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ORIGINAL ARTICLE

Dioxin, an endocrine disruptor, induces long term effects on DNA methylation in men after *in-utero* exposure

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ABSTRACT

BACKGROUND: Prenatal exposure to dioxin, a known endocrine disruptor, after the Seveso accident of 1976 has been associated with thyroid dysfunction, metabolic syndrome and semen quality reduction. Experimental exposure to dioxin *in utero* produced epigenetic endocrine modifications associated with reduction of semen quality, while in men epigenetic effects are not known. Our objective was to study, by a case control approach, the long-term epigenetic effects of prenatal dioxin exposure in 38 men whose mothers had been exposed to high doses of dioxin, serum median 52.0 ppt at exposure, and therefore who were exposed *in utero*, median 24.7 ppt at pregnancy, *vs.* 41 unexposed men.
METHODS: Bisulfite-converted DNA was hybridized onto illumina Infinium Methylation EPIC BeadChip and methylation differences were studied at both individual probe (DMPs) and gene region (DMRs) levels.
RESULTS: We identified hypomethylation of the SPAG1 gene region and a slightly hypermethylated region containing genes of the HOXA family associated with thyroid and skeletal development. An elevated level of epigenetic drift was noted in the exposed group potentially contributing to disease risk. Epigenetic age acceleration did not show significant association with *in-utero* dioxin exposure. Additionally, we found heightened neutrophils and diminished natural killer cells in blood of dioxin exposed men.
CONCLUSIONS: These observations are the first in the literature and align with the long-term semen quality reduction and alteration of thyroid homeostasic mechanisms reported in children exposed *in utero* to dioxin in Seveso. The actual dioxin background serum levels, 1.0-2.0 ppt, are much lower than those associated to these effects.

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KEY WORDS: Dioxins; Seveso accidental release; Prenatal exposure delayed effects; Epigenetics; Spermatogenesis.

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, or dioxin), primarily a by-product of industrial processes, is considered one of the most toxic man-made molecules and is a low-concentration, persistent environmental pollutant and is considered an endocrine disruptor chemical. Its actions are different in different animal species but prenatal/neonatal dioxin exposure is related to the most toxic effects in different organs in animals.[1-3] Information on humans result mainly from accidents and are often not consistent among studies related to immune, endocrine and reproductive systems.[4] Experimental studies also report an epigenetic effect on semen quality and hormones of second and third generation after dioxin exposure of the original relatives.[5] Near Seveso, on July 10, 1976, an accident in a chemical factory exposed a large population to TCDD. Except chloracne, especially in young people, no other significant sign of acute exposure has been reported.[6, 7] Serum samples were collected soon after the accident and therefore it has been possible thereafter, when serum dioxin measurement has been available, to directly associate acute and chronic adverse effects to dioxin serum concentrations. In dioxin exposed people in Seveso, pleiotropic effects have been observed such as the modification of sex ratio at birth, the reduction of semen quality and hormonal dysregulation especially after dioxin exposure during infancy, the endocrine dysregulation of thyroid, the modification of glucose metabolism, menstrual cycle and other effects.[8-13] In addition, *in-utero* and perinatal dioxin exposure in Seveso reduces sperm concentration and motility as well as increases FSH hormone and decreases inhibin B in serum of men born after the exposure of their mothers to dioxin in 1976.[14] Recently, some of us observed that the offspring born years after the exposure of their mothers to dioxin in 1976 have shown later, in adulthood, effects on metabolic syndrome in males, on BMI and glucose metabolism in females, and on thyroid homeostasis on both sexes.[15-17] The mechanisms underlying such diverse and broad-spectrum effects remain largely unknown. Among others, epigenetics has been recognized as a mechanism of action of TCDD in experimental animals.[5, 18] Investigating epigenetics in individuals exposed to TCDD *in utero* can provide relevant information on these mechanisms. Our objective, therefore, has been to study the epigenetic profile of adult men exposed to TCDD *in utero* in comparison with unexposed men as referents.[14] For this purpose we used a robust methylation profiling microarray for the genome-wide extensive coverage of CpG islands, genes, and enhancers providing the quantitative methylation of 850K unique methylation sites in the most biologically significant regions of the human methylome.

Materials and methods

This study has been approved by Institutional Review Boards. The study participants gave written informed consent. The study, a retrospective observational study, was designed to compare the methylation profile of 39 adult men exposed *in utero* to dioxin with 41 referent men born from mothers resident in municipalities away from areas contaminated by TCDD in 1976, and matched for age at the time of blood draw. Their characteristics are summarized in brief: the 39 *in-utero* exposed men were 18-26 years old (median 22.5±2.2 years) at the time of study, they were all sons of women 24.8±5.6 years old at the date of the accident, while at conception the median age of the mothers was 28.2±5.4 years (for more details see Mocarelli *et al.*).[14] Out of 39 for 1 subject it was not possible to retrieve DNA from biological specimens available, therefore we had DNA available for 38 subjects; of 41 referent men 31 were from clinical laboratory employees subjected annually to a health check up, and 10 of them are blood donors whose health has been monitored on a regular basis. The actual study involved 79 men in total.

Measure of the exposure to dioxin

For the *in-utero* exposed men TCDD was measured, in maternal serum samples stored frozen since 1976-1977, at CDCs (Centers for Disease Control and Prevention, Atlanta, USA) by isotope-dilution high resolution mass-spectrometry.[19] Serum TCDD concentrations are reported as parts per trillion on serum lipid basis (ppt). The median serum dioxin concentration at the time of the accident for the mothers of the 38 men

considered in this study was 52.0 ppt and the interquartile range was 26.9-108.2 ppt; the median maternal serum dioxin concentration estimated at conception was 24.7 ppt and the interquartile range was 16.4-57.8 ppt as already reported.[14] Exposure of the mothers of cases has been assessed directly while background exposure of the mother of controls has been estimated from the measure of four serum pools of females aged 20-40 years resident in the municipalities away from areas contaminated by TCDD in 1976. Each pool consisted of approximately 1 mL of serum from 20 or 21 females for a total of about 20 mL of serum. In 1976 the mean TCDD serum background of females aged 20 to 40 years was 10.4 ppt.[20]

DNA methylation assay

Whole blood was processed to extract DNA using an automated system and a magnetic bead-based kit. Initial quantification of genomic DNA was performed, followed by dilution to a concentration of 20 ng/μL using Tris-EDTA buffer. Subsequent quantification ensured accuracy, with samples showing abnormal ratios discarded or subjected to further purification. In accordance with the manufacturer's guidelines, 900 ng of high-quality genomic DNA underwent bisulfite conversion utilizing the EZ DNA Methylation Kit (Ref: D5001, Zymo Research Corporation). Illumina incubation conditions were employed to enhance the efficiency and reproducibility of the bisulfite conversion process. Quality control and quantification of bisulfite-converted DNA (bsDNA) were carried out using an N60 Implen Nanophotometer. Subsequently, approximately 200 ng of bisulfite-converted DNA were hybridized onto illumina Infinium Methylation EPIC BeadChips. The samples were processed following the manufacturer's semi-automated guidelines and scanned using the illumina iScan. Fluorescent signals were detected using the illumina iScan scanner equipped with a two-color laser (532 nm/660 nm) and saved as intensity data files (*.idat). The methylation level for each Cytosine-phosphate-Guanine (CpG) site was represented as β values, determined based on the fluorescent intensity ratio between methylated and unmethylated probes, with β values ranging between 0 (non-methylated) and 1 (fully methylated).

