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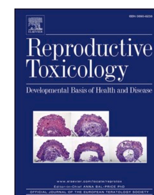

Epigenetic modifications in the ferroptosis pathway in cord blood cells from newborns of smoking mothers and their influence on fetal growth

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ABSTRACT

Maternal smoking during pregnancy increases oxidative stress and decreases antioxidant capacity in newborns. Uncontrolled oxidative stress plays a role in fetal development disorders and in adverse perinatal outcomes. In order to identify molecular pathways involved in low fetal growth, epigenetic modifications in newborns of smoking and non-smoking mothers were examined. Low birth weight newborns of mothers who smoked more than 10 cigarettes per day during the first trimester of pregnancy and normal birth weight newborns of mothers who did not smoke during pregnancy were included in the study. DNA was extracted from umbilical cord blood of term newborns. 125 differentially methylated regions were identified by MeDIP-Seq. Functional analysis revealed several pathways, such as ferroptosis, that were enriched in differentially methylated genes after prenatal smoke exposure. GPX4 and PCBP1 were found to be hypermethylated and associated with low fetal growth. These epigenetic modifications in ferroptosis pathway genes in newborns of smoking mothers can potentially contribute to intrauterine growth restriction through the induction of cell death via lipid peroxidation of cell membranes. The identification of epigenetic modifications in the ferroptosis pathway sheds light on the potential mechanisms underlying the pathophysiology of low birth weight in infants born to smoking mothers.

1. Introduction

Maternal smoking during pregnancy is associated with fetal growth restriction although its pathophysiological mechanisms currently remain unknown. There are more than 4000 chemical components in tobacco (more than 40 of which are carcinogenic), as well as benzopyrenes, nicotine, and carbon monoxide [1,2]. Nicotine crosses the placenta, and its concentration is 15% higher in fetal than in maternal blood [3]. Cigarette consumption is particularly harmful to the epigenome during the first trimester of gestation, and even more if it is maintained, and is dose dependent [4], even though isolated exposures can also have effects on the long-term development of newborns [5].

Smoking during pregnancy has important effects on fetal growth. Thus, several studies have shown that maternal smoking during pregnancy could decrease birth weight and significantly increase the risk of low birth weight and preterm births, also showing a dose dependent and time-specific association [6].

Maternal smoking could affect growth and development of the embryo, fetus, and newborn as a result of DNA epigenetic modifications. These modifications can lead to changes in the gene expression regulating cell pathways involved in the function of the different fetal organs and tissues [7,8]. Indeed, in utero exposure to tobacco smoke has been related with changes in DNA methylation of genes associated with growth restriction [9].

During prenatal life, cell proliferation and differentiation are very sensitive to any change that is harmful to the environment. Such changes can lead to permanent structural and functional alterations that may then persist into adult life. Compelling evidence shows that the period of development from conception, pregnancy, birth, and infancy is a key window of sensitivity for the early interactions between the genome and the environment with lifelong consequences. During early development, environmental stimuli determine not only short term but also lifelong and trans-generationally heritable effects due to epigenetic programming. Long lasting epigenetic imprinting is currently considered to be a

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form of molecular "memory", explaining why and how health and disease over the course of a person's life have developmental origins [10, 11]. These epigenetic changes could be active in adulthood even if tobacco consumption ceases, thereby increasing the risk of diseases such as diabetes, cardiovascular illness, and metabolic syndrome [12,13].

Maternal smoking has been associated with other obstetric issues [14] and childhood illnesses such as sudden infant death, reduced lung function, asthma, allergies, type II diabetes mellitus, otitis media, cancer, and neurocognitive alterations [2,5,15]. All these conditions are probably related to epigenetic modifications during the first few weeks of gestation. Indeed, the embryo is strongly sensitive to external factors such as undernutrition or tobacco, both of which can modify the DNA methylation patterns in a wide variety of embryo tissues. These epigenomic changes can have a detrimental effect on health in the subsequent development of the child [16,17].

Smoking pregnant women and their newborns have a higher concentration of oxidative stress parameters [18–20]. In this regard, some authors have revealed that cigarette smoking increases lipid peroxidation and decreases antioxidant capacity in umbilical cord blood cells, as they found lower levels of reduced glutathione (GSH) and glutathione peroxidase (GPX) in the cord blood cells from newborns of smoking mothers [21].

Smoking has been reported to decrease GSH concentration and the antioxidant enzyme glutathione peroxidase [22,23] and to increase reactive oxygen species (ROS). Uncontrolled oxidative stress (OS) can play a key role in fetal development disorders and contributes to adverse perinatal outcomes in newborns of smoking mothers with intrauterine growth restriction (IUGR). Given the epigenetic nature of such disorders, the aim of our study was to identify key genes epigenetically modified by in utero tobacco smoke exposure in small newborns, and the molecular pathways involved.

