The Oxygen Load Supplied during Delivery Room Stabilization of Preterm Infants Modifies the DNA Methylation Profile

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Objectives To determine whether the amount of oxygen provided during postnatal stabilization changes the DNA methylome in preterm infants.

Study design This prospective, observational study included 32 preterm infants ≤32 weeks of gestation who received oxygen in the delivery room. Patients were monitored using a respiratory function monitor to determine the amount of oxygen received upon stabilization. Blood samples were processed for comparison of DNA methylation before and after resuscitation using a DNA methylation high-resolution microarray Infinium Human DNA methylation EPIC 850K BeadChip.

Results The median amount oxygen provided to preterm infants during stabilization was 644 mLO_2/kg. Male sex and vaginal delivery were associated with increased oxygen needs. There were 2626 differentially methylated CpGs representing 1567 genes that showed an association with oxygen load selected and, of these, 85% were hypomethylated. We found that oxygen loads of >500 mLO_2/kg changed the methylation pattern of the selected CpGs. Genes associated with these CpGs were "enriched" in KEGG pathways involved in cell cycle progression, DNA repair, and oxidative stress.

Conclusions The oxygen load provided upon resuscitation modified the DNA methylome. Differential methylation may lead to altered expression of genes related to cell cycle progression, oxidative stress, and DNA repair. The reversibility of these early epigenetic changes is unknown but merits further study. *(J Pediatr 2018;■■:■■-■■).*

Prematurity is one of the leading causes of morbidity and mortality in the first year of life.[1] Epigenetic changes refer to modifications in the DNA that do not involve changes in the sequence but have an impact on gene transcription and function and have been linked to the development of specific diseases.[2-4] The most widely studied epigenetic change is DNA methylation, which is characterized by the addition of a methyl group to the 5′ position of a cytosine in a cytosine guanine dinucleotide (region of DNA where a cytosine nucleotide is followed by a guanine in the linear sequences of bases [CpG]) site. Increased methylation levels in promoters silence gene transcription and demethylation creates a transcriptionally permissive state by relaxing the chromatin.[5]

The stabilization of preterm infants after birth often requires oxygen. However, preterm infants frequently spend a substantial time either in hypoxia or hyperoxia.[6] Hyperoxia is associated with the generation of free radicals that cause structural and functional alterations to biomolecules.[7] Preterm infants have immature antioxidant defenses and are very susceptible to the harmful effects of oxidative stress, including DNA guanine base oxidation.[8,9] There is a relationship between oxygen, oxidative stress, and epigenetics during the perinatal period. Histone demethylases require oxygen as a cofactor, directly linking epigenetic processes to oxygen gradients during development.[10] In addition, oxygen free radicals cause changes in the cellular redox status and could also be considered environmental factors capable of modifying DNA methylation pattern, thus affecting the expression of specific genes after neonatal supplementation of oxygen.[10]

We hypothesized that oxygen load during postnatal stabilization might cause a general pattern of hypomethylation. In a proof-of-concept study, we calculated the oxygen load during stabilization in the delivery room using a respiratory function monitor (RFM) and assessed its impact on DNA methylation at the genomic

CpG	Region of DNA where a cytosine nucleotide is followed by a guanine in the linear sequences of bases
DMCpGs	Differentially methylated CpGs
RFM	Respiratory function monitor
V_T	Tidal volume

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level using a high-resolution microarray in the human genome.

Methods

This prospective, observational study was performed in the neonatal intensive care unit of the UPH La Fe (Valencia, Spain) between June 1, 2016 and May 31, 2017.

We studied 32 eligible infants <32 weeks of gestation who required positive pressure ventilation and oxygen in the delivery room. Spontaneously breathing babies or those who initiated effective spontaneous breathing during stabilization not requiring positive pressure ventilation and oxygen were excluded. In addition, infants with severe neonatal depression (Apgar score at 1 minute of <3), having severe malformations or chromosomopathies, or not following the study protocol were also excluded.

Immediately after birth, a pulse oximeter sensor was attached to the right wrist to monitor SpO_2 and heart rate. Positive pressure ventilation was provided using silicon facemasks coupled to a T-piece resuscitator (Giraffe T-piece resuscitator, GE Health Care, Helsinki, Finland) with an initial pressure of 6 cm H_2O. Pressure limits were set at 20-25 cm H_2O and the positive end-expiratory pressure was set at 5-6 cm H_2O. The initial FiO_2 of 0.3 was titrated to achieve SpO_2 targets of 70%-75%, 75%-80%, 80%-85%, and 85%-95% at 3, 4, 5, and 10 minutes, respectively.[11] Simultaneously, an RFM (Newlifebox, Weener, Germany) was connected between the inspiratory line and the face mask interface (**Figure 1**; available at www.jpeds.com). The RFM also received input from the air/oxygen blender, the oxygen analyzer (Teledyne Analytical Instruments, City of Industry, California) located in the inspiratory limb of the T-piece resuscitator, pulse oximeter (Masimo Corporation, Irvine, California) and retrieved respiratory graphs, respiratory rate, expiratory tidal volume (V_T), peak inspiratory pressure, end expiratory pressure, FiO_2, heart rate, and SpO_2. All the information was exported to a correlational database.