Bioinformatics and statistical analysis

Methylation data management

In the data management phase of DNA methylation studies, minimizing the impact of potential biological or technical biases is crucial. Our approach involved utilizing the Champ package for initial quality control, which entailed removing probes with extreme fluorescence values and filtering out sites with SNPs and known C/T polymorphisms.[21] While sex chromosomes are typically excluded after quality control to minimize gender bias and reduce potential background noise, they were retained in this study. The uniformity of the all-male cohort avoided concerns regarding gender differences, enabling a more inclusive analysis. Subsequently, SWAN normalization was conducted to diminish technical variability.[22] After completing the quality control and preprocessing phase, efforts shifted to identifying potential batch effects in the methylation data.[23] To visualize the presence of batch effects, an exploratory analysis using principal component analysis (PCA) was performed on the probes that had passed the quality control and preprocessing steps. This analysis helped to detect clusters that could be attributed to batch variability. Following this detection, Combat Normalization from the SVA package was employed to correct for these effects.[24]

Differential methylation analysis

We examined methylation differences between men exposed *in utero* to TCDD and men not exposed at both individual probe (DMPs) and gene region (DMRs) levels, using the limma and DMRcate packages.[25, 26] Firstly at probes levels, our model utilizes the methylation M value as the outcome variable. The M value is calculated as the $\log_2$ ratio of the intensities of methylated probe versus unmethylated probe with the Sample Group (men exposed *in utero* to TCDD – not exposed men) as the independent predictor.[27] Additionally the model was fixed by including the first three principal components as covariates containing information about chronological age, biological age, and immune cell composition estimated by the wateRmelon and epidish packages.[28-30] The obtained results underwent Bon-

ferroni correction for multiple testing to identify probes with individual significance, considering those with a Bonferroni-adjusted P value less than 0.05 as significant. Among these, probes exhibiting both statistical significance and a log fold change |logFC| exceeding 0.4, a threshold recognized as biologically significant, were selected.[27] For the DMRs analysis, we began the examination with the individually significant probes, employing the recommended λ smoothing and C value parameters of the DMRcate package. We considered genes regions as significant if they exhibited an aggregated Fisher's P value below 0.05.

Over representation analysis

To gain a comprehensive overview of potentially altered biological processes, we used WebGestalt to perform an over-representation analysis (ORA) on the significant findings from differential methylation analysis.[31] This analysis allows us to move beyond observations of individual genes to evaluate the presence of enriched biological processes pathways. We considered an enriched pathway significant if its adjusted P value with false discovery rate was below 0.05.

Epigenetic drift analysis

To identify Stochastic Epigenetic Mutations (SEMs), we employed a method previously described by Gentilini *et al.* and Spada *et al.*[32, 33] Each subject's methylation profile was assessed by comparing it to the methylation profiles of other subjects within the study. This comparison enabled the establishment of a reference methylation range, against which extreme outlier beta values, referred to as SEMs, were identified. The calculation for identifying these outliers was conducted as follows: the upper value was determined as Q3 + (k times IQR), and the lower value as Q1 - (k times IQR). Here, Q1 represents the first quartile, Q3 the third quartile, the Interquartile Range (IQR) is Q3 - Q1, and k is set to 3. Once SEMs were computed for all samples, we generated the burden of identified epimutations for each individual.

The analysis involved separately examining hypomethylated and hypermethylated epimutations, as well as their combined total burden. By employing a linear regression model with the same regressors utilized in the prior differential analysis, the relationship between these epimutation burdens and the sample groups was evaluated. This approach facilitates a comprehensive understanding of how various types of epimutations impact the study groups.

Age acceleration analysis

The purpose of this analysis was to evaluate if trans generational exposure to dioxin affects the aging process. We utilized age acceleration as a marker typically defined as the deviation between biological and chronological age.[34] We considered four distinct age acceleration metrics: Age Acceleration Residual, Age Acceleration Grim, Age Acceleration PhenoAge, and Age Acceleration Hannum. Each metric was computed using Steve Horvath's website application from raw .idat data (https://dnamage.genetics.ucla.edu/home). To determine if there is a significant difference in these age acceleration parameters based on the origin of the sample group, regression models were employed, with the sample group as the independent variable and age acceleration as the dependent variable.

Data availability

Raw data related to our study are available on the GEO repository under accession number GSE247869 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE247869). Public access to the GEO repository is open, and thus administrative permission to access and use the data is not needed.

Results

Differential methylation analysis

In data exploratory analysis, methylation values did not show batch effects and evidenced minor clustering influenced by the biological component (Supplementary Digital Material 1: Supplementary Figure 1). From the initial differential methylation analysis, conducted at the probe level, we identified 62 statistically sig-

nificant differentially methylated probes (DMP). Of these, 45 probes showed significant biological changes in methylation, with 35 displaying hypermethylation and 10 exhibiting hypomethylation (Supplementary Digital Material 2: Supplementary Figure 2; Supplementary Digital Material 3: Supplementary Table I).

From the differential methylation analysis at the level of genomic regions (DMRs), two significant regions were identified. The first region, DMR1, is consistently hypomethylated ($P=1.92e^{-25}$) in men exposed *in utero* to TCDD compared to those not exposed, and it is associated with the SPAG1 gene (Figure 1).

Seven out of the 9 probes in DMR1 are the most significant even at the individual level (Supplementary Digital Material 4: Supplementary Figure 3).

The second region, DMR2, is hypermethylated ($P=5.85e^{-08}$), consisting of probes that were not individually significant in the previous analysis. It involves a cluster of HOXA genes: HOXA-AS3, HOXA3, RP1-170O19.22, HOXA5, and HOXA6 (Figure 2).

Over representation analysis

The over-representation analysis performed on the significant probes did not reveal any enriched pathways. However, the application of the same analysis to genes identified in DMRs analysis highlighted significantly enriched biological processes associated with embryogenesis and the general development of the organism. These findings are shown in Figure 3.

Notably, the significant aggregation of pathways is primarily attributed to the combination of genes HOXA3, HOXA5, and HOXA6 corresponding to the gene region that starts from 27182435 to 27185732 on chromosome 7. For more detailed information, such as the size of

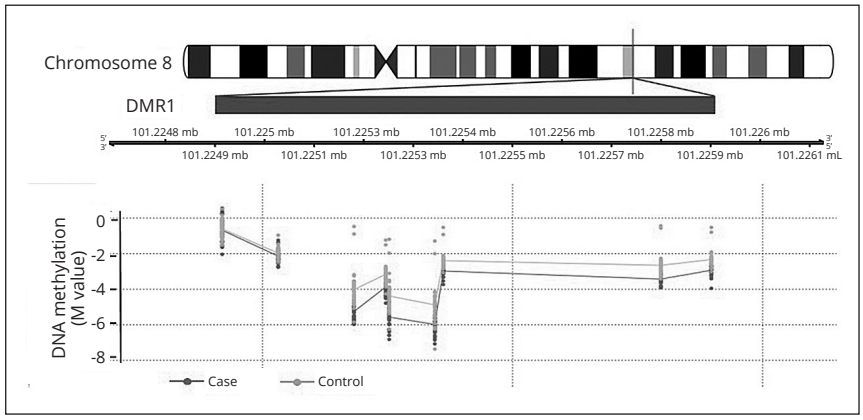


Figure 1.—Methylation of DMR1 in men exposed *in utero* to dioxin.

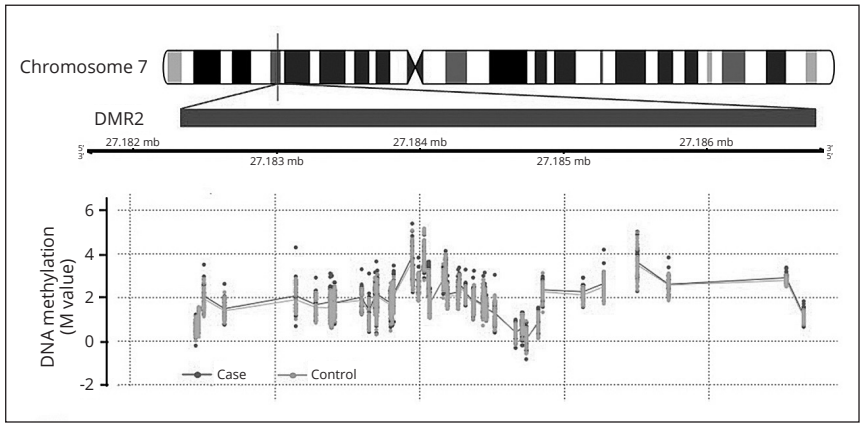


Figure 2.—Methylation of DMR2 in men exposed *in utero* to dioxin.