2. Materials and Methods

2.1. Samples

Samples were collected at the Lozano Blesa University Clinical Hospital in 2018 and 2019. DNA was extracted from the umbilical cord blood of 10 newborns according to the following inclusion criteria:

1. Five newborns exposed to tobacco smoke: children of mothers who smoked more than 10 cigarettes per day during the first trimester of pregnancy and with low birth weight.
2. Five newborns not exposed to tobacco smoke: children of mothers who did not smoke during pregnancy with adequate birth weight [24,25].

Exclusion criteria were:

1. Maternal diseases that may cause intrauterine growth restriction
2. Preeclampsia
3. Uterine infections
4. Fetal malformations
5. Chromosomal alterations
6. Preterm labor
7. Not signing the informed consent

2.2. Procedure

2.2.1. Maternal and Newborn Characteristics

Maternal sociodemographic characteristics (age, race, nationality, employment status, educational level), smoking and drug use (including alcohol) during pregnancy were assessed using an interview administered questionnaire.

Clinical data on the course of pregnancy, gestational age, mode of delivery, placental weight, condition of the newborn— including sex, birth weight, head circumference and Apgar score—were collected from medical records.

2.2.2. Blood Collection and DNA Extraction

Cord blood samples were collected in EDTA tubes (BD Vacutainer™ Hemogard™ Closure Plastic K3-Edta Tube, Fisher Scientific SL, Spain) by hospital providers. The samples were stored at 4ºC until they could be transported to the molecular laboratory at the University of Zaragoza School of Medicine, where they were processed. DNA was isolated using Norgen's Blood DNA Isolation Midi Kit (Cat. 51400) (Norgen Biotek Corp., Canada).

2.2.3. Library Preparation

After extraction and purification, DNA samples were sonicated to a size range of 200–800 bp using a Diagenode Bioruptor. About 1 ug of fragmented DNA was prepared for Illumina HiSeq 4000 (Illumina, Inc San Diego, EEUU) sequencing as follows:

1) End repair of DNA samples;
2) A single 'A' base was added to the 3'-ends;
3) Ligation of Illumina's genomic adapters to DNA fragments;
4) MeDIP to enrich methylated DNA using the anti-5-methylcytosine antibody;
5) PCR amplification to enrich precipitated fragments;
6) Size selection of 300–900 bp DNA fragments using AMPure XP beads.

Quality control (QC) was performed to assess the quality of the MeDIP experiment and sequencing library. The samples were diluted to a final concentration of 8 pM and a cluster was generated on the Illumina cBot.

The completed libraries were quantified using an Agilent 2100 Bioanalyzer.

Reagents: Genomic DNA Sample Kit (#FC-102–1002, Illumina) Diagenode® Anti-5-Methylcytosine antibody Equipment: NanoDrop ND-1000

2.2.4. Sequencing

The libraries were denatured with 0.1 M NaOH (Sodium Hidroxyde, 500 mg, reagent grade, ≥98%, pellets (anhydrous), Merck KGaA, Darmstadt, Deutschland) to generate single-stranded DNA molecules, which were captured on an Illumina flow cell and amplified in situ. The libraries were then sequenced on the Illumina HiSeq 4000 following the HiSeq 3000/4000 SBS Kit (300 cycles) protocol. Image analysis and base calling were performed using Off-Line Basecaller software (OLB V1.8) after generation of the sequencing images. Reagents: HiSeq 3000/4000 PE Cluster Kit (#PE-410–1001, Illumina) HiSeq 3000/4000 SBS Kit (300 cycles) (#FC-410–1003, Illumina)

2.2.5. Data Analysis

The raw sequencing data generated using the Illumina HiSeq 4000 were analyzed as follows. After passing the Solexa CHASTITY quality filter, the clean reads were aligned to the human genome (UCSC hg19) using Hisat2/2.1.0 software. Aligned reads were used for peak calling, and statistically significant mRNA-associated MeDIP-enriched regions (peaks) were identified for each sample using a q-value threshold of 10–4 and the MACS2 software [26,27].

2.2.6. Gene Ontology (GO) Analysis

The gene ontology analysis covered three domains, namely biological process, cellular component and molecular function [28]. Fisher's exact test was used to determine whether there was more overlap between the DE list and the GO annotation list than would be expected by chance. The p-value denotes the significance of GO terms enrichment in the DE genes.

2.2.7. Pathway Analysis

Pathway analysis is a functional analysis mapping of genes to KEGG pathways [29]. The p-value (EASE-score, Fisher-Pvalue or Hypergeometric-Pvalue) denotes the significance of the pathway correlated to the conditions.