The oxygen load provided during the initial resuscitation was retrieved using an RFM. DNA methylome analyses before and after oxygen supplementation were completed. Oxygen load was defined as the milliliters of O_2 per kilogram provided during delivery room stabilization (heart rate >100 bpm; SpO_2 ≥90% for >1 minute). We retrieved the V_T per breath and total breaths. The oxygen analyzer provided information of the oxygen content in the inspired gas. If babies were on room air, V_T was multiplied by 0.21. Oxygen load per breath (B) was calculated as: $OL_B = [V_T \times FiO_2]/kg$, and the total oxygen load during stabilization was calculated as: $\Sigma\ (B_1 + B_2 + \ldots B_n)$, where n is the number of breaths during stabilization. Investigators were trained in the use of the RFM in mannequins and with live patients. Reproducibility in 3 successive measurements of RFM variables were assessed at the end of the training period. Moreover, readings of the monitor were stored and the total oxygen load calculated as breath × breath to confirm algorithm's accuracy.

Blood (0.5 mL) was sampled using a heparinized syringe at (t_1) before oxygenation in umbilical artery and (t_2) after admission in the neonatal intensive care unit. Blood was centrifuged (1500*g* × 10 minutes) at 4°C to separate plasma from the cell pellet. Cell fractions were stored at -80°C until processed. Epigenomic studies were performed with the Infinium Human DNA Methylation EPIC 850K BeadChip arrays (Illumina Inc, San Diego, California) used for the interrogation of >850 000 CpG sites. The protocol followed is detailed in the Supplementary Methods (**Appendix**; available at www.jpeds.com).

Statistical Analyses

Clinical data were summarized using mean, median, and first and third quartiles for continuous variables and relative and absolute frequencies for categorical variables.

Changes in the methylation status of specific CpGs were assessed using mixed beta regression models.[12] Beta regression naturally accommodates responses between 0 and 1 such as percent methylation values (beta values) obtained by the methylation 850K array.[12] The methylation score for each CpG was represented as a beta value according to the fluorescent intensity ratio. Beta values may take any value between 0 (no methylated) and 1 (completely methylated). The raw data (IDAT files) were normalized using functional normalization as implemented in the R-package minfi (version 1.22.1, The R Foundation for Statistical Computing, Vienna, Austria). Because observations were paired, the individuals were added as a random factor to the models to account for the non-independence of observations in our pre–post design. All *P* values were adjusted using the Benjamini-Hochberg procedure to control the False Discovery Rate. Adjusted *P* values of <.05 were considered statistically significant.

For the search of the KEGG Pathways of Gene Ontology, we used the annotations found by GeneCodis3.[13] *P* values of <.03 have been obtained through hypergeometric analysis corrected by the false discovery rate method.

Results

A total of 32 eligible preterm infants <32 weeks of gestation were included in the study (**Figure 2**; available at www.jpeds.com). The clinical characteristics of the population and perinatal interventions and the individual gestational age, sex, birthweight, and type of delivery and oxygen load are shown in **Tables I** and **II**, respectively. The mean and standard deviation for delivery room stabilization was 11.5 ± 2.5 minutes. Oxygen load ranged from 275 to 976 O_2 mL/kg, with a median of 644 mL O_2/kg. No significant differences were found in relation to the oxygen load during stabilization relative to birth weight (**Figure 3**, A and B). However, male sex (**Figure 3**, C) and vaginal delivery (**Figure 3**, D) were associated with significantly greater oxygen load during stabilization ($P < .01$).

We assessed the effect of oxygen load on epigenetic status, considering oxygen load as a continuous variable to improve

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Table I. Clinical characteristics and perinatal interventions in preterm infants ≤32 weeks of gestation enrolled in the study

Variables	Preterm infants (n = 32)
Gestational age (weeks)	28 (25-32)
Birth weight (g)	1273.3 ± 468.4
Apgar at 1 min	7 (4-9)
Apgar at 5 min	8 (6-10)
Umbilical artery pH	7.31 ± 0.05
Male sex	15 (46.9)
Type of delivery: vaginal/cesearean	10/22 (31.1/68.9)
Twin pregnancy	20 (62.5)
Antenatal steroids*	29 (90.6)
Hypertensive state during pregnancy†	6 (18.8)
Chorioamnionitis‡	10 (31.2)
Intubation in the delivery room	12 (37.5)
Time after birth for obtaining second blood sample (min)†	125 ± 18