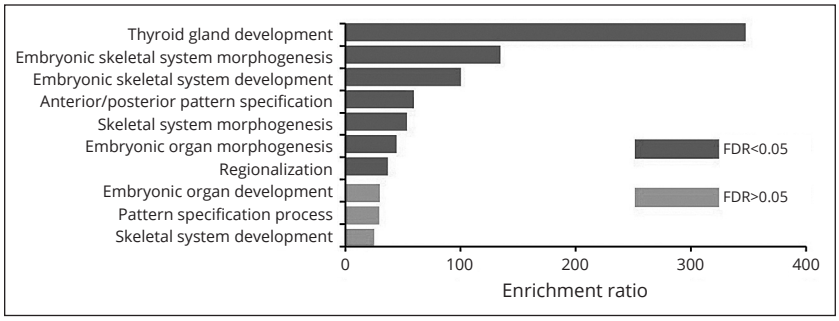


Figure 3.—Enriched biological processes pathways.

each pathway, its significance, and the level of enrichment refer to Supplementary Digital Material 5, Supplementary Table II.

Immune circulating cells components

Linear regression models were utilized to explore potential variances in the estimated levels of immune circulating cells. The analysis revealed a statistically significant increase in neutrophils (Beta value=0.05 [Confidence Interval 95%: 0.01, 0.10]) and a decrease in natural killer (NK) cells (Beta value=-0.03 [Confidence Interval 95%: -0.05, -0.01]) in men *in-utero* exposed to TCDD. Supplementary Digital Material 6, Supplementary Table III and Supplementary Digital Material 7, Supplementary Figure 4.

Epigenetic drift analysis

The epigenetic drift analysis found variations in SEMs burden between men exposed *in utero* to TCDD and not exposed men (Table I, Figure 4).

The adjustment for the set of covariates was the same adopted in the differential methylation analysis. Notably, the burden of SEMs was log-transformed to achieve a parametric distribution. The covariates adopted encompassed the first three principal components, derived from combining estimates of immune cell counts and age.

TABLE I.—*Stochastic epigenetic mutation burden in 38 men exposed* in utero *to dioxin compared with 41 not exposed men.*

SEM regression model (a)	Adjusted P value (b)	Beta Coefficient (CI 95%) (c)
Log (total SEM) ~ sample group + Dim1 + Dim2 + Dim3 (d)	0.03	0.71 (0.09, 1.32)
Log (hypermethylated SEM) ~ sample group + Dim1 + Dim2 + Dim3	0.01	1.18 (0.32, 2.04)
Log (hypomethylated SEM) ~ sample group + Dim1 + Dim2 + Dim3	0.18	0.33 (-0.14, 0.80)

(a) SEM: stochastic epigenetic mutation; (b) P value adjusted for Benjamini Hoechberg multiple test correction; significant results are reported in bold; (c) CI 95%: Confidence Interval at 95% probability level; (d) Dim1, Dim2, Dim3: PCA first three principal components.

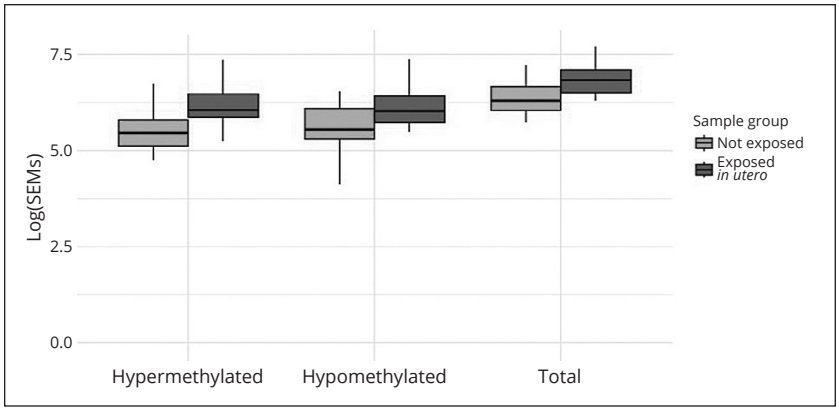


Figure 4.—Epimutation burden in men exposed *in utero* to dioxin.

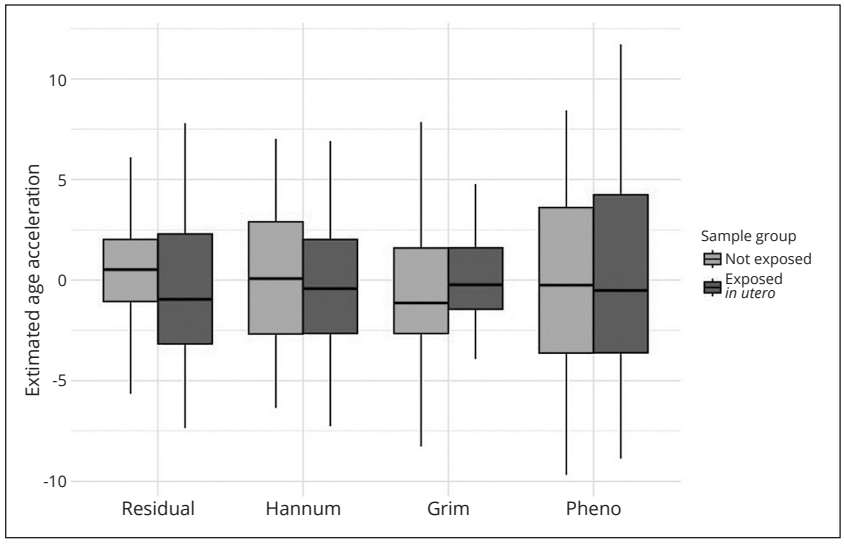


Figure 5.—Age acceleration in men exposed *in utero* to dioxin.

A statistically significant increase in SEM burden was observed among men exposed *in utero* to TCDD compared to non exposed men for both hyper methylated and total SEMs.

Age acceleration analysis

The impact of dioxin exposure on the aging process was evaluated by employing age acceleration as an indicator. We utilized four distinct age acceleration metrics: Age Acceleration Residual, Age Acceleration Hannum, Age Acceleration Grim, and Age Acceleration PhenoAge. Differences were examined using a regression model. Age acceleration served as the dependent variable, while the group was the independent variable. However, no statistically significant difference in epigenetic aging emerged between the two groups, as depicted in Figure 5 and supported by Supplementary Digital Material 8, Supplementary Table IV.

Discussion

This study examined how *in-utero* exposure to TCDD affects the epigenomes of adult men. It is crucial to highlight that in this distinctive scenario, we had the availability of the original individual TCDD serum concentration from 1976 samples collected at the time of the mother's exposure and therefore we calculated its value at conception.[14] This estimation served as a proxy for the TCDD serum concentration during pregnancy, when TCDD crosses from mother to embryo.

The DNA methylation profiles of men exposed *in utero* to TCDD (median maternal serum TCDD concentration 52.0 ppt at exposure, and 24.7 ppt at conception) were compared to those of referents who had been exposed to a mean of 10.4 ppt TCDD maternal serum background.[20]

The results revealed significant epigenetic differences between men exposed *in utero* to dioxin, and non exposed ones. These differences were observed after about two decades following their *in-utero* TCDD exposure. The differential methylation analysis identified 45 probes with significant biological changes in methylation and 2 DMRs, with both results remaining significant after adjusting for multiple testing. When delving into the first of the two regions displaying differential methylation, we observed that this specific locus, encompassing the SPAG1 gene, stands out for its consistent demethylation. The probes associated with SPAG1 produced robust results, with 7 out of the 9 probes showing significant demethylation in the group of men born to mothers exposed to TCDD. The SPAG1 gene, alternatively known as Sperm-associated antigen 1, contributes to sperm motility and fertilization by participating in the development of cilia and flagella. It is relevant for the assembly of the cili-

ary dynein arms, and their function by binding guanosine triphosphate (GTP), and GTPase activity. It also plays a role in mammalian gametogenesis and fertility, in particular in the inhibition of cell growth of immature Sertoli cells.[35] The reduced DNA methylation within the DMR1 is supposed to increase the expression of SPAG1 gene so modifying Sertoli cells and sperm functions. Can we hypothesize that the reduced sex ratio observed in Seveso and originated exclusively by TCDD exposed men could be associated with the demethylation of SPAG1?[9] While specific evidence on how dioxin directly affects SPAG1 is lacking in the existing literature, it is widely acknowledged that TCDD can impact fertility, sperm motility, and sperm count.[10, 14, 36] In particular, Mocarelli *et al.* assessed sperm quality in the same men explored in the current study, albeit with a larger number of control subjects.[14] Even if epigenetic effects are hypothesized in men reproduction, limited information is available.[37] Recent studies support the idea that epigenetic changes may be involved, considering the demonstrated ability of dioxins to induce such alterations in experimental animals, therefore it should be relevant to investigate the sons of these men.[5, 38]