2.2.8. Real-Time qPCR for Expression Validation

First, methylated DNA was immunoprecipitated and real-time qPCR was performed (Arraystar, Inc.

Rockville, Maryland 20850. USA).

MeDIP -qPCR Data Analysis (△△Ct method):

a) To account for chromatin sample preparation differences, the MeDIP DNA fraction CT values were normalized to Input DNA fraction CT values for the same qPCR Assay (ΔCt). The % Input for each MeDIP fraction was calculated: %Input=2(Ct Input-Ct MeDIP)×Fd×100%, where Fd is Input dilution factor. Fold Enrichment = [%(MeDIP/Input)] / [%(Negative control/Input)]

b) The normalized MeDIP fraction Ct value was adjusted for the normalized back-ground (mock IP) fraction Ct value. ΔΔCt [MeDIP /mock IP] = ΔCt [normalized MeDIP] - ΔCt [normalized mock IP] [26, 27].

3. Results

3.1. Characteristics of study participants

All mothers were from Spain of Caucasian race/ethnicity. None of them used alcohol or other drugs during pregnancy. Smoking mothers consumed a mean of 12.60 (SD 2.51) cigarettes per day. The average age of all mothers at delivery was 33.14 years (SD 5.14) (31.51 years (SD 5.97) for smoking mothers and 34.78 years (SD 4.26) for non-smoking mothers ($p=0.348$)). The gestational age was similar in both groups (40 weeks (SD 1.41) for smoking mothers and 39.27 weeks (0.79) for non-smoking mothers ($p=0.349$)). All non-smoking mothers worked during pregnancy, compared with 40% of smoking mothers ($p=0.038$)

Children of smoking mothers had a lower mean birth weight (2688 g (SD 98.15)) than those of non-smoking mothers (3582 g (SD 562.81)) ($p=0.008$). All newborns exposed to in utero tobacco smoke were diagnosed with IUGR (birth weight <10th percentile). The percentage of girls and boys was similar in both groups, with 40%/60% in the non-smoking group and 60%/40% in the smoking group ($p=0.527$). The 5-minute Apgar score was higher than 7 points in all cases and did not differ significantly between the studied groups ($p=0.545$). Table 1 presents the main clinical characteristics of the sample.

3.2. Identification of Differentially DNA Methylated Regions in Promoters

To identify new candidate genes epigenetically modified by tobacco and related to low birth weight, genome-wide DNA extracted from umbilical cord blood of term newborns was compared between smoking and non-smoking mothers.

A total of 125 differentially hypermethylated sequences were identified in promoter regions (supplementary table S1). Table 2 shows a summary of some of the hypermethylated DMR-associated genes. Fig. 1 illustrates a volcano plot presenting the differentially hypermethylated genes in low birth weight newborns of smoking mothers vs normal birth weight newborns of non-smoking mothers.

3.3. GO Functional Analysis

To examine possible cell components and processes associated with tobacco exposure and low birth weight, differentially hypermethylated genes were studied using a GO functional analysis. The enrichment analysis is summarized in Table 3.

A total of 77 cellular components, 307 biological processes and 68 molecular functions were enriched among differentially hypermethylated genes. According to these results, cytosolic ribosomes, the structural constituent of ribosomes, and SRP-dependent co-translational protein targeting to membrane translation were the most enriched terms in their respective categories.

3.4. KEGG Enrichment Analysis

Functional enrichment analysis revealed several pathways that were enriched in differentially methylated genes by prenatal smoke exposure. Table 4 shows the pathways identified using KEGG. These pathways included ribosome (42 genes), legionellosis (4 genes), ferroptosis (3 genes), antigen processing and presentation (4 genes), RNA transport (6 genes), and the spliceosome (5 genes).

These findings highlight a pathway that could be potentially affected by prenatal smoke exposure and related to fetal growth restriction, specifically ferroptosis, with three hypermethylated genes involved (GPX4, FTL and PCBP1 (Fig. 2)). Due to its apparent broad impact on different pathways, we aim to continue further analysis and studies on ferroptosis, given its possible relationship with iron intake during pregnancy and the currently available knowledge about this emerging phenomenon.

3.5. Validation of Methylation Status

Immunoprecipitation and qPCR confirmed that the two candidate genes, GPX4 and PCBP1, were differentially hypermethylated in small for gestational age smoking exposed vs. adequate gestational age non-tobacco exposed newborns ($p=0.0004$ and $p=0.002$ respectively). FTL was discarded for further analysis due to lack of significance in this step ($p=0.18$).

These two hypermethylated genes validated in the ferroptosis pathway are discussed below.