Values are median (IQR), mean (SD), or n (%).
*Full course of antenatal steroids: 12-mg doses of betamethasone given intramuscularly 24 hours apart or four 6-mg doses of dexamethasone administered intramuscularly every 12 hours.[14]
†Following the definition of the Canadian Obstetric Guidelines.[15]
‡Chorioamnionitis or intra-amniotic infection was defined as an acute inflammation of the membranes and chorion of the placenta with clinical, histologic, or microbiological findings.[16]

Table II. Preterm infants ≤32 weeks of gestation postnatal characteristics and oxygen load retrieved with an RFM in the delivery room

Case numbers	Gestational age (weeks)	Birth weight (g)	Sex (M/F)	Oxygen load (mL O_2 per kg)	Type of delivery
1	25	750	Female	359	Cesearean
2	25	940	Female	393	Cesearean
3	27	795	Male	393	Vaginal
4	28	1200	Female	781	Vaginal
5	30	1000	Female	297	Cesearean
6	27	940	Female	475	Cesearean
7	32	1930	Female	408	Cesearean
8	32	2610	Male	546	Vaginal
9	32	1660	Female	470	Cesearean
10	30	1400	Male	275	Cesearean
11	25	850	Male	665	Vaginal
12	25	875	Female	857	Cesearean
13	27	925	Female	925	Cesearean
14	28	1025	Male	831	Vaginal
15	28	1150	Female	678	Cesearean
16	27	1090	Male	673	Vaginal
17	25	965	Male	819	Cesearean
18	32	1500	Male	408	Cesearean
19	32	1840	Female	398	Cesearean
20	28	1315	Female	955	Cesearean
21	29	1600	Male	885	Vaginal
22	29	1320	Male	623	Vaginal
23	25	870	Male	966	Vaginal
24	27	970	Male	848	Vaginal
25	26	680	Male	611	Cesearean
27	31	1685	Female	708	Vaginal
28	32	1860	Female	340	Cesearean
29	26	990	Female	389	Cesearean
30	32	1955	Female	343	Cesearean
31	32	1980	Female	674	Cesearean
32	27	730	Male	944	Cesearean

statistical power and accuracy and allow for better interpretability of the results. A total of 2626 differentially methylated CpGs (DMCpGs) were identified (false discovery rate-adjusted $P < .05$) and were associated with 1567 RefSeq genes (**Supplement**; available at www.jpeds.com).

We assessed the methylation profile of preterm infants relative to oxygen load and found a gradual effect of oxygen load on methylation status, with a diffusely defined threshold around 500 mL O_2/kg of weight (**Figure 4**, A). At oxygen loads of ≥500 mL O_2/kg of weight, we identified a clear loss of methylation of 2196 CpGs (representing 83.6%) and a gain of methylation of 430 CpGs (representing 16.4%; **Figure 4**, B). Interestingly, the distribution of DMCpGs was not random ($P < .001$).

To address the functional implications of the signature found in DNA methylation, we performed an enrichment analysis of functions using GeneCodis[13] with the DMCpGs with potential role in gene expression that is found in islands and shores. Enrichment analysis of genes associated with the loss of methylation DMCpGs for annotations involving KEGG pathways revealed a significant enrichment for genes involved in pathways in cancer, interaction between cells or between cells and the extracellular matrix, and actin cytoskeleton (**Figure 5**, A). Pathways in cancer included relevant genes involved in the maintenance of genomic integrity and the DNA damage checkpoint, such as *BRCA1*, *CDKN2B*, *PERP*, *ATMIN*, *FOXM1*, and *TP53BP1*, and DNA repair pathways including *XAB2*, *XRCC6*, *GTF2H1*, *POLR2H*, *MTHFD1*, *ERCC5*, and *RAD51AP1* (**Supplement**). In genes associated with gain of methylation DMCpGs, we found enrichment for functions involving cell adhesion and cell cycle progression (**Figure 5**, B) such as *CHEK1*, *POLD2*, *TP53*, *TOP2A*, and *RASSF1* (**Supplement**). Finally, we further identified several genes encoding proteins involved in mitochondrial electron transport such as *NDUFS2*, *NDUFB4*, *ATP5F1*, *ATP5A1*, and *COX15*; and response to oxidative stress such as *DUOX1*, *DUOXA1*, *PRDX3*, and *ATOX1*.