The second DMR, was slightly hypermethylated and associated with a group of genes prevalent in the HOXA genes family: HOXA-AS3, HOXA3, RP1-170O19.22, HOXA5, HOXA6. Based on the list of significant genes, an Over Representation Analysis was performed, revealing significant associations with different biological processes, including thyroid development, skeletal development, embryonic organ development and regionalization. These findings support earlier results of some of us showing that prenatal TCDD exposure may impact thyroid hormone levels later in life, potentially leading to thyroid dysfunction.[17] The primary mechanism proposed for thyroid disruption by TCDD is via up-regulating the expression in the liver of thyroid hormone metabolizing enzyme, UDP-GT resulting in increased clearance of T4 or T3.[39] Other mechanisms include inhibition of deiodinases, altered binding to serum transport proteins including thyroid-hormone binding globulin and TCDD immunotoxicity.[39-42] Our findings suggest for the first time that endocrine disruption of dioxin on thyroid homeostasis could be sustained also by the increase of methylation of HOXA genes family associated with the pathway of thyroid development.

Prenatal exposure to TCDD has been shown to have also significant effects on skeletal development in various studies as demonstrated in rats and monkeys where TCDD exposure during pregnancy and lactation led to bone defects, particularly in the most sensitive rat line, resulting in decreased bone length.[43, 44] This could be related to the observation in humans in Seveso that dioxin produced developmental enamel defects in subjects who had been exposed to high doses of dioxin when younger than 5 years of age at the time of the accident.[45]

Another interesting finding emerged from the analysis of blood cellular components estimates. Using methylation data to estimate cellular components, we observed an increase in neutrophils and a decrease in NK cells in men with *in-utero* exposure to TCDD. Neutrophil upregulation and NK cell downregulation play crucial roles in various scenarios, particularly in the context of inflammatory responses, and it would be interesting to additionally investigate these findings.[46,]

The epigenetic analysis has extended beyond the examination of differential methylation, exploring additional intriguing facets of epigenetics such as epigenetic age acceleration and epigenetic drift. Epigenetic age acceleration specifically quantifies the difference between epigenetic age and chronological age, serving as a marker for biological age. Conversely, epigenetic drift represents a stochastic process involving global DNA methylation changes, influenced by genetic and environmental factors. Both these epigenetic changes lead to alterations in methylation patterns that may impact biological aging and disease risk.[47] Epigenetic age was analyzed using four different epigenetic clocks: Hannum DNA methylation age, PhenoAge, GrimAge, and Horvath DNAm Age to estimate biological age, with GrimAge being noted for its superior performance in predicting disease risk and mortality compared to other clocks. No significant difference emerged between the two groups for any of the age acceleration estimates used. This finding is intriguing as it indicates, for the first time,

TABLE II.—*Main findings on methylation changes and their potential biological implications in men exposed to high doses of dioxin* in utero.

DNA region	Variation	Associated genes	Potential biological implications
DMR1 (a)	Hypo-methylation	SPAG1	Modification of spermatogenesis and fertilization
DMR2 (b)	Hyper- methylation	HOXA genes family	Modification of thyroid development
All DNA	Increased Epigenetic drift (c)	All genes	Risk of chronic diseases later in life

(a) DMR1: first differential methylated region; (b) DMR2: second differential methylated region; (c) increased epigenetic drift: higher number of stochastic epigenetic errors.

that *in-utero* exposure to TCDD does not seem to have a significant impact on biological aging, unlike other lifetime exposure factors, such as airborne chemicals, phthalates, per- and polyfluoroalkyl substances, and toxic metals do.[48] Conversely, significant differences emerged when considering epigenetic drift. Specifically, the group of men exposed *in utero* to TCDD exhibited a higher number of stochastic epigenetic errors. Assessing the clinical implications of increased epigenetic drift in exposed men poses a challenge. However, we can argue that an elevated drift could potentially contribute to a broader escalation in epigenetic dysregulation, a known factor associated with various chronic pathologies. Furthermore, this observation suggests that the epigenetic consequences of dioxin *in-utero* exposure might not be confined to specific genes but stochastically involves the entire genome and that this effect could endure into adulthood.

The observed epigenetic DNA modifications can be the result of the action of methylated non coding RNAs. DNA methylation can affect the histone epigenetic modification and the histone retention. It should be very interesting to study epigenetic modifications of non coding RNAs and histones in the future.[49] The reduced methylation of the CPGs associated to SPAG1 can be considered as an epigenetic signature for exposure *in utero* to dioxin in men. It can also be very interesting to study the HOXA gene family as key regulator of endocrine disruption of the thyroid homeostasis.

The main results of our work are synthesized in Table II.

These observations warrant to be replicated on a validation cohort, and the study to be extended to the cohort of females exposed *in utero* to dioxin. The study of long term epigenetic consequences of prenatal dioxin exposure should be continued and expanded to the second generation.

Strengths and limitations of the study

One primary limitation is the small sample size and the lack of a validation cohort. Among the strengths, the principal one is the rather unique condition of the investigated cases: in fact we know the original serum level of TCDD of the mothers at the moment of the accident in 1976 and we could therefore calculate it at the time of pregnancy.

Conclusions

In conclusion, we found persistent epigenetic alterations in men prenatally exposed to high doses of TCDD. Notably, DNA methylation, within a genomic region encompassing the SPAG1 gene, critical for sperm production and function, was significantly reduced, supposed to increase the expression of SPAG1 gene. This finding suggests a potential mechanism by which TCDD exposure might influence sperm production and maturation. The hypermethylation within the genomic region encompassing HOXA genes family could influence thyroid and skeletal development. The elevated level of epigenetic drift in these exposed men may contribute to an increased risk of chronic diseases later in life, warranting further investigation into the long-term health consequences of prenatal TCDD exposure. Interestingly, epigenetic age acceleration was not detected. The actual background serum level of TCDD of about 1.0-2.0 ppt in industrialized countries is much lower than TCDD serum levels associated to these results.

References

1. Schecter A, Birnbaum L, Ryan JJ, Constable JD. Dioxins: an overview. Environ Res 2006;101:419–28.

2. WHO. Overview: State of the science of endocrine dis-

rupting chemicals 2012. In: Bergman Å, Heindel JJ, Jobling S, Kidd KA, Zoeller RT, editors [Internet]. Available from: https://www.who.int/publications/i/item/9789241505031 [cited 2024, Oct 4].

3. Gore AC, Chappell VA, Fenton SE, Flaws JA, Nadal A, Prins GS, *et al.* EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals. Endocr Rev 2015;36:1–150.

4. National Center for Environmental Health (U.S.). Division of Laboratory Sciences; National Health and Nutrition Examination Survey (U.S.). 2009. Fourth national report on human exposure to environmental chemicals [Internet]. Available from: https://stacks.cdc.gov/view/cdc/21813 [cited 2024, Oct 24].

5. Ben Maamar M, Nilsson EE, Skinner MK. Epigenetic transgenerational inheritance, gametogenesis and germline development. Biol Reprod 2021;105:570–92.

6. Mocarelli P, Marocchi A, Brambilla P, Gerthoux P, Young DS, Mantel N. Clinical laboratory manifestations of exposure to dioxin in children. A six-year study of the effects of an environmental disaster near Seveso, Italy. JAMA 1986;256:2687–95.

7. Bertazzi PA, Bernucci I, Brambilla G, Consonni D, Pesatori AC. The Seveso studies on early and long-term effects of dioxin exposure: a review. Environ Health Perspect 1998;106(Suppl 2):625–33.

