

EXPLORING THE LONG-TERM EPIGENETIC CONSEQUENCES OF PRENATAL DIOXIN EXPOSURE: AN EPIGENOME-WIDE ASSOCIATION STUDY WITHIN THE SEVESO COHORT EXPOSED IN UTERO

Sacco Davide^{1,3}, Brambilla Paolo^{5,6}, Calzari Luciano², Cavagnola Rebecca¹, Baldrighi Giulia Nicole¹, Costantino Lucy³, Ferrara Fulvio⁴, Signorini Stefano^{5,6}, Besana Silvia^{5,6}, Siracusa Claudia^{5,6}, Cattaneo Katia^{5,6}, Leoni Valerio^{5,6}, Mocarelli Paolo^{5,6}, Gentilini Davide^{1,2}

¹Department of Brain and Behavioral Sciences, Università di Pavia, 27100 Pavia, Italy

²Bioinformatics and Statistical Genomics Unit, Istituto Auxologico Italiano IRCCS, 20095 Cusano Milanino, Italy

³Medical Genetics Laboratory, Centro Diagnostico Italiano, 20147 Milan, Italy

⁴Integrated Laboratory Medicine Services, Centro Diagnostico Italiano, 20147 Milan, Italy

⁵Laboratory of Clinical Pathology and Toxicology, Hospital Pio XI of Desio, Azienda Socio Sanitaria Territoriale della Brianza (ASST-Brianza), 20832 Desio, Italy

⁶Department of Medicine and Surgery, University of Milano-Bicocca, 20126 Monza, Italy

Introduction: 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) is recognized as one of the most toxic substances within the dioxin family, a prominent class of endocrine disruptors widely spread in the environment and highly persistent in human tissues, with a half-life of approximately 7 years ^[1,2]. The evidence of the damage that this substance can cause to human health of directly exposed individuals and their offsprings is derived from studies conducted on the victims of the Seveso incident, where on July 10, 1976, a massive amount of TCDD was released from the ICMESSA chemical plant due to a temperature regulation accident ^[3]. Prenatal exposure has been linked to thyroid dysfunction, impaired glucose regulation, and male reproductive problems ^[4,5]. Epigenetics, which involves mechanisms that regulate gene expression and are influenced by environmental factors, may play a significant role in these outcomes ^[6]. However, the specific epigenetic changes in individuals exposed to TCDD in utero, where gene expression and epigenetic processes exert significant influence, remain largely unknown. The primary epigenetic marker used for monitoring the environment's effects on the epigenome is DNA methylation due to its ease of acquisition through array technology and its ability to be dynamically shaped by environmental factors. ^[7]

Aim: The study, categorized as a retrospective observational study, aims to explore the presence of epigenetic alterations caused by TCDD exposure on the offspring of mothers who were exposed during the Seveso incident and became pregnant a few years after this event. Our research compared DNA methylation patterns in blood samples collected from 38 adult men (median 22.5 +/- 2.2 years) whose mothers had been exposed to high doses of TCDD (median serum level of 52.0 ppt) and were therefore exposed in utero to a median of 24.7 ppt, with samples from 41 unexposed matched men.

Methods: The obtaining of DNA methylation patterns was performed on whole blood samples using the Illumina Methylation EPIC array, which utilizes specific probes to measure the methylation intensity of 866'000 CpG sites due to their association with gene expression. Raw methylation data underwent quality control and preprocessing using R, where the Champ package was employed to remove probes with poor or unclear signal quality and to reduce potential instrument error. ^[8,9]

As an initial analysis, to assess the differences between the two groups, we conducted a differential methylation analysis at the probe level using the Limma package. This analysis compared whether there were differences in the mean methylation values between the two groups by applying hierarchical linear models with methylation values per probe as the outcome and Sample Group as the independent predictor ^[10]. Additionally, the model was fixed by including the first three principal components as

covariates, which contained information about chronological age, biological age, and immune cell composition. The obtained p-values were corrected for multiple testing using Bonferroni correction, and differentially methylated probes were considered significant with adjusted p-value < 0.05. Subsequently, using smoothing techniques, integrated in the DMRcate package we investigated differentially methylated genomic regions, considering regions significant if they had a Fisher p-value < 0.05 [11].

In the second analysis, we studied Stochastic Epigenetic Mutations (SEMs), defined as extreme outlier values relative to a reference population, using the entire study population as the reference [12]. Probes were considered SEMs if they exhibited values greater than three times the interquartile range above the third quartile or below three times the interquartile range below the first quartile. We then established the presence of an association between the burden of SEMs and the sample group using linear regression models evaluating significant associations with a p-value < 0.05.

Finally, to assess potential implications of in utero TCDD exposure on early aging, we utilized age acceleration as a marker. Age acceleration is defined as the disparity between chronological age and biological age, with the latter inferred through deconvolution algorithms [13]. We then used linear models to evaluate the association between the age acceleration and Sample Group, considering the association significant if the p-value was < 0.05.