DMCpGs of the MethylationEPIC array were classified based on their gene name and gene region of UCSC annotations and CpG context previously defined[17] (**Supplement**). With respect to CpG context, CpG sites can be located in islands, shores (regions flanking CpG islands ≤2 kb distant with lower CpG density), shelves (regions ≤2 kb long flanking shores with a lower density of CpG sites), and open sea (very low CpG density regions throughout the genome).[18] Almost one-half of the selected DMCpGs are found in the open sea (47%), one-third in the shores of CpG islands (32%), 15% within CpG islands, and the rest in shelves (**Figure 6**, A). We focused on DMCpGs within CpG islands and shores because shelves and open sea play a relatively minor role in the control of gene transcription.[5] The overall distribution of these DMCpGs revealed similar enrichment

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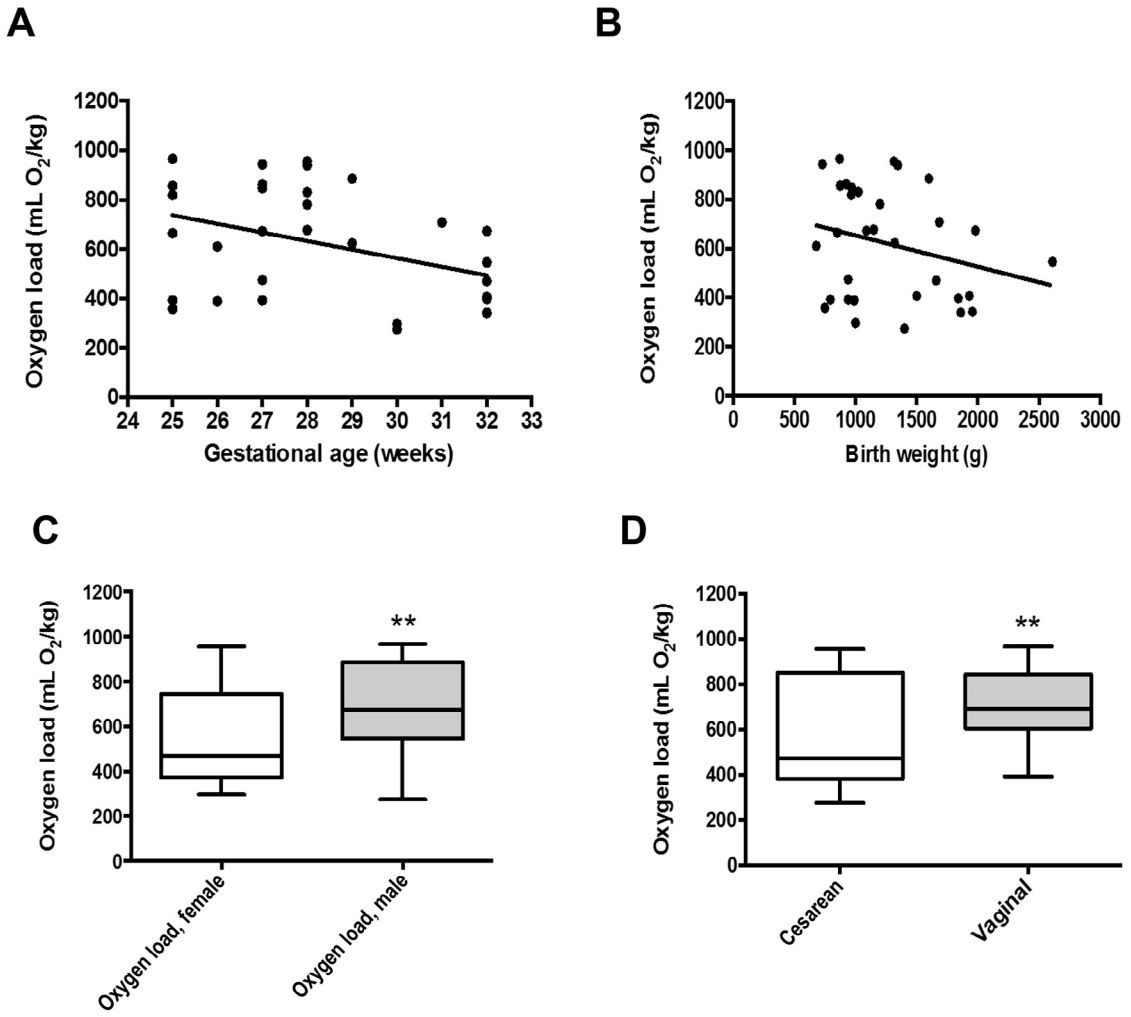

Figure 3. Correlation between oxygen received upon stabilization in preterm infants with clinical characteristics of the population and perinatal interventions. **A,** Gestational age. **B,** Birth weight. **C,** Sex. **D,** Type of delivery. $^{**}P < .01$ by 2-tailed *t* test.

of loss and gain of methylation CpGs in promoters, body gene, and intergenic regions (**Figure 6**, B).

