

## RESEARCH ARTICLE

## Male obesity effects on sperm and next-generation cord blood DNA methylation

Ramya Potabattula<sup>1\*</sup>, Marcus Dittrich<sup>1</sup>, Martin Schorsch<sup>2</sup>, Thomas Hahn<sup>2</sup>, Thomas Haaf<sup>1</sup>, Nady El Hajj<sup>1,3\*</sup>**1** Institute of Human Genetics, Julius Maximilians University, Würzburg, Germany, **2** Fertility Center, Wiesbaden, Germany, **3** College of Health and Life Sciences, Hamad Bin Khalifa University, Education City, Doha, Qatar\* [ramya.potabattula@uni-wuerzburg.de](mailto:ramya.potabattula@uni-wuerzburg.de) (RP); [nady.elhajj@uni-wuerzburg.de](mailto:nady.elhajj@uni-wuerzburg.de), [nelhajj@hbku.edu.qa](mailto:nelhajj@hbku.edu.qa) (NEH)

## Abstract

The prevalence of metabolic disorders, in particular obesity has dramatically increased worldwide. Genetic variants explain only a minor part of the obesity epidemic induced by physical inactivity and over-nutrition. Epidemiological studies in humans and animal models indicate that epigenetic changes associated with adverse parental and/or intrauterine factors may contribute to the missing heritability of metabolic disorders. Possible adverse paternal effects are likely transmitted by sperm to the next-generation. To investigate this hypothesis, we have systematically analyzed the effects of male body mass index (BMI) on sperm epigenome and its association with next-generation fetal cord blood (FCB) DNA methylation. Methylation levels of maternally imprinted (*PEG1*, *PEG4*, *PEG5*, and *PEG10*), paternally imprinted (*H19-IG DMR*, *IGF2-DMR0*, and *MEG3-IG DMR*) regions, and obesity-related non-imprinted *HIF3A* gene were quantified by bisulphite pyrosequencing in sperm samples of 294 human donors undergoing *in vitro* fertilization or intracytoplasmic sperm injection, and in 113 FCBs of the resulting offspring. Multivariable regression analyses revealed that *MEG3* intergenic differentially methylated region (IG DMR) showed positive correlation between sperm methylation and donor's BMI. A gender-specific correlation between paternal BMI and FCB methylation was observed for *MEG3-IG DMR*, *HIF3A*, and *IGF2-DMR0*. The former two genes displayed same directional nominal association (as sperm) between paternal BMI and FCB methylation in male offspring. Hypomethylation of *IGF2-DMR0* with increased paternal BMI was observed in FCBs from female offsprings. Our results suggest that male obesity is nominally associated with modification of sperm DNA methylome in humans, which may affect the epigenome of the next-generation. Nevertheless, it is important to note that none of the associated p-values survived multiple testing adjustments. Future work should test the effect of associated methylation aberrations in the offspring as DNA methylation was shown to control expression and/or imprint establishment across the studied genes.

## OPEN ACCESS

**Citation:** Potabattula R, Dittrich M, Schorsch M, Hahn T, Haaf T, El Hajj N (2019) Male obesity effects on sperm and next-generation cord blood DNA methylation. PLoS ONE 14(6): e0218615. <https://doi.org/10.1371/journal.pone.0218615>

**Editor:** Yvonne Böttcher, University of Oslo, NORWAY

**Received:** January 7, 2019

**Accepted:** June 5, 2019

**Published:** June 27, 2019

**Copyright:** © 2019 Potabattula et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Data Availability Statement:** All relevant data are within the manuscript and its Supporting Information files.

**Funding:** R.P. was supported by a fellowship of the German Excellence Initiative to the Graduate School of Life Sciences, University of Würzburg. This study was supported by the German Research Foundation (grant EL 742/1-1 to N.E.H.) and the European Union (ERAofART to T.Hf.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing interests:** The authors have declared that no competing interests exist.