8. Centers for Disease Control (CDC). Preliminary report: 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure to humans—Seveso, Italy. MMWR Morb Mortal Wkly Rep 1988;37:733–6.

9. Mocarelli P, Gerthoux PM, Ferrari E, Patterson DG Jr, Kieszak SM, Brambilla P, *et al.* Paternal concentrations of dioxin and sex ratio of offspring. Lancet 2000;355:1858–63.

10. Mocarelli P, Gerthoux PM, Patterson DG Jr, Milani S, Limonta G, Bertona M, *et al.* Dioxin exposure, from infancy through puberty, produces endocrine disruption and affects human semen quality. Environ Health Perspect 2008;116:70–7.

11. Chevrier J, Warner M, Gunier RB, Brambilla P, Eskenazi B, Mocarelli P. Serum dioxin concentrations and thyroid hormone levels in the Seveso Women's Health Study. Am J Epidemiol 2014;180:490–8.

12. Mocarelli P. Seveso: a teaching story. Chemosphere 2001;43:391–402.

13. Eskenazi B, Warner M, Brambilla P, Signorini S, Ames J, Mocarelli P. The Seveso accident: A look at 40 years of health research and beyond. Environ Int 2018;121:71–84.

14. Mocarelli P, Gerthoux PM, Needham LL, Patterson DG Jr, Limonta G, Falbo R, *et al.* Perinatal exposure to low doses of dioxin can permanently impair human semen quality. Environ Health Perspect 2011;119:713–8.

15. Warner M, Rauch S, Ames J, Mocarelli P, Brambilla P, Signorini S, *et al.* In utero dioxin exposure and cardiometabolic risk in the Seveso Second Generation Study. Int J Obes (Lond) 2019;43:2233–43.

16. Warner M, Rauch S, Brambilla P, Signorini S, Mocarelli P, Eskenazi B. Prenatal dioxin exposure and glucose metabolism in the Seveso Second Generation study. Environ Int 2020;134:105286.

17. Warner M, Rauch S, Ames J, Mocarelli P, Brambilla P, Signorini S, *et al.* Prenatal dioxin exposure and thyroid hormone levels in the Seveso second generation study. Environ Res 2020;183:109280.

18. Mai X, Dong Y, Xiang L, Er Z. Maternal exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin suppresses male reproductive functions in their adulthood. Hum Exp Toxicol 2020;39:890–905.

19. Patterson DG Jr, Hampton L, Lapeza CR Jr, Belser WT, Green V, Alexander L, *et al.* High-resolution gas chromatographic/high-resolution mass spectrometric analysis of human serum on a whole-weight and lipid basis for 2,3,7,8-tetrachlorodibenzo-p-dioxin. Anal Chem 1987;59:2000–5.

20. Eskenazi B, Mocarelli P, Warner M, Needham L, Patterson DG Jr, Samuels S, *et al.* Relationship of serum TCDD concentrations and age at exposure of female residents of Seveso, Italy. Environ Health Perspect 2004;112:22–7.

21. Tian Y, Morris TJ, Webster AP, Yang Z, Beck S, Feber A, *et al.* ChAMP: updated methylation analysis pipeline for Illumina BeadChips. Bioinformatics 2017;33:3982–4.

22. Welsh H, Batalha CM, Li W, Mpye KL, Souza-Pinto NC, Naslavsky MS, *et al.* A systematic evaluation of normalization methods and probe replicability using infinium EPIC methylation data. Clin Epigenetics 2023;15:41.

23. Ross JP, van Dijk S, Phang M, Skilton MR, Molloy PL, Oytam Y. Batch-effect detection, correction and characterisation in Illumina HumanMethylation450 and MethylationEPIC BeadChip array data. Clin Epigenetics 2022;14:58.

24. Leek JT, Johnson WE, Parker HS, Jaffe AE, Storey JD. The sva package for removing batch effects and other unwanted variation in high-throughput experiments. Bioinformatics 2012;28:882–3.

25. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, *et al.* limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res 2015;43:e47.

26. Peters TJ, Buckley MJ, Statham AL, Pidsley R, Samaras K, V Lord R, *et al.* De novo identification of differentially methylated regions in the human genome. Epigenetics Chromatin 2015;8:6.

27. Du P, Zhang X, Huang CC, Jafari N, Kibbe WA, Hou L, *et al.* Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis. BMC Bioinformatics 2010;11:587.

28. Zhang Q, Vallerga CL, Walker RM, Lin T, Henders AK, Montgomery GW, *et al.* Improved precision of epigenetic clock estimates across tissues and its implication for biological ageing. Genome Med 2019;11:54.

29. Zheng SC, Breeze CE, Beck S, Dong D, Zhu T, Ma L, *et al.* EpiDISH web server: Epigenetic Dissection of Intra-Sample-Heterogeneity with online GUI. Bioinformatics 2019;36:1950–1.

30. Jones MJ, Islam SA, Edgar RD, Kobor MS. Adjusting for Cell Type Composition in DNA Methylation Data Using a Regression-Based Approach. Methods Mol Biol 2017;1589:99–106.

31. Liao Y, Wang J, Jaehnig EJ, Shi Z, Zhang B. WebGestalt 2019: gene set analysis toolkit with revamped UIs and APIs. Nucleic Acids Res 2019;47(W1):W199–205.

32. Gentilini D, Garagnani P, Pisoni S, Bacalini MG, Calzari L, Mari D, *et al.* Stochastic epigenetic mutations (DNA methylation) increase exponentially in human aging and correlate with X chromosome inactivation skewing in females. Aging (Albany NY) 2015;7:568–78.

33. Spada E, Calzari L, Corsaro L, Fazia T, Mencarelli M, Di Blasio AM, *et al.* Epigenome Wide Association and Stochastic Epigenetic Mutation Analysis on Cord Blood of Preterm Birth. Int J Mol Sci 2020;21:5044.

34. Horvath S. DNA methylation age of human tissues and cell types. Erratum in: Genome Biol 2015;16:96.

35. Faraji S, Sharafi M, Shahverdi AH, Fathi R. Sperm Associated Antigens: Vigorous Influencers in Life. Cell J 2021;23:495–502.

36. Faiad W, Soukkarieh C, Murphy DJ, Hanano A. Effects

of dioxins on animal spermatogenesis: A state-of-the-art review. Front Reprod Health 2022;4:1009090.

37. Aitken RJ, Gibb Z. Sperm oxidative stress in the context of male infertility: current evidence, links with genetic and epigenetic factors and future clinical needs. Minerva Endocrinol 2022;47:38–57.

38. Viluksela M, Pohjanvirta R. Multigenerational and Transgenerational Effects of Dioxins. Int J Mol Sci 2019;20:2947.

39. Miller MD, Crofton KM, Rice DC, Zoeller RT. Thyroid-disrupting chemicals: interpreting upstream biomarkers of adverse outcomes. Environ Health Perspect 2009;117:1033–41.

40. Boas M, Feldt-Rasmussen U, Main KM. Thyroid effects of endocrine disrupting chemicals. Mol Cell Endocrinol 2012;355:240–8.

41. Zoeller TR. Environmental chemicals targeting thyroid. Hormones (Athens) 2010;9:28–40.

42. Gogal RM Jr, Holladay SD. Perinatal TCDD exposure and the adult onset of autoimmune disease. J Immunotoxicol 2008;5:413–8.

43. Miettinen HM, Pulkkinen P, Jämsä T, Koistinen J, Simanainen U, Tuomisto J, *et al.* Effects of in utero and lactational TCDD exposure on bone development in differentially sensitive rat lines. Toxicol Sci 2005;85:1003–12.

44. Hermsen SA, Larsson S, Arima A, Muneoka A, Ihara T, Sumida H, *et al.* In utero and lactational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) affects bone tissue in rhesus monkeys. Toxicology 2008;253:147–52.

45. Alaluusua S, Calderara P, Gerthoux PM, Lukinmaa PL, Kovero O, Needham L, *et al.* Developmental dental aberrations after the dioxin accident in Seveso. Environ Health Perspect 2004;112:1313–8.