Discussion

The amount of oxygen provided during delivery room stabilization was titrated to avoid hyperoxia or hypoxemia[1] and quantified as an oxygen load using an RFM and an ad hoc algorithm. This approach, in conjunction with the use of beta regression models, which improve the detection of differential DNA methylation and take advantage of the continuous nature of both the response and the predictor variables,[19] allowed us to establish an association between oxygen load and induced epigenetic changes. The median oxygen load (644 mL O_2/kg; range, 275-976 mL O_2/kg) was significantly higher than oxygen load reported for term healthy newborn infants (~300 mL O_2/kg).[20]

We found that male infants or those born vaginally needed significantly more oxygen to achieve stabilization (**Figure 3**, C). This finding may reflect the greater pulmonary and vascular maturity of female neonates.[21] Cesarean delivery has been advocated as protective for very preterm infants, although results have not been conclusive.[22] Our results suggest one effect is a decreased oxygen load in the delivery room.

The effects of hyperoxia have been widely studied in animal models. For instance, hyperoxia-exposed newborn rats have arrested lung alveolarization secondary to epigenetic disruption of signaling pathways relevant to lung and neural development and neuroprotection.[23-25] We now show that human infants display short-term epigenetic disruption after neonatal resuscitation with oxygen.

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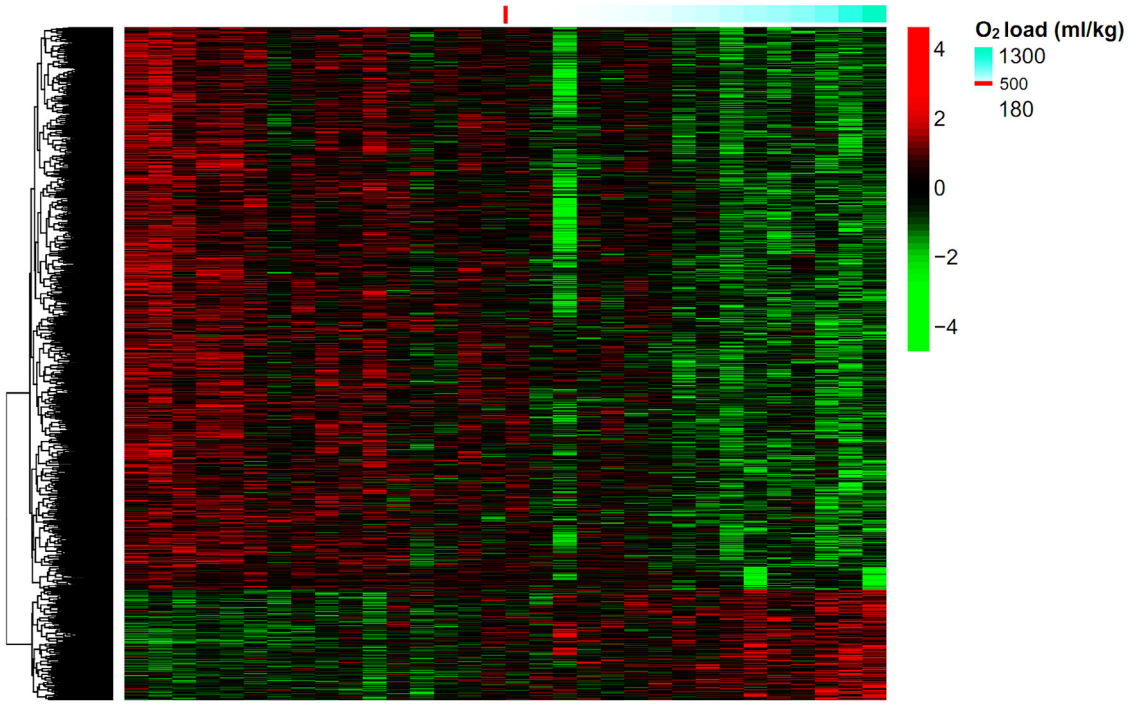

Figure 4. Selection of DMCpGs in preterm newborns depending on the oxygen load received during stabilization. **A,** Heatmap including the CpGs displaying statistically significant differences in beta regression models. Rows (CpGs) and columns (individuals) are ordered according to the results of hierarchical clustering. Color scale ranges from red for hypermethylation to green for the hypomethylation. Different methylation profiles clustered higher and lower doses of oxygen load. Changes in methylation appear progressively as oxygen load increases with a diffuse common threshold for most CpGs around 500 mL O_2/kg. **B,** The number of loss and gain of methylation DMCpGs with higher oxygen load.