GPX4: Glutathione peroxidase 4

The family of selenium dependent peroxidases includes the phospholipid hydroperoxide glutathione peroxidase (GPX4), which was initially reported in 1982. Unlike the classical cellular glutathione peroxidase, GPX1, GPX4 is a monomeric enzyme that exists in two distinct forms: a mitochondrial form of approximately 22 kd and a non-

Table 1
Main clinical characteristics of the sample.

Sex	Smoking	Gestational Age (week+day)	Weight (g)	APGAR test	Delivery	Height (cm)	Head circumfe-rence (cm)	Placental weight (g)
F	Yes	39+2	2680	10	Vaginal	46.5	32.5	420
M	Yes	38	2600	10	Cesarean	50	33	530
M	Yes	39+1	2725	9	Vaginal	49	33	465
M	Yes	40	2835	10	Vaginal	50	33.5	480
F	Yes	39+6	2600	10	Vaginal	50	33	510
F	No	39	2885	10	Vaginal	49	34.5	520
M	No	38	3070	10	Vaginal	51	34	515
F	No	41	4005	9	Vaginal	51	34	635
F	No	41	4100	10	Cesarean	51	35	700
M	No	41	3850	9	Cesarean	51.5	35.5	525

Table 2
Sumary of differentially hypermethylated promoter regions in newborn from smoking mothers.

GeneName	DMR_To_TSS	DMR_Locus	DMR_ Length	log2FC	p-value	q-value
RPS29	-176	chr14:50053121–50053500	379	6.15	4.1371E-08	0.00035558
RPS12	1723	chr6:133137281–133137580	299	5.95	3.2742E-08	0.00034868
FTL	1105	chr19:49469481–49469860	379	5.43	9.1329E-08	0.00039162
RPS27A	1227	chr2:55460861–55461220	359	5.19	4.93E-08	0.00035922
RPL41	797	chr12:56511001–56511340	339	5	1.5051E-07	0.00047901
RPL15	55	chr3:23959061–23959440	379	4.99	3.6564E-08	0.00034868
RPLP1	-78	chr15:69744901–69745260	359	4.9	5.3224E-08	0.00036263
RPL27	1335	chr17:41151601–41151960	359	4.87	5.3488E-08	0.00036263
RPL36	795	chr19:5690961–5691320	359	4.8	5.7902E-08	0.00036391
EEF1A1	1265	chr6:74229281–74229700	419	4.45	1.4677E-07	0.00047744
HNRNPA1	533	chr12:54674841–54675200	359	4.06	4.4342E-08	0.00035922
PABPC1	335	chr8:101733821–101734140	319	2.96	1.0353E-07	0.00041271
HSP90AA1	1062	chr14:102552261–102552640	379	2.96	7.7764E-08	0.00038222
EIF1	1784	chr17:39846761–39847060	299	2.92	7.0445E-08	0.00037246
RGPD5	577	chr2:113190341–113190720	379	2.67	6.1643E-07	0.00129708
HNRNPU	717	chr1:245027001–245027220	219	2.55	3.5952E-05	0.03609227
HSPA1B	949	chr6:31795661–31797260	1599	2.52	2.1973E-06	0.00362391
PCBP1	916	chr2:70315321–70315680	359	2.49	8.9152E-08	0.00039162
HSPA1A	990	chr6:31783441–31785120	1679	2.48	5.2049E-06	0.00751118
HLA-E	1888	chr6:30458941–30459200	259	2.33	2.4445E-07	0.0006399
GPX4	1672	chr19:1106161–1106480	319	1.77	9.1747E-05	0.07109925
SMN2	-799	chr5:69344221–69344880	659	1.24	6.3802E-05	0.05534301
SMN1	-799	chr5:69344221–69344880	659	1.24	6.3802E-05	0.05534301

DMR, Differentially Methylated Regions. **DMR_To_TSS**, the distance of DMR (middle position) to TSS (Transcription Start Site). Positive value indicates downstream, and negative value indicates upstream. **DMR_Locus**, the genomic locus of DMR. **DMR_Length,** the length of DMR. **log2FC**, Fold Change of normalized tag counts between two groups (log2 transformed). **p_value**, p-value of the DMR. **q-value**, BH FDR-corrected p-value.

