatilization from pond and lake surface water
16 days	Calculated volatilization from river surface water
118 hours (winter) 27 hours (spring) 21 hours (summer) 51 hours (fall)	Calculated sunlight photolysis in water at 40 latitude
40 hours	Photolysis in near-surface waters
1.15–1.62 years	Estimated unacclimated aqueous aerobic biodegradation in surface water
2.29–3.23 years	Estimated unacclimated aqueous aerobic biodegradation in groundwater
	Soil and Sediment
1.15–1.62 years	Soil die-away test data for two soils
10–12 years	Degradation in soil
9–15 years	Surface soils
25–100 years	Subsurface soils
>10 years	In sewage sludge applied to land
>97 years	Sediment core data

Table 2. Half-life of TCDD in environment

A difference in DNA methylation profile in only one CpG is unlikely to be associated with an altered gene expression and the biological relevance of fluctuations in DNA methylation levels of individual CpG is still debated (Wessely and Emes, 2012). On the contrary changes in DNA methylation in groups of adjacent CpG sites are more likely to have a biological role and to be associated with altered gene expression, because they potentially affect chromatin structure (Bacalini et al., 2015; Jones, 2012).

Our results show significant differences in DNA methylation profiles of the CYP1A1 region when we compared the 29 individuals whose parents participated in the war in the spray zones before 1972 (CASES_F_P) with the 65 individuals whose parents did not participate in the war in the spray zones (CASES_NO_F_P). Individuals who live in sprayed areas but whose parents did not participate in the war in the spray zones (CASES_NO_F_P) and CTRL (*i.e.* individuals who lived in areas never sprayed) show no differences in DNA methylation profile of these regions thus suggesting that there is no significant effect of past dioxin releases on present day populations. The main changes in DNA methylation are observed instead in individuals whose parents participated in the war in the spray zones. They present a general hypomethylation of the CYP1A1 region. This result is most interesting because it is in agreement with studies showing that in rodents the exposure during embryonal development to dioxin, which is the prototype ligand for the Aryl Hydrocarbon Receptor (ARH) alters DNA methylation patterns in different tissues such as testes, mammary tissue, muscle, liver, and at the same time elevates DNA MethylTransferases (DNMT)

activity in pre-implantation embryos (Manikkam et al., 2012; Papoutsis et al., 2015; Somm et al., 2013; Winans et al., 2015; Wu et al., 2004). It is noteworthy that DNA methylation of CYP1A1 has been associated with maternal smoking during pregnancy (Joubert et al., 2012). However we do not have information on smoking habits for the individuals here analyzed but it is unlikely that maternal smoking influences our results because Vietnam GATS 2015 (a nationally representative survey performed in 2015) reported that the prevalence of smoking in Vietnam was 1.1% among adult women (>15 years old). In particular among females the prevalence of current smoking was 1,9 % among those 45 years and older and lowest (0,5%) among those aged 15–24 (Van Minh et al., 2017).

The experimental studies in rodents make plausible the hypothesis of an inter- or trans-generational effect of dioxin and are in keeping with our observation of modifications in methylation profiles in peripheral blood. It is important to underline that the topic of inter- or trans-generational effect is still highly controversial as it is difficult to identify the molecular basis of information transferred through gametes (Daxinger and Whitelaw, 2012). It is known that DNA methylation is a tissue specific process (Lokk et al., 2014) but the main limitation of human epigenetic studies is the difficulty to measure methylation in a specific tissue involved in the process. The observed changes in DNA methylation in peripheral blood are in agreement with the hypothesis that they may be transmitted across generations. Defects in complete erasure or in maintenance of the DNA methylated protected regions, could be the first potential effect deriving from internal or external factors (such as exposure to dioxin) during gametogenesis (Popp et al., 2010; Stuppia et al., 2015). Recent studies support the role of non-coding RNAs in reconstituting epigenetic states that may escape the two demethylation cycles that occur between generations and a potential mechanism based on the transferring of RNAs from somatic epididymal cells directly to maturing post-testis sperm through vesicles was identified (Sharma, 2015).

Further study of additional descendants of individuals exposed to dioxin and more extensive historical research to pinpoint sites where parents might have been exposed to documented spray events are needed. Since we could not investigate the correlation between methylation and gene expression we can only hypothesize the consequences of the observed changes. Our data cannot indicate any relationships between the adaptive and maladaptive consequences of the epigenetic modifications observed and their impact on diseases. However, the results show that AO/dioxin spraying is associated to hypomethylation of CYP1A1 regions that is usually associated with an increase in the expression of the CYP1A1 gene. The CYP1A1 enzyme is responsible for the oxidation of lipophilic into less lipophilic compounds, which helps in discarding xenobiotics from the body. The induction of CYP1A1 represents therefore a protective mechanism against the accumulation of dioxin and other chemicals in mammalian cells (Ma 2001). We cannot exclude an indirect exposure of the fetus to dioxin during the war because the age of individuals is compatible with the spraying period. However, it is interesting to note that the fathers of 23 out of 29 individuals (CASES_F_P) were exposed to dioxin during the war, which supports the recent hypothesis that epigenetic modifications could be transmitted through the paternal lineage (Ferguson-Smith and Patti, 2011;

Ng et al., 2010; Stuppia et al., 2015). A deep overview of theories and studies that support our observations through paternal inheritance is reported in a review published in 2017 by Pilsner and colleagues (Pilsner et al., 2017).