Our epigenome-wide approach identified a signature of DMCpG sites with an association with the oxygen load during resuscitation. We found that exceeding a boundary of approximately 500 mL O_2/kg was associated with altered methylation status; most of DMCpG sites lost methylation and others gained it. It has been reported that DNA oxidative damage provokes global hypomethylation and aberrant hypermethylation of some gene promoter regions,[26] which is in agreement with our results. Base oxidation of the CpGs modifies the interactions between the CpGs sites and transcription factors leading to changes in gene expression.[27] In this scenario, the use of a higher FiO_2 of 0.9 vs 0.3 in extremely preterm infants caused significant oxidative damage to DNA and led to increased respiratory morbidity.[9] Interestingly, preterm infants have limited nucleotide excision repair capacity.[28] Persistent oxidized DNA triggers the genome-wide hypomethylation that has been associated with the development of tumors, obesity, diabetes, or autoimmune diseases.[29-31] Epidemiologic studies in the US and Sweden established an association between the use of pure oxygen in the first minutes after birth and childhood cancer.[32-34]

Widespread methylation differences between term and extremely preterm infants have been reported, and these differences may interact with environmental and social risk factors to establish risk for future complications. However, follow-up studies of preterm and term infants through the end of adolescence showed that methylation differences are largely resolved by 18 years of age, although specific probes associated with preterm individuals remain and reflect a long-term epigenetic legacy of preterm birth.[35]

We found that a brief exposure to oxygen caused specific enrichment of functions involved in defense against oxidative stress, interaction between cells or between cells and the extracellular matrix and actin cytoskeleton, cell adhesion, cell cycle progression, and cancer. We identified several genes that have been previously associated with oxidative stress and oxygen regulation such as *DUOX1* responsible for the synthesis of dual oxidases and NADPH oxidase and associated with the production of reactive oxygen species[36]; and *PRDX3* that encodes a mitochondrial protein involved in redox regulation and has an antioxidant function protecting cells against oxidative stress,[37] and *ATOX1* which encodes a copper chaperone that plays a role in the incorporation of copper to Cu-dependent antioxidant enzymes and also functions as an antioxidant against superoxide and hydrogen peroxide.[38]

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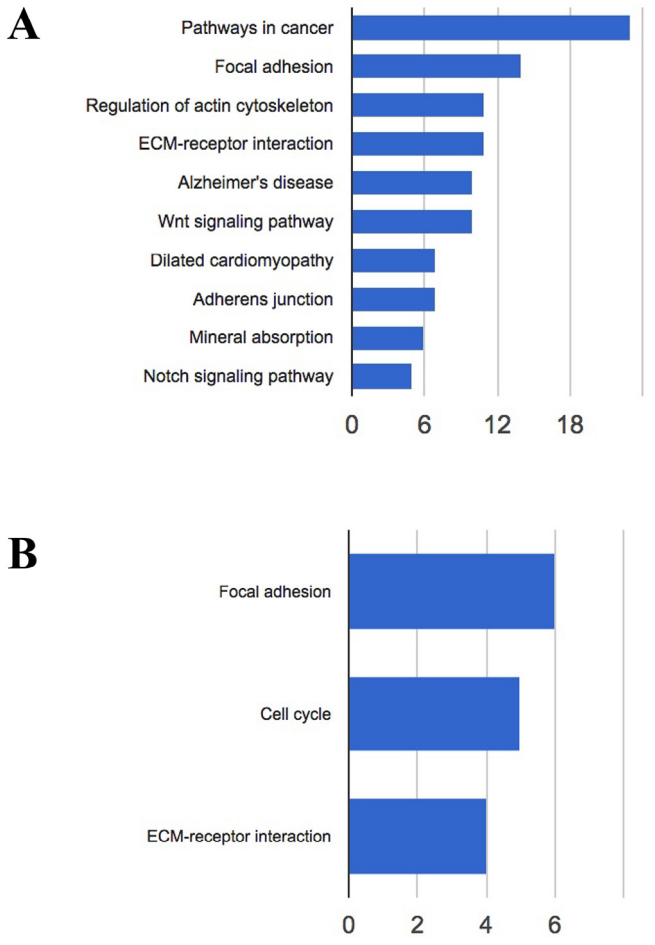

Figure 5. Gene ontology analysis of the genes associated to the selected DMCpGs present in CpG islands and shores. KEGG pathways significantly enriched in genes associated to **A,** loss of methylation and **B,** gain of methylation DMCpGs. All the analyses were performed using GeneCodis3 web tool with an adjusted *P* value from false discovery rate of <.03. ECM, extracellular matrix.