46. Sun R, Xiong Y, Liu H, Gao C, Su L, Weng J, *et al.* Tumor-associated neutrophils suppress antitumor immunity of NK cells through the PD-L1/PD-1 axis. Transl Oncol 2020;13:100825.

47. Martin GM. Epigenetic drift in aging identical twins. Proc Natl Acad Sci USA 2005;102:10413–4.

48. Dutta S, Goodrich JM, Dolinoy DC, Ruden DM. Biological Aging Acceleration Due to Environmental Exposures: An Exciting New Direction in Toxicogenomics Research. Genes (Basel) 2023;15:16.

49. Skinner MK, Ben Maamar M, Sadler-Riggleman I, Beck D, Nilsson E, McBirney M, *et al.* Alterations in sperm DNA methylation, non-coding RNA and histone retention associate with DDT-induced epigenetic transgenerational inheritance of disease. Epigenetics Chromatin 2018;11:8.

Conflicts of interest

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Authors' contributions

Davide Gentilini, Paolo Brambilla and Paolo Mocarelli conceived the study; Davide Sacco and Davide Gentilini contributed to the analysis, and co-wrote the first draft of the manuscript; the recruitment of patients (and clinical information) was managed by Paolo Mocarelli, Paolo Brambilla, Stefano Signorini, Silvia Besana, Claudia Siracusa, Katya Cattaneo; Luciano Calzari performed the DNA methylation Illumina microarray assay; Davide Sacco, Davide Gentilini, Rebecca Cavagnola and Giulia N. Baldrighi developed the informatics pipeline for this study; Davide Sacco, Davide Gentilini, Luciano Calzari, Rebecca Cavagnola and Giulia N. Baldrighi analyzed the data; Lucy Costantino, Fulvio Ferrara, Paolo Mocarelli, Paolo Brambilla, Stefano Signorini and Valerio Leoni contributed by providing assistance in writing the final version of the manuscript and in interpreting the results. All authors read and approved the final version of the manuscript.

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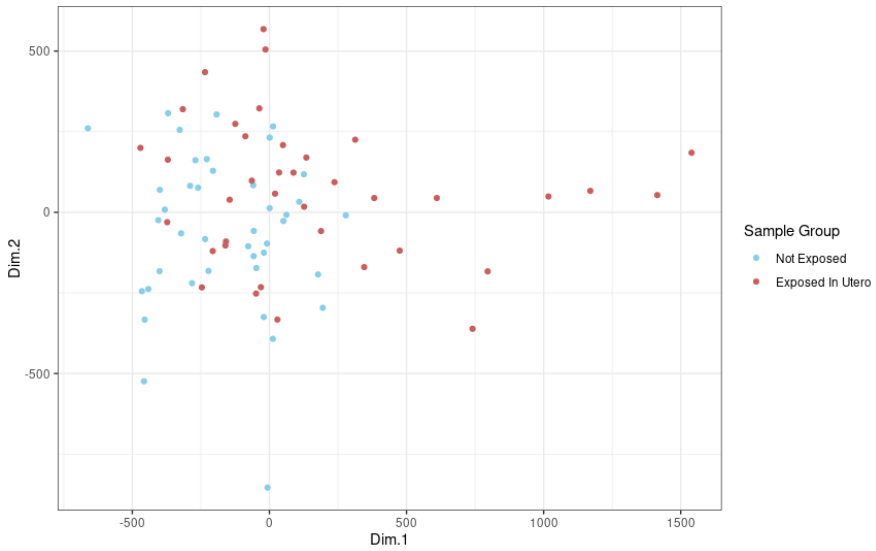
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History

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SUPPLEMENTARY DIGITAL MATERIAL 1



Supplementary Figure 1.—Principal component analysis: scatter plot of results of methylation probe across men "Exposed In Utero*"* to TCDD and "Not Exposed" referent men in the two principal dimensions.

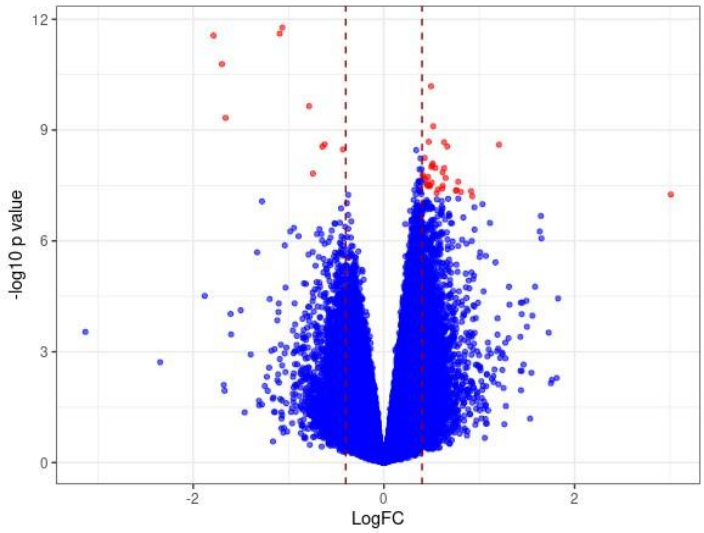
SUPPLEMENTARY DIGITAL MATERIAL 2

Supplementary Table I.—PCA covariates.

Dim.1	Dim.2	Dim.3
2.886888	-0.99078	0.909784
1.310968	1.609722	-1.152474
-1.897466	1.89334	-1.003348
-0.887766	-0.691757	-0.768881
4.334859	1.677648	0.876045
3.557097	0.196262	-1.14624
-1.25212	2.60954	-0.682937
4.764624	-1.644173	-0.793945
-0.559068	3.829702	-2.089479
1.058575	-1.847184	-0.839401
-0.402898	1.605129	-0.682072
1.679337	0.310861	0.846354
0.988542	1.95036	0.971496
2.242765	-0.419522	-0.830157
1.395516	1.300571	-0.943939
3.455837	1.080961	0.239959
-1.11781	-1.683382	-0.299781
2.341844	0.762746	-0.823942
0.023388	-0.948594	-0.546328
-1.044306	1.619756	0.553597
-1.496464	1.913043	0.139186
-1.114088	1.028337	-0.76896
3.562092	0.697823	1.80508
-0.993866	1.05631	-0.217277
3.697047	-0.323433	-0.196693
4.308143	1.218381	-0.743241
0.1519	-0.515553	-0.835056
0.478868	0.613601	-1.03732
0.987307	0.646161	-0.797474
1.369296	0.496889	-1.21214
1.451331	-0.455623	-1.244587
-0.59823	-1.510941	-0.108899
0.825176	2.641321	-0.149372
-0.071957	0.69211	0.092167
0.886229	-0.575638	-0.434711
0.087137	-1.085859	2.449245
2.261685	0.881201	-0.953988
-1.311639	-1.207911	1.875641
0.791827	0.413976	-0.486258
0.473049	1.43051	-0.716622
0.301172	1.529588	-0.289885
-1.831522	-0.89206	-0.912724
-2.971565	2.950931	0.135253
-0.552276	-0.598734	0.749036
1.145952	1.3302	2.677407
-1.201798	-2.000872	-0.576647
-3.232932	0.605058	-0.239357

-0.411145	0.674416	3.078775
-0.143365	-1.498529	-0.133373
-1.34335	-0.721108	-0.402862
-1.504903	1.19484	1.181363
1.232484	-1.035744	1.198729
-0.601901	-1.828892	-1.940955
-2.051722	0.818671	-1.467371
0.4008	-3.446103	0.490799
-0.102024	-1.366882	-0.215888
-1.700142	-1.620381	0.645921
-1.720161	-2.479927	-0.019662
-0.123478	-1.267059	-0.92007
-1.510887	-0.012035	1.007895
-2.841352	-0.24377	-1.165072
-0.64086	-1.423377	-0.276289
-1.827454	-3.29805	-0.433403
-3.413364	1.368371	-0.330447
-2.478968	-0.302007	-1.245278
-1.722045	0.630184	1.48906
-0.754279	-0.408136	2.613357
1.47837	-2.681642	-0.835742
-1.460462	1.530042	1.77272
-0.480164	-1.278647	0.336148
-3.425733	0.483813	-0.952831
-0.866465	0.93798	2.423786
1.441464	-1.635114	-0.804654
-0.527532	-2.504125	-0.57275
0.9054	-0.178459	2.552543
-1.965008	-0.184846	-0.227212
-1.000032	0.059606	-0.738449
-1.384715	-0.482483	1.746478
0.262318	-1.00063	2.348616

SUPPLEMENTARY DIGITAL MATERIAL 3



Supplementary Figure 2.—Volcano plot of differential methylation analysis at the probe level, between men “Exposed In Utero“ to TCDD *vs*“Not Exposed” referent men. Significant probes with a log fold change |LogFC| greater than 0.4 and after Bonferroni's correction for multiple tests are indicated in red.