**Abbreviations:** ART, Assisted Reproductive Technology; BMI, body mass index; DLK1, delta-like 1 homolog; dNTPs, deoxyribose nucleoside triphosphates; EDTA, ethylenediamine tetra acetic acid; EWAS, epigenome wide association study; FCB, fetal cord blood; FDR, false discovery rate; HCl, hydrogen chloride; HIF3A, hypoxia inducible factor 3 alpha; HNA, hypermethylation of the non-imprinted allele; ICSI, intracytoplasmic sperm injection; IG DMR, intergenic differentially methylated region; IGF2, insulin like growth factor 2; IUGR, intrauterine growth restriction; IVF, *in vitro* fertilization; MEGs, maternally expressed genes; MEST, mesoderm specific transcript; MgCl<sub>2</sub>, magnesium chloride; NaCl, sodium chloride; NNAT, neuronatin; PCR, polymerase chain reaction; PEGs, paternally expressed genes; POMC, pro-opiomelanocortin gene; SDS, sodium dodecyl sulfate; SGCE, epsilon sarcoglycan; SNRPN, small nuclear ribonucleoprotein polypeptide N; WHO, world health organization.

## Introduction

According to the World Health Organization (WHO), the worldwide prevalence of obesity has nearly doubled since 1980. Obesity is viewed as a complex multifaceted disease with a wide range of determinants including behavioral and heritable causes [1]. The estimated heritability of obesity is approximately 75% nevertheless the identified genetic variants so far can explain less than 30% of individual body mass index (BMI) levels and weight variation [2]. Recently, several research efforts focused on determining whether there is an epigenetic basis influencing the risk for metabolic disorders [3]. A large multicenter epigenome-wide association study (EWAS) has identified increased methylation at the hypoxia-inducible factor 3 alpha (*HIF3A*) gene in blood and adipose tissue to be associated with increased BMI [4]. Furthermore, a variably methylated region in the pro-opiomelanocortin gene (*POMC*) was reported to regulate human body weight [5].

The environmental influence experienced by an individual can be transmitted to the next-generation and have an effect on the offspring's phenotype [6, 7]. It is recently becoming clear that parental obesity has a negative influence on the health and well-being of the offspring. Children born to obese mothers are well documented to have an increased predisposition for obesity, insulin resistance, and metabolic perturbations in later life [8]. These increased risks are not limited to maternal influences as epidemiological evidence has also shown that the offspring of obese fathers are at a higher risk of developing metabolic disorders [9, 10]. Newborn males of obese fathers exhibit a significant increase in birth weight as well as a change in several anatomical parameters including head circumference, abdominal diameter, and abdominal circumference [9]. Furthermore, a child born to an obese father and normal weight mother showed an increased risk of becoming obese whereas an obese mother and normal weight father could not predict whether the child would be overweight or obese [10]. Paternal effects were additionally reported to have transgenerational influences where an increase in nutritional abundance was significantly associated with increased rates of diabetes and cardiovascular problems in the children and grandchildren [11, 12]. Elegant rodent studies provide evidence for non-genetic germline transmission of obesity and insulin resistance in the progeny of mice fed a high fat diet [13]. Fathers exposed to nutritional perturbations including high fat diet, low protein diet, and caloric restriction were reported to sire offspring with pancreatic  $\beta$ -cell dysfunction and impaired glucose-insulin homeostasis [14–16]. In humans, recent reports have shown that paternal obesity and aging can epigenetically reprogram the gametes and that these epigenetic alterations might be subsequently transmitted to the children [17, 18]. For instance, weight loss after bariatric surgery was shown to remodel sperm DNA methylation patterns particularly in regions/genes that play a role in appetite control [19].

Imprinted genes are known to escape the wave of methylation reprogramming during early embryogenesis and have been proposed as carriers of epigenetic information into the next-generation [20]. As a result, any epigenetic alterations at imprinted genes can be transmitted via the germ cells thus contributing to future disease risk. Imprinted loci show a parent-of-origin dependent expression regulated by epigenetic silencing of either the maternal or paternal allele. The methylation marks are stably inherited via the germline ensuing mono-allelic expression in all somatic tissues [21]. Imprinted genes are well-known to control fetal growth where the parental 'conflict theory' proposes that paternally expressed genes (PEGs) promote nutrition acquisition from the mother whereas maternally expressed genes (MEGs) limit the usage of resources by the offspring [22]. Imprinted genes are also known to play an important role in a wide array of biological processes and diseases including intrauterine growth restriction (IUGR), obesity, and diabetes mellitus [23].