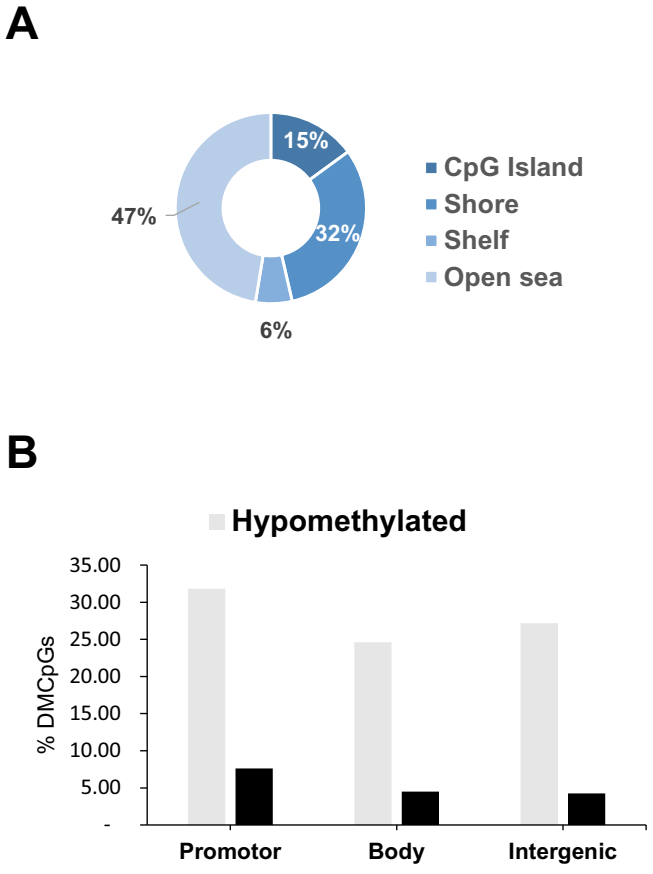

Figure 6. Genomic distribution of selected DMCpGs according to the effect of oxygen load. **A,** Number of the selected DMCpGs by CpG context: CpG islands, shores, shelves and open sea. **B,** Relative abundance of loss and gain of DMCpGs according to gene promoters, gene bodies or intergenic regions.

Our study has some limitations. The sample size is relatively small. However, the lack of waiver of consent and the need for mask ventilation coupled with the RFM favored the exclusion of a substantial number of newborn infants. In addition, we only evaluated some of the specific antenatal stressors influencing DNA methylation at a single point in time.

Our results show that oxygen in the immediate postnatal period potentially induces changes in the DNA methylation pattern, especially when oxygen supplementation is >500 mL O_2/kg. The durability of these methylome changes remains an important question and warrants further study. ■

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Appendix

Oxygen Load during Stabilization of Preterm Infants Modifies Epigenetic Profile

Supplementary Methods.

DNA Extraction. Total DNA was isolated from the cell pellet with MagNA Pure Compact Nucleic Acid Isolation Kit 1-Larg Volume (03730972001, Roche Molecular Systems Inc., Basel, Switzerland) and MagNA Pure Compact Instrument (03731146001, Roche Molecular Systems Inc.), following the manufacturer's instructions. Purified DNA was quantified with NanoDrop and RNA was removed with ribonuclease A from a bovine pancreas for molecular biology (R6513, Sigma-Aldrich Co., Saint Louis, Missouri) at 10 μg/mL during 30 minutes at 60°C, was quantified by the fluorometric method (Quant-iT PicoGreen dsDNA Assay, Life Technologies, Carlsbad, California), and assessed for purity with NanoDrop (Thermo Scientific, Waltham, Massachusetts) 260/280 and 260/230 ratio measurements. The DNA integrity of fresh frozen samples was checked by electrophoresis in 1.3% agarose gel.

Epigenomic Studies. Infinium DNA MethylationEPIC beadchip array shares the Infinium HD chemistry Assay (Illumina Inc, San Diego, California) were used to interrogate the cytosine markers with HumanMethylation450 beadchip. Thus, the applicable protocol for MethylationEPIC is the same as for HumanMethylation450, which is the Infinium HD Methylation Assay Protocol that has been previously established as a reliable technology to detect epigenetic alteration in our and other laboratories.[1] The EPIC array used interrogates >850 000 CpG sites (dinucleotides that are the main target for methylation) that covers 99% of genes described and 95% of CpG islands. The 850K array includes the information of projects such as ENCODE and FANTOM5, which have identified regions as critical sites for differential methylation. This technology is highly reliable detecting epigenetic alterations.[2]

There were 600 ng of purified DNA distributed randomly on a 96-well plate and processed using the EZ-96 DNA Methylation kit (Zymo Research Corp., Irvine, California) following the manufacturer's recommendations for Infinium assays. We processed 4 μL of bisulfite-DNA following the Illumina Infinium HD Methylation Assay Protocol, as previously described.[2] This full protocol consisted of a whole genome amplification step followed by enzymatic endpoint fragmentation, precipitation, and resuspension. The resuspended samples were hybridized on Human Methylation 850K EPIC BeadChips at 48°C for 16 hours. Then, unhybridized and nonspecifically hybridized DNA were washed away, followed by a single nucleotide extension using the hybridized bisulfite-treated DNA as a template. The nucleotides incorporated were labeled with biotin (ddCTP and ddGTP) and 2,4-dinitrophenol (DNP) (ddATP and ddTTP). After the single base extension, repeated rounds of staining were performed with a combination of antibodies that differentiated DNP and biotin by fixing them different fluorophores. Finally, the BeadChip was washed and protected to scan it.