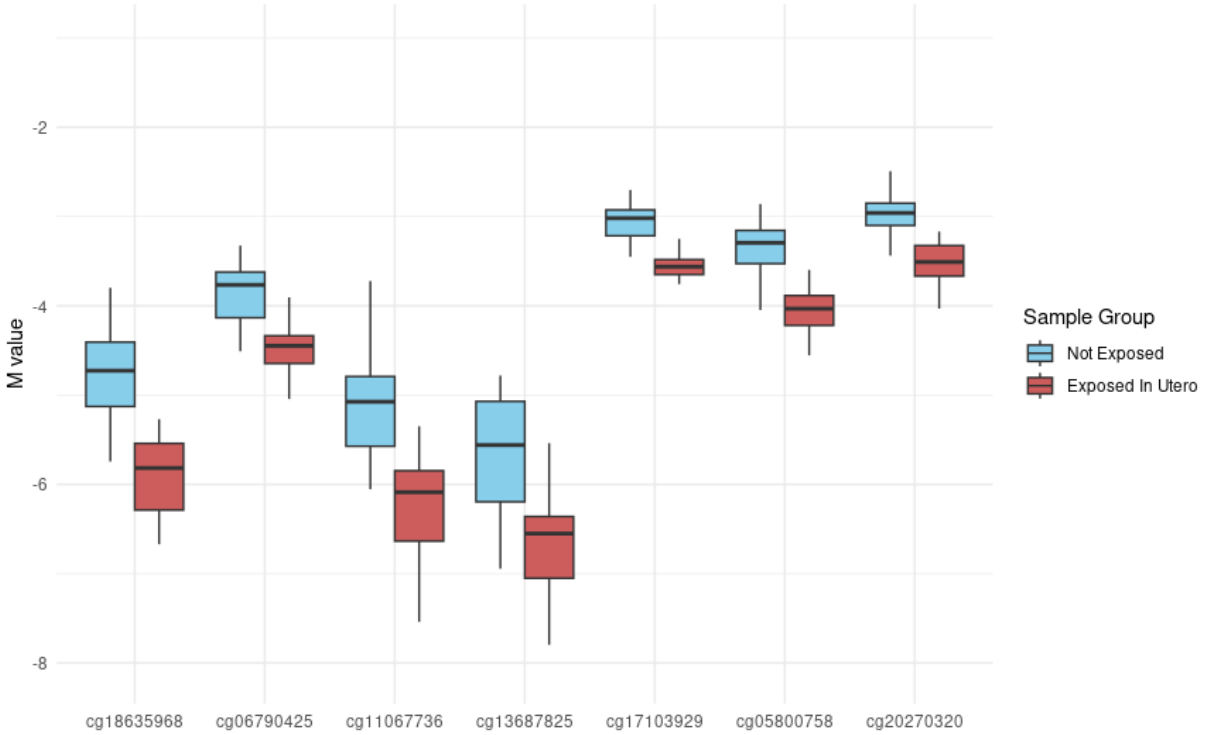
SUPPLEMENTARY DIGITAL MATERIAL 4

Supplementary Table II.—DMPs results.

chr	pos	strand	probes	Type	Gene Name	logFC	adj.P.Val
chr8	101225245	+	cg06790425	Island	SPAG1	-1.06468	1.28E-06
chr8	101225800	+	cg05800758	Island	SPAG1	-1.09333	1.86E-06
chr8	101225252	+	cg11067736	Island	SPAG1	-1.78695	2.09E-06
chr8	101225181	-	cg18635968	Island	SPAG1	-1.69785	1.24E-05
chr6	33577711	-	cg13775306	OpenSea		0.494155	4.93E-05
chr8	101225361	+	cg17103929	Island	SPAG1	-0.78318	0.000169
chr8	101225344	+	cg13687825	Island	SPAG1	-1.66018	0.000354
chr13	52419714	+	cg16096766	OpenSea		0.517858	0.000594
chr14	97492133	-	cg15418593	OpenSea		0.47002	0.001565
chr7	1654491	+	cg00631272	OpenSea		0.631413	0.001608
chr2	210825932	+	cg12622096	OpenSea	UNC80	-0.62027	0.00183
chr18	72146124	+	cg03835428	OpenSea		1.207584	0.001868
chr13	32659085	+	cg07776980	OpenSea	FRY	0.664387	0.002103
chr19	732849	-	cg05999731	N_Shelf		-0.64558	0.002111
chr7	25991107	+	cg15263946	Island		-0.42918	0.002526
chr1	1045506	+	cg26405545	N_Shore	C1orf159	0.339898	0.002629
chr3	188987241	+	cg21684532	OpenSea	TPRG1	0.428473	0.004263
chr8	22786360	-	cg01966160	OpenSea	PEBP4	0.383076	0.004404
chr5	135732754	+	cg20383841	OpenSea	AC063980.1	0.511789	0.006112
chr12	114138803	+	cg22857373	OpenSea	RP11-438N16.1	0.508173	0.00687
chr18	56238142	-	cg25045618	OpenSea		0.496923	0.007487
chr1	10601579	-	cg09165170	OpenSea	PEX14	0.539031	0.007978
chr2	112433296	-	cg21729825	OpenSea		0.633176	0.008094
chr8	116976019	-	cg09251168	OpenSea	LINC00536	0.395003	0.008625
chr15	40228471	+	cg01460585	S_Shore		0.37371	0.008724
chr12	116775313	-	cg21590782	OpenSea		0.616079	0.010439
chr8	101225902	+	cg20270320	S_Shore	SPAG1	-0.74398	0.011314
chr1	116250018	-	cg20810993	OpenSea		0.406148	0.012373
chr1	2329052	-	cg11070929	OpenSea	RER1	0.462987	0.013978
chr19	47461896	-	cg22914112	OpenSea		0.647733	0.015029
chr12	132887175	+	cg12510087	S_Shore	GALNT9	0.419395	0.015138
chr3	42886621	+	cg03628061	OpenSea	KRBOX1;RP11-70C1.3;CCBP2	0.439213	0.016595
chr15	40883950	+	cg05111383	N_Shelf		0.383055	0.017744
chr17	61767711	+	cg11776773	OpenSea		0.362941	0.018319
chr9	136538372	-	cg14313373	OpenSea		0.778672	0.018966
chr20	586425	-	cg24182304	N_Shelf		0.368151	0.018998
chr2	206586414	+	cg01355046	OpenSea	NRP2	0.395161	0.019574
chr16	50003457	+	cg09621708	OpenSea		0.50286	0.019962
chr3	160717062	+	cg02043033	OpenSea	PPM1L	0.345401	0.022428
chr15	91411495	+	cg23208881	N_Shelf	FURIN	0.449068	0.022658
chr10	82168743	+	cg16751764	Island	FAM213A	0.456884	0.023082
chr1	106765205	+	cg08433912	OpenSea		0.477006	0.023383
chr9	124506555	-	cg13537416	OpenSea	DAB2IP	0.616429	0.024377
chr8	11519192	+	cg03975306	OpenSea		0.46914	0.024563
chr5	172714232	-	cg24254552	S_Shelf		0.482105	0.0249
chr4	184367267	+	cg02109734	S_Shore	CDKN2AIP	0.368033	0.025178

chr10	128359945	-	cg26446974	OpenSea	C10orf90	0.6107	0.028919
chr10	33446364	-	cg01017800	OpenSea		0.567666	0.03079
chr4	23288013	+	cg07162914	OpenSea	RP11-453O5.1	0.319288	0.031671
chr1	147225220	-	cg10669374	OpenSea		0.363076	0.03192
chr5	139118687	-	cg11349797	N_Shore	CTB-35F21.1	0.762985	0.032202
chr13	67874129	-	cg02822908	OpenSea		0.754665	0.032648
chr12	49203017	-	cg02233606	OpenSea	Y_RNA	0.379045	0.033176
chr12	133015982	+	cg20973476	S_Shore		0.914746	0.034009
chr10	71122534	+	cg23610394	OpenSea	HK1	0.385446	0.035527
chr4	2009215	-	cg00679383	N_Shore	WHSC2	0.807649	0.035665
chr13	52419728	+	cg10390211	OpenSea		0.554717	0.038562
chr1	17694382	+	cg08978456	OpenSea		0.39368	0.041103
chr14	101069717	+	cg10829391	OpenSea		3.009428	0.041607
chr2	25966986	-	cg02562678	OpenSea		0.334532	0.042007
chr17	74100054	+	cg18586095	Island	EXOC7	-0.37407	0.042768
chr4	89829387	-	cg17065901	OpenSea	FAM13A	0.927156	0.046832