We finally observed a significant difference in the methylation level of CYP1A1 and IGF2 genes when we considered the place of living of the parents (64 living in contaminated area, called PcPL and 30 living in non-contaminated area, called PncPL. Statistically significant differences in 4 CpG sites/units of CYP1A1 gene (CpG 5, CpG 6, CpG 9, CpG 18.19) and in 5 CpG sites/units of IGF2 (CpG 1.2, CpG 4, CpG 5, CpG 20, CpG 21) were observed which support the hypothesis that ancestral environmental exposures are the main forces in driving epigenetic differences. In this last comparison methylation differences were observed also in the IGF2 gene a result which might be correlated with the clustering by place of living. This clustering could highlight a set of environmental conditions (such as diet, nutrient consumption, food availability, climate etc) that were reported to affect methylation profiles of the offspring (Waterland et al., 2010, 2008). In particular Kovacheva and colleagues (Kovacheva et al., 2007) reported changes in DNA methylation of the same IGF2 region in response to altered maternal dietary choline indicating a possible effect of diet in wartime on DNA methylation of the offspring and supporting the role of DNA methylation as environmental and ecological biosensor (Hoyo et al., 2009). The present study could measure methylation in peripheral blood DNA, which is a mixture of white blood cell types characterized by distinct methylation profiles. This - on one side - can be a positive aspect for biomarkers identification as peripheral blood has the advantage of being easily accessible in the clinical setting. On the other side we cannot infer cell count on the basis of gene-candidate methylation as usually done in epigenome wide studies. However to have a general idea of the impact of cellular composition on the results observed, we selected the CpG sites of the Illumina 450k that are located in the regions analysed. Only few CpGs change according to cell count (cg12101586 that is CYP1A1 CpG_2.3.4, cg11924019 that is CYP1A1 CpG_6 and cg11717189 that is IGF2_CpG_26 with p-values 0.009, 0.002 and $4.2 \cdot 10^{-7}$ respectively). However it is likely that the differences observed are not influenced by cell counts because 1) for CYP1A1 the results here presented involved many CpGs sites that are not influenced by cell composition (not only the site CpG_2.3.4 and CpG_6) and 2) for IGF2 gene the CpG site that is associated to cell composition with the smallest p-value ($4,2 \cdot 10^{-7}$) is CpG 26 and any comparison showed differences in this site. However further studies are needed to evaluate the impact of dioxin in different cell types.

The absence of correlations between DNA methylation levels and the score (s) calculated on the basis of the number of runs, the duration of the stimulus (dioxin sprayed) and the time each individual spent in that area indicates that DNA methylation of the two genes in blood is not affected by these parameters.

CONCLUSIONS

Our data indicate that the whole blood epigenetic profile of the present-day Vietnamese population is shaped by historical events that exposed many individuals to environmental pollutants and stressors. The novelty of

the present study, which combines expertise in historical and molecular research fields, is the observed association between past exposure to AO/dioxin and the DNA methylation profile of the CYP1A1 region in the present-day Vietnamese populations and the effect of parents' place of residence on DNA methylation of the offspring. Despite the limited number of samples analysed, this seminal findings open the way to the use of DNA methylation as a biomarker for environmental and ecological changes. Eventually new studies based on such biomarker(s) and on epigenome wide data may lead to the identification of individuals at risk for the long-term effects of dioxin, a strategy which could complement projects aiming at the bioremediation of dioxin in the environment.

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FIGURE LEGENDS

Figure 1. Overview of the geography considered in this study. "DMZ" indicates Vietnamese Demilitarized Zone.

Figure 2. DNA methylation analysis of CYP1A1 and IGF2 gene. DNA methylation levels of individuals presently residing in formerly sprayed districts (CASES: grey line) and individuals who live in areas never sprayed (CTRL: black line) for the regions located in the CYP1A1 (A) and IGF2 (B) genes. In C and D the methylation data in the CYP1A1 and IGF2 genes of CASES were further divided in two subgroups: CASES_F_P (red line) represent the average methylation levels of individuals who lived in regions exposed to dioxin and whose parent(s) participated in the war in the spray zones before 1972; and CASES_NO_F_P (gray line) representing individuals who presently live in formerly sprayed areas but whose parent(s) did not participate in the war in the spray zones; CTRL (black line) refers to individuals who lived in non-sprayed areas and whose parents were never present in the sprayed areas). Red shadows indicates significant p-values (<0.01) in the pairwise t-test in A and B comparing CASES vs CTRL and in C and D comparing CASES_F_P vs CASES_NO_F_P.

Figure 3. Location of A Lưới and Nam Đông Towns (former tactical zones) and Spray Runs. Map by D. Biggs using spray data from HERBS Tape: Defoliation Missions in South Vietnam, 1965-1971, Data by Province, 1985, Special Collections, USDA National Agricultural Library.

Figure 4. Principal Component analysis for methylation data of CYP1A1 region. Individuals were divided according to the place of living of the parents. In blue 30 PncPL (Parents non contaminated Place of Living) are individuals whose parents lived in non contaminated area and in red 64 PcPL (Parents contaminated Place of Living) are individuals whose parents lived in contaminated area. The proportion of the variance is reported in x-axis and y-axis.

Figure 5. Principal Component analysis for methylation data of IGF2 region. Individuals were divided according to the place of living of the parents. In blue 30 PncPL (Parents non contaminated Place of Living) are individuals whose parents lived in non contaminated area and in red 64 PcPL (Parents contaminated Place of Living) are individuals whose parents lived in contaminated area. The proportion of the variance is reported in x-axis and y-axis.

Supplementary Material 1. Detailed maps of spray run used for calculating the score for each area.

Supplementary Table 1S. 94 individuals presently residing in sprayed districts, living place of the father and of the mother and data regarding the participation of the parents to the war at the spraying zone during 1961-1972 were reported. In red were indicated contaminated area.