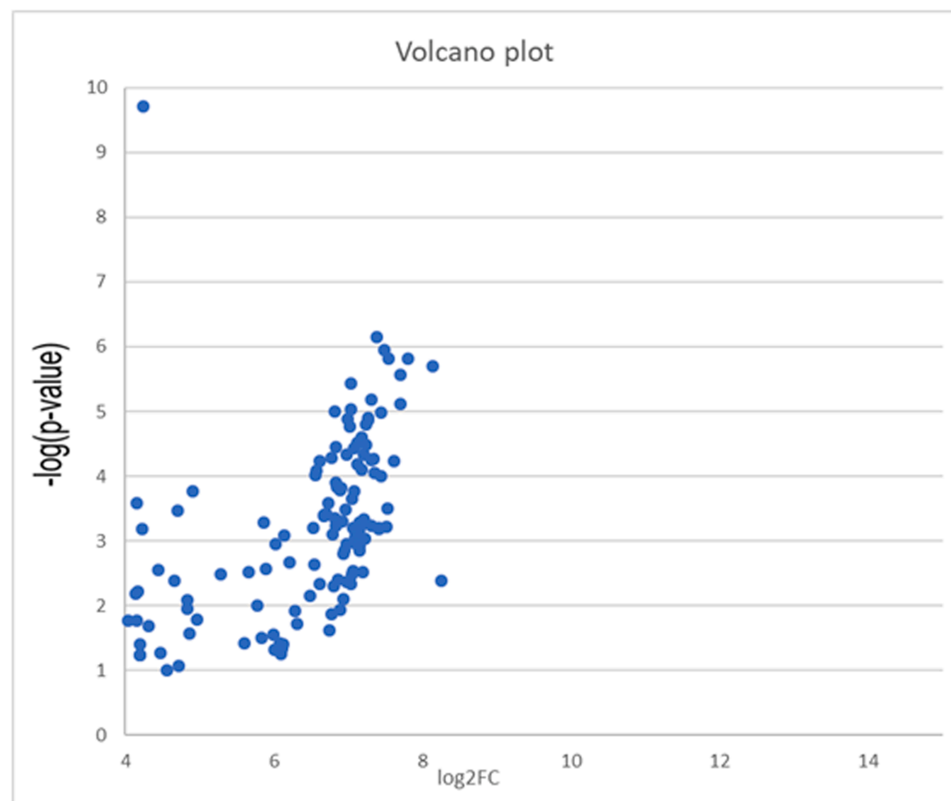

Fig. 1. Volcano plot of differentially hypermethylated genes in low birth weight newborns of smoking mothers vs normal birth weight newborns of non-smoking mothers (log2FC≥4, -log(p-value)≥1).

mitochondrial form of approximately 19 kd. GPX4 is prominently expressed in the testes and thyroid [30].

The GPX4 gene encodes a protein that facilitates the reduction of hydrogen peroxide, organic hydroperoxides, and lipid hydroperoxides, thereby safeguarding cells against oxidative stress. This protein exhibits a strong affinity for lipid hydroperoxides and shields cells from membrane lipid peroxidation and cell death. Various transcript variants of this gene, resulting from alternative splicing or alternate promoter usage, have been identified to encode isoforms with distinct subcellular localization.

Table 3
Summary of most enrichment terms using Go functional analysis.

Ontology	Term	Number of DE genes associated	Number of total genes associate	P value
Cellular Component	Cytosolic ribosome	42	127	2.85E-66
Cellular Component	Ribosomal subunit	42	197	4.46E-57
Cellular Component	Ribosome	43	249	3.20E-54
Molecular Function	Structural constituent of ribosome	41	172	2.76E-57
Molecular Function	Structural molecule activity	47	766	2.43E-37
Molecular Function	RNA binding	60	1879	4.36E-33
Molecular Function	Nucleic acid binding	66	4386	5.06E-18
Molecular Function	Organic cyclic compound binding	71	6436	2.62E-12
Biological Process	SRP dependent cotranslational protein targeting to membrane	40	93	2.57E-68
Biological Process	Cotranslational protein targeting to membrane	40	99	6.27E-67
Biological Process	Translational initiation	43	143	1.70E-65
Biological Process	Protein targeting to ER	40	106	1.92E-65

Table 4
Pathways identified using KEGG in genes differentially hypermethylated.

Term	p_value	Genes
Ribosome	<0.001	RPL10//RPL13//RPL14//RPL15//RPL17-C18orf32//RPL18A//RPL19//RPL23//RPL23A//RPL26//RPL27//RPL27A//RPL28//RPL3//RPL30//RPL31//RPL35A//RPL36//RPL36AL//RPL37//RPL37A//RPL41//RPL7A//RPL8//RPLP1//RPS10//RPS11//RPS12//RPS15//RPS17//RPS2//RPS21//RPS23//RPS24//RPS25//RPS26//RPS27//RPS27A//RPS29//RPS7//RPS8//RPSA
Ferroptosis	0.011	FTL//GPX4//PCBP1
Antigen processing and presentation	0.012	HLA-E//HSP90AA1//HSPA1A//HSPA1B
RNA transport	0.014	EEF1A1//EIF1//PABPC1//RGPD5//SMN1//SMN2
Spliceosome	0.019	HNRNPA1//HNRNPU//HSPA1A//HSPA1B//PCBP1