SUPPLEMENTARY DIGITAL MATERIAL 5



Supplementary Figure 3.—Methylation of DMR2 in men *in utero* exposed to dioxin: Boxplots showing the M values from the DMP analysis for the seven probes within the gene SPAG1 that showed a significant difference, at the individual level, between men “Exposed in Utero” to dioxin in Red, and “Not Exposed” men in Blue.

Notes: The M-value is calculated as the log2 ratio of the intensities of methylated probe

SUPPLEMENTARY DIGITAL MATERIAL 6

Supplementary Table III.—Significantly enriched biological processes identified by over representation analysis.

Gene Set GO (a)	PathwayDescription	Size (b)	Expect	Ratio	P-value (c) adj. (FDR)
GO:0048706	Embryonic skeletal system development	128	0.02	131.23	0.01
GO:0009952	Anterior/posterior pattern specification	218	0.03	77.05	0.01
GO:0030878	Thyroid gland development	31	0.01	361.25	0.03
GO:0003002	Regionalization	426	0.07	39.43	0.04
GO:0007389	Pattern specification process	470	0.08	35.74	0.04
GO:0001501	Skeletal system development	529	0.09	31.75	0.04
GO:0043009	Chordate embryonic development	661	0.11	25.41	0.07
GO:0009792	embryo development ending in birth or egg hatching	682	0.12	24.63	0.07
GO:0048704	Embryonic skeletal system morphogenesis	94	0.01	119.13	0.10
GO:0035270	Endocrine system development	137	0.02	81.74	0.17

(a): (GO): Gene Ontology; (b): (size): number of genes in the Pathway; (c): (FDR): false discovery rate.

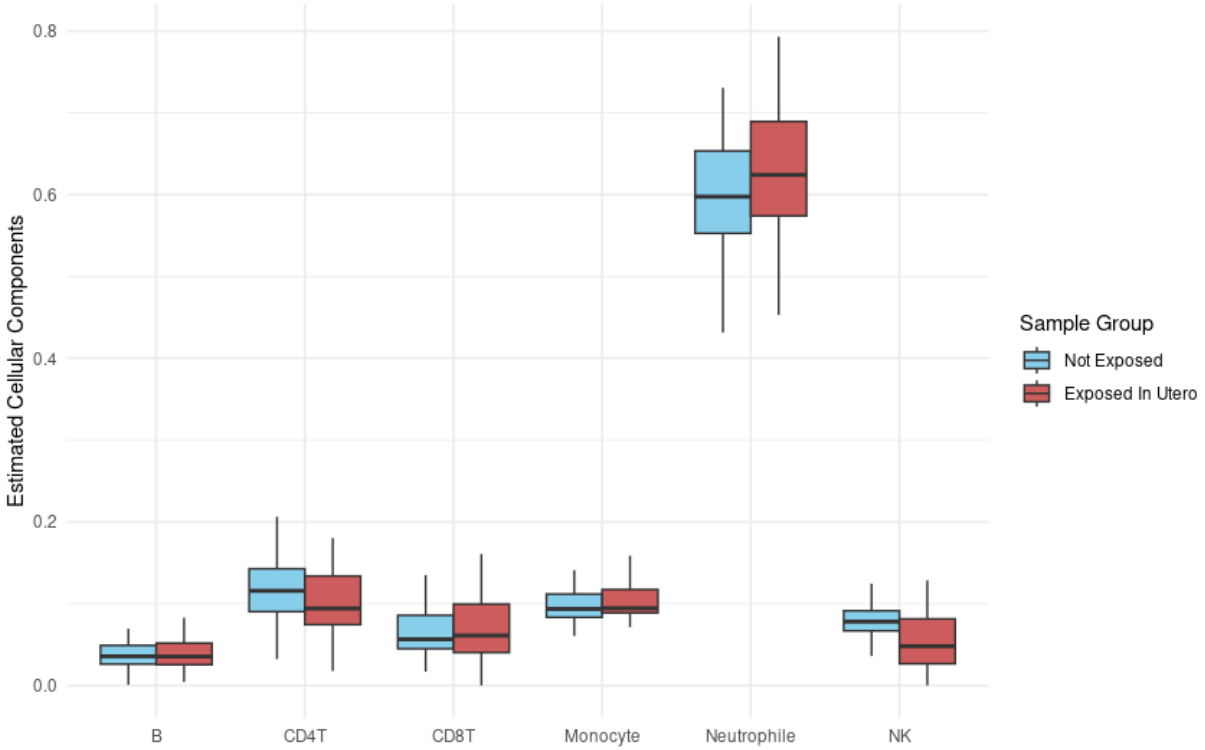
SUPPLEMENTARY DIGITAL MATERIAL 7

Supplementary Table IV.—Linear regression model results from estimated cellular components analysis.

Regression Model	Adjusted p-value	Beta Coefficients (CI 95%)
NK ~ Sample Group + Age	<0.01	-0.03 (-0.05, -0.01)
Neutrophil ~ Sample Group + Age	0.03	0.05 (0.01, 0.10)
CD4T ~ Sample Group + Age	0.11	-0.02 (-0.05, 0.01)
Monocyte ~ Sample Group + Age	0.38	0.01 (-0.01, 0.02)
B ~ Sample Group + Age	0.59	<-0.01 (-0.01, 0.01)
CD8T ~ Sample Group + Age	0.87	<-0.01 (-0.02, 0.02)

Notes. Regression Model Results for Blood Leukocytes Components in men “Exposed In Utero“ to TCDD *vs* “Not Exposed” referent men. Adjusted p-value are obtained by BenjaminiHoechberg multiple test correction. Significant results are reported in bold.

SUPPLEMENTARY DIGITAL MATERIAL 8



Supplementary Figure 4.—Immune Circulating Cells: boxplot of the estimated proportion of Immune Circulating Cells Components. Men "Exposed in Utero*"* to dioxin are represented in Red, and "Not Exposed" in Blue.
Notes. B: B lymphocytes, CD4T: helper T lymphocytes, CD8T: suppressor T lymphocytes, NK: natural killer cells.

SUPPLEMENTARY DIGITAL MATERIAL 9

Supplementary Table V.—Linear regression model results from age acceleration analysis.

Age Acceleration approaches (a)	p Value	Beta Coefficient (CI 95%)
Residual	0.10	-1.25 (-2.73, 0.22)
Hannum	0.69	-0.30 (-1.85, 1.23)
Grim	0.18	1.04 (-0.47, 2.57)
PhenoAge	0.69	0.44 (-1.77, 2.67)

Notes. (a): Aging process in men exposed *in utero* to dioxin *vs* referents were evaluated by different Age acceleration approaches: Residual, Hannum, Grim, and PhenoAge.Each metric was computed using Steve Horvath's website application from raw .idat data (https://dnamage.genetics.ucla.edu/home). To determine if there is a significant difference in these age acceleration parameters based on the origin of the sample group, regression models were employed, with the sample group as the independent variable and age acceleration as the dependent variable.