Results: The study identified 62 differentially methylated probes and 2 gene regions, highlighting hypomethylation of the SPAG1 gene and slight hypermethylation of a region associated with HOX box family genes. Pathway analysis revealed associations with thyroid and skeletal development confirming the existing literature. Subsequently, focus turned to SPAG1 due to its role in sperm functions, suggesting a mechanism through which TCDD exposure may affect spermiogenesis [14,15,16,17].

Association of methylation values of this gene with seminal parameters showed that increased gene hypomethylation intensity corresponded to an increase in spermatozoa's angular neck toward a pathological threshold in individuals exposed in utero. Furthermore, through the study of SEMs, we found an increase of the mutations in exposed individuals compared to unexposed ones, suggesting an accumulation of potentially harmful epigenetic mutations for health [18]. However, no significant associations were found between age acceleration and exposure to TCDD.

Conclusion: The study identified epigenetic alterations potentially involved in phenotypic aspects highlighted in the literature, suggesting mechanisms potentially linked to male infertility.

Additionally, further studies are needed to understand the consequences of the connection between the increase in SEMs on health.

Bibliography

1. L S Birnbaum., The mechanism of dioxin toxicity: relationship to risk assessment. en. In: Environ. Health Perspect, Nov 1994; 102 Suppl 9, pp. 157 – 167
2. Kerger BD., Leung HW., Scott P. et al., Age and concentration-dependent elimination half-life of 2,3,7,8-tetrachlorodibenzo-p-dioxin in Seveso children. Environ Health Perspect, 2006 Oct; 114(10):1596-602.
3. Eskenazi B., Warner M., Brambilla P. et al., The Seveso accident: A look at 40 years of health research and beyond. Environ Int, 2018 Dec;121(Pt 1):71-84.
4. Mocarelli P., Gerthoux PM., Patterson DG Jr. et al., Dioxin exposure from infancy through puberty, produces endocrine disruption and affects human semen quality. Environ Health Perspect, 2008 Jan; 116(1): 70-7.
5. Warner M., Rauch S., Brambilla P. et al., Prenatal dioxin exposure and glucose metabolism in the Seveso Second Generation study. Environ Int, 2020 Jan;134:105286.
6. Torano EG., García MG., Fernández-Morera JL. et al., The Impact of External Factors on the Epigenome: In Utero and over Lifetime. Biomed Res Int, 2016 May; 2016:2568635.
7. Colwell ML., Townsel C., Petroff RL. et al., Epigenetics and the Exposome: DNA Methylation as a Proxy for Health Impacts of Prenatal Environmental Exposures. Exposome. 2023;3(1):osad001.
8. Tian Y., Morris T.J., Webster A.P. et al., ChAMP: updated methylation analysis pipeline for Illumina BeadChips. Bioinformatics 33, 2017 Dec; 33(24):3982-3984.
9. Welsh H., Batalha C.M.P.F., Li W. et al., A systematic evaluation of normalization methods and probe replicability using infinium EPIC methylation data. Clin Epigenetics, 2023 Mar; 15(1):41.
10. Ritchie M.E., Phipson B., Wu D. et al., limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res, 2015 Apr; 43(7):e47.
11. Peters T.J., Buckley M.J., Statham A.L. et al., De novo identification of differentially methylated regions in the human genome. Epigenetics Chromatin, 2015 Jan; 27;8:6.
12. Gentilini D., Garagnani P., Pisoni S. et al., Stochastic epigenetic mutations (DNA methylation) increase exponentially in human aging and correlate with X chromosome inactivation skewing in females. Aging, 2015 Aug; 7(8):568-78.
13. Horvath S., DNA methylation age of human tissues and cell types. Genome Biol, 2013; 14(10):R115.
14. Kanazawa R., Komori S., Sakata K. et al., Isolation and characterization of a human sperm antigen gene h-Sp-1. Int J Androl, 2003 Aug; 26(4):226-35
15. Faraji S., Sharafi M., Shahverdi AH et al., Sperm Associated Antigens: Vigorous Influencers in Life. Cell J., Oct 2021; 23(5):495-502.
16. Faiad W., Soukkarieh C., Murphy D.J., et al., Effects of dioxins on animal spermatogenesis: A state-of-the-art review. Front Reprod Health,2022 Oct; 4:1009090.
17. Mocarelli P., Gerthoux, P.M., Needham, L.L., et al., Perinatal Exposure to Low Doses of Dioxin Can Permanently Impair Human Semen Quality. Environ Health Perspect., 2011 May; 119(5):713-8
18. Martin G.M., Epigenetic drift in aging identical twins. Proc Natl Acad Sci, 2005 Jul; 102(30):10413-4.