PCBP1: t(poly-(rC)-binding protein)

This intronless gene is thought to have been generated by retrotransposition of a fully processed PCBP-2 mRNA. This gene, along with PCBP-2, has paralogs (PCBP3 and PCBP4) that are believed to have originated through gene duplication. The protein product of this gene appears to have multiple functions and has been observed to contribute to the formation of a sequence specific alpha-globin mRNP complex, which is linked to the stability of alpha-globin mRNA [31]

4. Discussion

Our study found hypermethylation of the genes GPX4 and PCBP1 in cord blood cells obtained from fetuses of smoking mothers in the fetal growth restriction group. These genes are involved in intracellular iron homeostasis, which could be altered and lead to excessive ferroptosis, and cell death mediated by lipid peroxidation of cell membranes.

Ferroptosis is a type of cell death mediated by the iron-dependent lipid peroxidation of cell membranes, which is caused by excess iron. Iron is essential for many biological processes within all cells, including energy production and the correct functioning of DNA. The term “ferroptosis” was first used in 2012 to describe a mechanism of iron-mediated programmed cell death that is distinct from other known

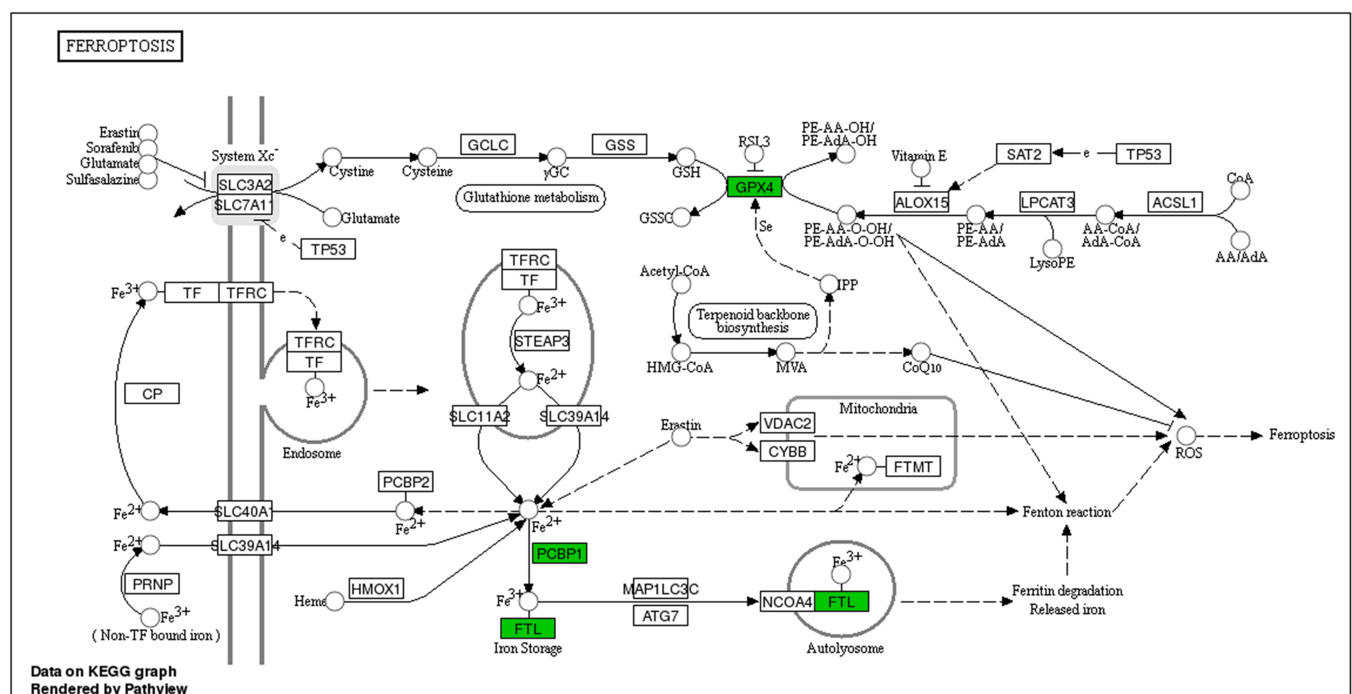

Fig. 2. Ferroptosis pathway. Green marked nodes are hypermethylated genes in small new born from smoking mothers, white nodes have no significance in methylation between two groups.

forms of apoptosis [32,33]. Several publications have since confirmed the existence of ferroptosis and characterized its impact on both health and disease [34,35].