To study male obesity associated changes in sperm and their epigenetic transmission into the next-generation, we performed an in-depth methylation analysis at the regulatory regions of four maternally imprinted genes, three paternally imprinted loci, as well as the hypoxia inducible factor 3 alpha subunit (*HIF3A*) in a large cohort of 294 human sperm samples undergoing *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) as well as in the collected fetal cord blood (FCB) of their respective offspring when a live birth was achieved.

## Materials and methods

### Ethics approval and consent to participate

The Ethics committee at the medical faculty of the University of Würzburg approved this study on human sperm and fetal cord blood samples (no. 117/11 and 212/15). Written informed consent was collected from all participating couples.

### Study samples

The left-over swim-up sperm fractions (excess material) after IVF/ICSI were collected at the Fertility Center Wiesbaden, pseudonymized and frozen at  $-80^{\circ}\text{C}$  until further use. In this study, the human sperm cohort consisted of a total of 294 samples including sperm from 103 males where a live birth was achieved after assisted reproductive technology (ART). We were successful in acquiring the fetal cord blood samples from collaborating obstetric clinics throughout Germany. Following live birth, a total of 113 FCB samples (including 10 pairs of twins) were collected from new-borns after a successful IVF/ICSI.

### Genomic DNA isolation and sodium bisulphite conversion

After thawing, the swim-up sperm fraction was purified by Silane-coated silica density gradients PureSperm 40/80 (Nidacon, Mölndal, Sweden). Purified sperm was resuspended in 300  $\mu\text{l}$  buffer whose stock consisted of 5 ml of 5 M NaCl, 5 ml of 1 M Tris-HCl (pH 8), 5 ml of 10% SDS (pH 7.2), 1 ml of 0.5 M EDTA (pH 8), 1 ml of 100%  $\beta$ -mercaptoethanol and 33 ml of  $\text{dH}_2\text{O}$ . Upon addition of 100  $\mu\text{l}$  proteinase K (20mg/ml), the samples were initially incubated for 2 h at  $56^{\circ}\text{C}$  (on a thermomixer). Additional 20  $\mu\text{l}$  proteinase K was added and samples were incubated for another 2 h at  $56^{\circ}\text{C}$ . Subsequently, sperm DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) following the recommendations of the manufacturer. The FlexiGene kit (Qiagen, Hilden, Germany) was used for FCB DNA isolation. The concentration and quality of DNA were measured with the NanoDrop 2000c spectrophotometer (Thermo Scientific, Massachusetts, USA). Bisulphite conversion of sperm and FCB DNA (1  $\mu\text{g}$ ) was performed using the EpiTect Fast 96 Bisulphite kit (Qiagen, Hilden, Germany) following the manufacturer's protocol and the converted DNA was stored at  $-20^{\circ}\text{C}$ . In our experience, the average methylation variation between the technical replicates (including bisulphite conversion, PCR, and pyrosequencing) is approximately 1–2 percentage points.

### Bisulphite pyrosequencing

For primer design (S1 Table), the Pyro-Mark Assay Design 2.0 software (Qiagen, Hilden, Germany) was used. Methylation levels of four paternally expressed genes *PEG1*/mesoderm specific transcript (*MEST*), *PEG4*/small nuclear ribonucleoprotein polypeptide N (*SNRPN*), *PEG5*/neuronatin (*NNAT*), and *PEG10*/epsilon sarcoglycan (*SGCE*), three paternally imprinted loci *H19* intergenic differentially methylated region (IG DMR), insulin like growth factor 2 (*IGF2*) differentially methylated region 0 (DMR0), and *MEG3*-IG DMR, and one non-imprinted gene *HIF3A* were quantified. Assays were established by using methylation

standards with 0%, 25%, 50%, 75%, and 100% methylation. Polymerase chain reaction (PCR) for each sample was performed in 25  $\mu$ l reaction consisting of 2.5  $\mu$ l 10x PCR buffer with  $\text{MgCl}_2$ , 0.5  $\mu$ l (10mM) dNTPs, 1.25  $\mu$ l (10pmol/ml) of each reverse and forward primer, 0.2  $\mu$ l (5 U/ $\mu$ l) FastStart Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany), 1  $\mu$ l (~25 ng) bisulphite converted DNA and 18.3  $\mu$ l  $\text{dH}_2\text{O}$ . Pyro-sequencing was carried out using Pyro Q-CpG software (Qiagen, Hilden, Germany) and PyroMark Gold Q96 CDT reagent kit on the PyroMark Q96 MD system. Unmethylated and fully methylated DNA standards (Qiagen, Hilden, Germany) were used as controls in each pyro-sequencing run.