Scanning Beadchips. The Illumina HiScan SQ scanner is a 2-color laser (532 nm/660 nm) fluorescent scanner with a 0.375-μm spatial resolution capable of exciting the fluorophores generated during the staining step of the protocol.

References

1. Sandoval J, Heyn H, Moran S, Serra-Musach J, Pujana MA, Bibikova M, et al. Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. Epigenetics 2011;6:692-702.
2. Moran S, Arribas C, Esteller M. Validation of a DNA methylation microarray for 850,000 CpG sites of the human genome enriched in enhancer sequences. Epigenomics 2016;8:389-99.

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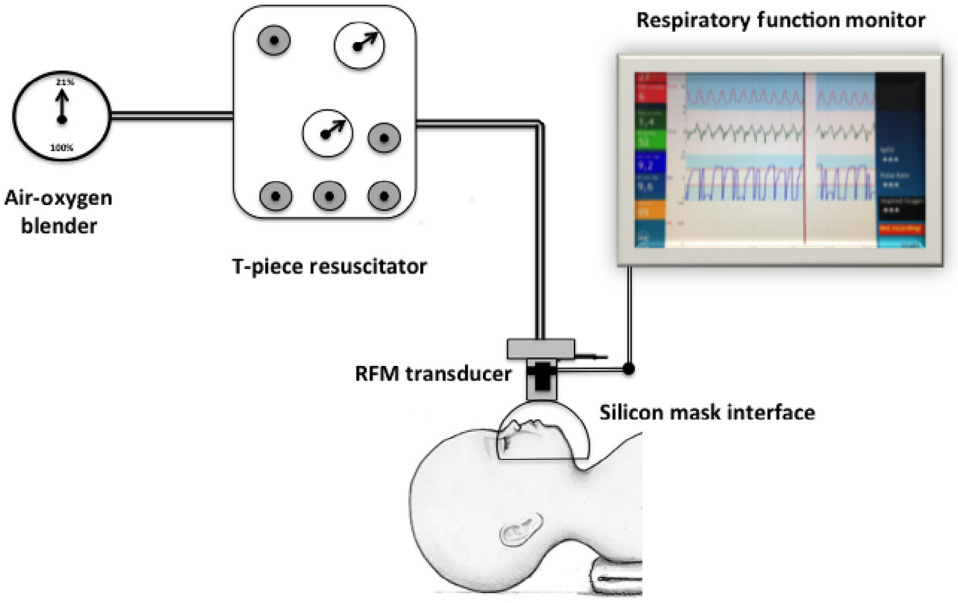

Figure 1. Ventilation and monitoring set up in the delivery room. Ventilation was provided from an air/oxygen blender, passed to a T-piece resuscitator and to the RFM transductor with a silicon mask interface. RFM stored videotaping of the resuscitation and clinical measures.

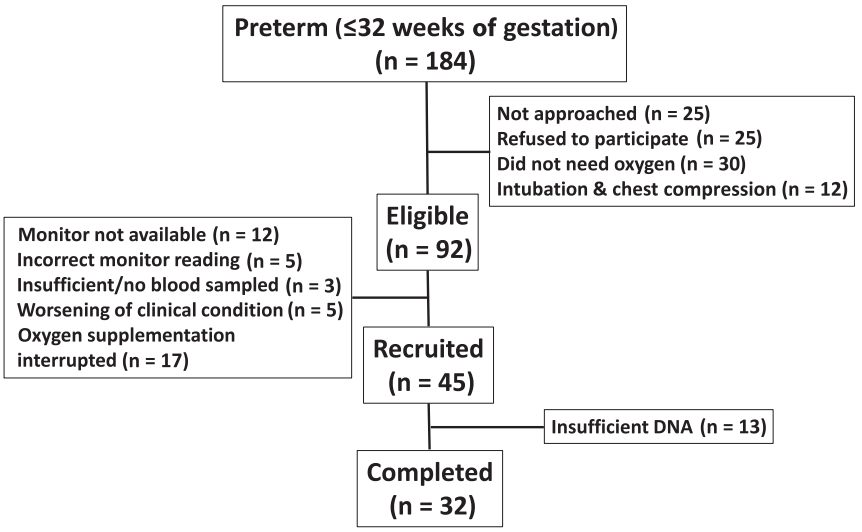

Figure 2. Selection process for the patients studied in the present work. In a 12-month period, 184 preterm newborns of ≤32 weeks of gestation were attended in the delivery room. Of these, 92 were eligible for the study, 45 were recruited, and 32 completed the epigenomic study.

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