An increasing body of evidence suggests that epigenetic regulation modulates the expression levels of ferroptosis-associated genes, thereby contributing to the determination of cell sensitivity to ferroptosis. Ferroptosis helps to maintain tissue homeostasis under physiological conditions, while its aberration is closely linked with numerous pathophysiological processes, such as acute tissue injury, chronic degenerative disease, and tumorigenesis [36]. In adult tobacco-related diseases, such as chronic obstructive pulmonary disease (COPD), an increase in lipid peroxidation and in induction of ferroptosis is also observed [37]. Hypermethylation of genes involved in this pathway protects against COPD-related oxidative stress and inflammation in the lung and inhibits GPX4 [38]. The selenium-dependent GPX4 inhibits ferroptosis by converting unstable ferroptotic lipid hydroperoxides into nontoxic lipid alcohols in a tissue-specific manner. In other organs, such as the human cornea, extensive nicotine consumption has been found to induce oxidative stress, and cigarette smoke to promote ferritin cleavage and iron accumulation in human corneal epithelial cells [39].

Ferroptosis has been associated with some of the main obstetric syndromes, includ-ing preeclampsia [33,40,41], gestational diabetes [42], and preterm birth [43]. Moreover, excessive iron intake and high iron status can be detrimental to pregnancy and have been associated with reproductive disorders such as preeclampsia and fetal growth restriction [44–46].

A high hemoglobin concentration has also been associated with adverse pregnancy outcomes and appears to remain elevated throughout all three trimesters. In addition, some studies suggest that a high iron status in late pregnancy is associated with a significantly lower birth weight [47]. In this regard, a randomized controlled trial to assess the effect of iron supplementation on pregnancy outcomes in iron-replete women found that women with higher hemoglobin levels in the third trimester were significantly more likely to develop hypertensive disorders and deliver small-for-gestational age (SGA) infants [48].

Ferroptosis has also been described as a cell death inducer in vascular smooth muscle cells in smoking patients with aortic aneurysm and dissection and, interestingly, cigarette smoke extract (CSE)-induced cell death in rat vascular smooth muscle cells (VSMCs) was completely inhibited by specific ferroptosis inhibitors and an iron chelator [49]. If confirmed in human pregnancies, this could lead to a new target treatment in vasculopathy-mediated obstetric illnesses such as preeclampsia and fetal growth restriction.

We have found two genes of the ferroptosis pathway, namely PCBP1 and GPX4, to be hypermethylated in small newborns exposed to tobacco smoke. The former (PCBP1; t(poly-(rC)-binding protein)) is a chaperone protein that binds iron inside cells to avoid iron-mediated cytotoxicity and hence become a part of the chelatable and redox-active intracellular labile iron pool. Iron-loaded PCBPs form a stable ternary complex with ferritin during iron transfer [50,51].

The effect of siRNA-mediated downregulation of PCBP1, PCBP2, and YBX1 genes on the differentiation of trophoblasts has been related to preeclampsia [52]. To the best of our knowledge, this study is the first to relate these alterations in expression of the PCBP1 gene to fetal growth restriction. The expression of iron homeostatic and ferroptotic genes, such as FTL and PCBP1, has been described in different cell types at the maternal-fetal interface. At term, FTL is highly expressed in almost all cell types, thus suggesting that intracellular iron storage and prevention of iron toxicity is likely to be particularly important at the maternal-fetal interface [33,53].

A deficit or inactivation of GPX4, the other hypermethylated gene found in our study, results in membrane lipid peroxidation damage and cell death [32,54]. In the human placenta, trophoblasts are particularly sensitive to ferroptosis caused by depletion or inhibition of GPX4. Ferroptosis is promoted by an excess of iron and lipoxygenation, glutathione depletion, and deletion or inactivation of the phospholipid peroxidase GPX4. Ferroptosis has been implicated in pathological cell death, such as ischemia-reperfusion injury, neurodegenerative disease, and cancer-related cell death. Moreover, several recent studies have implicated placental ferroptosis in pregnancy complications, thus adding a broader dimension to our understanding of the placental lipotoxic injury that underlies common diseases during human pregnancy [55].

In addition, reduced or mutated GPX4 has been associated with human placental dysfunction and preeclampsia, and ferroptosis inhibitors have been found to attenuate preeclampsia-like symptoms in rats [56]. Physiologically, the placenta is exposed to hypoxia and re-oxygenation during normal development, when blood supply to the intervillous space is established and the placenta transitions from a histotrophic to a hemotrophic support at the end of the first trimester of human pregnancy. The placenta is also normally exposed to cycles of reduced and restored blood flow similar to the hypoxia-reperfusion associated with ferroptosis in the brain, kidneys, and other organs. This type of injury has been implicated in the placental dysfunction underlying intrauterine growth restriction, preeclampsia, and related conditions [57].