## Statistical analysis

Statistical analyses have been performed with the computational statistical software R (version 3.2.2). The methylation measurements (beta values) for the imprinted genes in haploid cells (sperm) are usually close to the border of the possible ranges in the standard unit interval (0; 1). To properly model this data, a multivariable regression analysis using beta model has been applied as implemented in the R package 'betareg' (version 3.1). As described in detail [24], this approach inherently allows to model heteroskedasticity or skewness which is often observed in data with values in the standard unit interval (e.g. rates or proportions) such as methylation rates. For the beta model, we used the default logit link and thus estimates can be back-transformed using the inverse logit function. Due to the logit link function, the estimates of the beta regression can be interpreted similar to logistic regression models and thus denote the additional increase (or decrease in case of a negative coefficient) in the log-odds of observing an allele in a methylated state. Average sperm DNA methylation values (the dependent variable in the model) of each amplicon have been regressed against donor's BMI adjusting for potential confounding factors, which have been selected based on known factors influencing DNA methylation. The multivariable beta regression model for the sperm methylation data contained donor's BMI and age, sperm concentration and whether or not a live birth was achieved after ART.

For FCB data with methylation values of imprinted genes in the mid-range, multivariable regression analysis using classical linear models were used. Multiple testing correction for false discovery rate (FDR) has been performed using the method of Benjamini and Hochberg. The regression models included birth weight and gestational week of the child, paternal and maternal age, paternal and maternal BMI, and gender of the child including the interaction between paternal as well as maternal BMI and child's gender to allow for a varying effect of paternal and maternal BMI between the FCB genders.

## Results

### Characteristics of samples in the study

The body mass index (BMI) was calculated from the measured weight (in kilograms) and height (in centimeters) of the participating individuals. Based on the WHO guidelines of BMI categorization for adults, out of 294 sperm samples, one sample (0.34%) with 17.30 BMI fell in 'underweight' class; 144 samples (48.97%) in 'normal weight' class (BMI: 19.00–24.90); 149 samples (50.68%) in 'Pre-obesity/obesity' class (BMI: 25.00–40.30). To be more specific, 120 samples were 'Pre-obese' (BMI: 25.00–29.90) and the remaining 29 samples were classified as obese (BMI: 30.00–40.30). The FCB study cohort consisted of a total of 113 collected FCB samples (including 10 pairs of twins) from offspring fathered by 103 of the 294 sperm donors after a successful IVF/ICSI. Among them, 54 samples (47.79%) were female and 59 samples (52.21%) were male. Birth weight significantly correlated with gestational week of the child ( $\rho = 0.67$ ,  $p < 0.0001$ ). Father's age (103 out of 294 samples) significantly correlated with

mother’s age ( $\rho = 0.56, p < 0.0001$ ). Clinical parameters of the sperm and fetal cord blood study samples are summarized in [Table 1](#).

The relevant demographics that were considered in the regression models for the analyzed sperm and fetal cord blood samples.

### Sperm DNA methylation signatures correlating with donor’s BMI

Imprinted genes are the best candidates to study inter-generational epigenetic inheritance since they bypass the genome wide epigenetic reprogramming mechanism occurring after fertilization. In this regard, we selected four maternally imprinted genes (*MEST/PEG1*, *SNRPN/PEG4*, *NNAT/PEG5*, and *SGCE/PEG10*), three paternally imprinted loci (*H19-IG DMR*, *IGF2 DMR0*, and *MEG3-IG DMR*) and one obesity related gene *HIF3A* to check the correlation of male BMI with sperm DNA methylation. To determine the average methylation levels of these loci, bisulphite pyrosequencing was performed. The number of CpGs quantified for each gene is provided in [S1 Table](#). For sperm, multivariable regression analysis using beta model was applied in order to adjust for potential confounding factors. Because of limited amounts of DNA, we analyzed 261 sperm samples for *MEST*, 263 for *SNRPN*, 262 for *NNAT*, 261 for *PEG10*, 255 for *H19-IG DMR*, 271 for *IGF2 DMR0*, 265 for *MEG3-IG DMR*, and 260 for *HIF3A*. *MEG3-IG DMR* sperm DNA methylation showed a positive correlation (nominal significance without multiple testing adjustment) with male BMI (regression estimate +0.014,  $P = 0.043$ ). [Table 2](#) shows the result of the regression models when sperm DNA methylation was correlated with donor’s BMI. In additional [S1 File](#), we provide statistics without adjusting to any confounding factors.

The correlation of donor’s body mass index ( $\text{kg}/\text{m}^2$ ) with DNA methylation (%) of eight analyzed amplicons in sperm. The model was adjusted for sperm concentration, donor’s age, and ART success.

### Transmission of paternal BMI effects from sperm to next-generation in a gender specific manner

To examine the probable transmission of BMI induced sperm epigenetic signatures to the next-generation, we performed bisulphite pyrosequencing on 113 fetal cord blood samples

**Table 1. Clinical parameters of the sperm and fetal cord blood samples.**

Parameters	Sperm samples	Fetal cord blood samples
Sample size (N)	294	113
Sex of the child (Female; Male)	-	47.79% F (n = 54); 52.21% M (n = 59)
Paternal BMI ( $\text{kg}/\text{m}^2$ ) (range; mean $\pm$ SD)	17.30–40.30; 25.75 $\pm$ 3.1	17.30–40.30; 25.78 $\pm$ 3.3
Paternal age (years) (range; mean $\pm$ SD)	25.71–65.82; 38.95 $\pm$ 5.9	28.08–52.85; 38.63 $\pm$ 5.1
Sperm concentration (million/ml) (range; mean $\pm$ SD)	0.20–210.0; 31.81 $\pm$ 36.32	0.20–200.0; 35.99 $\pm$ 39.97
Maternal BMI ( $\text{kg}/\text{m}^2$ ) (range; mean $\pm$ SD)	-	17.00–39.80; 23.19 $\pm$ 4.0
Maternal age (years) (range; mean $\pm$ SD)	-	22.08–42.68; 34.62 $\pm$ 3.7
Birth weight (grams) (range; mean $\pm$ SD)	-	1510.00–4950.00; 3154.17 $\pm$ 602.1
Gestational weeks (range; mean $\pm$ SD)	-	34–42; 39.53 $\pm$ 1.8

<https://doi.org/10.1371/journal.pone.0218615.t001>

**Table 2. Multivariable regression analysis using beta model: sperm DNA methylation in relation to donor’s body mass index.**

Amplicon	Estimate	Standard error	p value
<i>MEST</i> (n = 261)	-0.004	0.009	0.661
<i>SNRPN</i> (n = 263)	-0.001	0.009	0.939
<i>NNAT</i> (n = 262)	-0.010	0.010	0.324
<i>PEG10</i> (n = 261)	-0.001	0.011	0.936
<i>H19-IG DMR</i> (n = 255)	0.012	0.009	0.191
<i>IGF2 DMR0</i> (n = 271)	0.003	0.005	0.632
<b><i>MEG3-IG DMR</i> (n = 265)</b>	<b>0.014</b>	<b>0.007</b>	<b>0.043</b>
<i>HIF3A</i> (n = 260)	0.008	0.004	0.080