Pregnancy is defined by a state of moderate oxidative stress associated with the intensification of metabolic processes and increased demand for oxygen in the maternal fetal compartment. Maternal smoking during pregnancy and, therefore, in utero tobacco smoke exposure, causes an additional increase in free radical processes in both the mother and the developing fetus. The resulting increase in oxidative damage could be a main factor in pregnancy complications such as fetal growth restriction and preeclampsia [21].

Expression of GPX in the placenta has been found to be lower in preeclampsia pregnancies than in a control group [58], and cigarette smoking has been associated with decreased serum vitamin C and GPX levels [59]. Furthermore, a case-control study involving plasma and placental tissue samples from 25 IUGR patients and 25 healthy pregnant women found that oxidative stress markers differed significantly in IUGR samples, and that GPX activities and their placental mRNA transcriptional levels were significantly lower [60]. These examples of GPX under-expression could be caused by epigenetic events such as that described in our study in IUGR newborns exposed to tobacco. However, other authors found that although active smoking during pregnancy contributed to higher maternal cadmium and lead concentrations in maternal and cord blood, its contribution to the variability of GPX was not significant [61]. A study on 60 late pregnancies [30 preeclampsia vs. 30 healthy) found significantly higher GPX4 mRNA levels ($P < 0.001$) in both the mother and in fetal circulation in the preeclampsia group [62].

GPX gene alterations are related to ferroptosis through several mechanisms. DNA methylation is the most common epigenetic modification to have been studied in gene regulation. Homocysteine treatment is known to induce DNA methylation of the GPX4 gene in nucleus pulposus, thus leading to ferroptosis sensitivity [63]. As such, DNA methylation is involved in the epigenetic silencing of ferroptosis-associated genes. These studies therefore support our findings by linking hypermethylation of ferroptosis-related genes with under-expression.

In addition to methylation, other epigenetic mechanisms, such as histone acetylation, could affect gene expression. Histone acetylation marks are written by histone acetyltransferases (HATs), read by bromodomains (BRDs), and erased by histone deacetylases (HDACs). This also affects the expression of ferroptosis-associated genes such as GPX4, which are down-regulated in breast and lung cancer cell lines upon BRD4 knockdown [64]. Finally, numerous miRNAs have also been found to participate in regulation of the key genes in ferroptosis, including the miRNA 4715–3p, which induces ferroptosis by inhibiting GPX4 expression [65].

The oral supplementation of iron and other micronutrients is a universal practice in pregnancy care because of the increased needs during this period and the frequent onset of anemia [66,67]. However, this practice has been questioned recently by some authors [44,45,68], and

our study also suggests that this practice should be reconsidered, at least in some pregnancies. Furthermore, some studies on preeclampsia, an obstetric syndrome that shares physio-pathological mechanisms with fetal growth restriction, have found a high level of iron and ferritin that could lead to increased ferroptosis in the placenta [69–71]. Finally, some ferroptosis-related drugs have been developed in cancer therapy. Thus, as5-Aza-cd treatment has been shown to release the DNA methylation-mediated epigenetic silencing of GPX4 and CDH1 genes, thereby restoring the resistance of cells to ferroptosis [63,72]. If the relationship between ferroptosis and fetal growth restriction is confirmed, a new treatment for this important obstetric syndrome could become available

Strengths and limitations

The main strengths of our study are the rigorous methodology used to select the cases and the wide-ranging analysis of the fetal cord blood methylome.

The main limitation of our study is the low number of cases. While the sample size in our study may be considered small, we believe that the robustness of our results can be attributed to the rigorous methodology we employed. We utilized validated techniques and rigorous data analysis, which allowed us to minimize bias and increase the validity of our findings.

5. Conclusions

Cigarette smoking during pregnancy could cause fetal growth restriction by triggering epigenetic changes as a result of the hypermethylation and downregulation of the GPX4 and PCBP1 genes, which could cause ferroptosis in fetal cells. The relationship between excessive iron consumption and ferroptosis during pregnancy therefore needs to be investigated further. A better understanding of the function of ferroptosis-mediated cell death during fetal growth and of the epigenetic regulatory mechanism underlying ferroptosis will offer new opportunities for early diagnosis and for the development of preventive and therapeutic tools.

Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of Aragon CEICA (protocol code PI16/0208 and date of approval, 14/09/2016)

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

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CRediT authorship contribution statement

José Ignacio Labarta: Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition. **Ana Gascón-Catalán:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Diego Lerma-Puertas:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **José Javier Jaulín-Pueyo:** Writing – review & editing, Writing – original draft, Formal analysis. **Eva Barrio:** Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jose Ignacio Labarta reports financial support was provided by Gobierno de Aragón. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

We have shared the code at the attached file

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.reprotox.2024.108581.

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