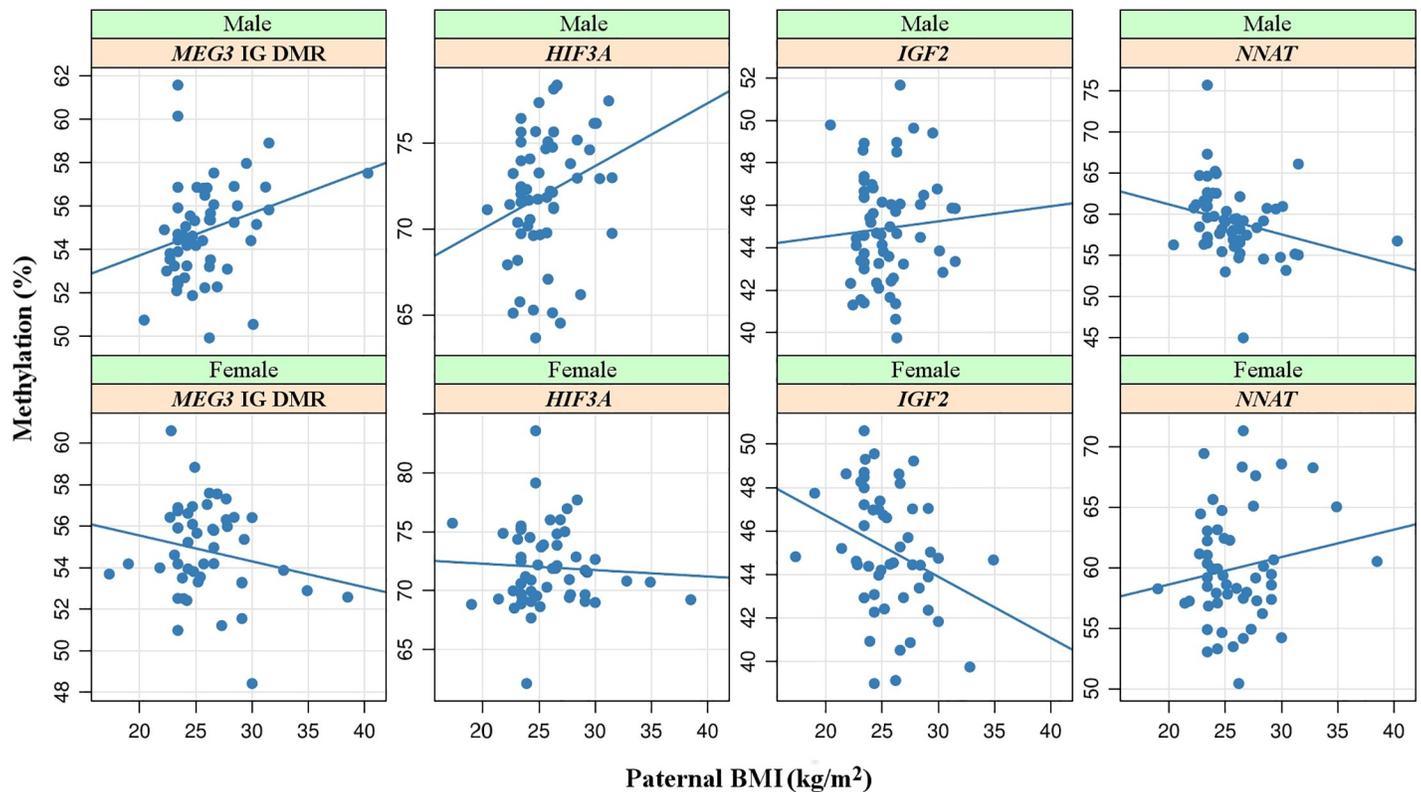
<https://doi.org/10.1371/journal.pone.0218615.t002>

which were retrieved after successful live birth. Similar to sperm samples, bisulphite pyrosequencing was also performed in the above-mentioned genes on the cord blood DNA. For FCB, multivariable regression analysis using linear models was used to adjust for potential confounding factors. Because of limited amounts of DNA, we analyzed 111 cord blood samples for *MEST*, 109 for *SNRPN*, 112 for *NNAT*, 113 for *PEG10*, 110 for *H19-IG DMR*, 110 for *IGF2 DMR0*, 103 for *MEG3-IG DMR*, and 112 for *HIF3A*. Interestingly, the influence of father’s BMI on the FCB DNA methylation varied depending on the gender of the child. Here, *MEG3-IG DMR* (regression estimate +0.002, P = 0.020) and *HIF3A* (regression estimate +0.004, P = 0.041) cord blood DNA methylation of males but not females showed a positive correlation with the BMI of the father. In addition, *IGF2 DMR0* (regression estimate -0.003, P = 0.034) showed a negative correlation with paternal BMI in female FCBs (Table 3 and Fig 1). However, none of the correlated genes survived multiple testing adjustment. It is noteworthy to mention that the methylation levels of none of the eight studied genes correlated with maternal BMI (Table 4). In additional S1 File, we present statistics without adjusting to any confounding factors. We additionally tested the extent to which DNA methylation in sperm correlates with cord blood methylation (additional S2 File). Here, the *MEG3-IG DMR* revealed the strongest correlation (p-value = 0.070) between methylation values across both studied tissues.

**Table 3. Multivariable regression analysis using linear models: Fetal cord blood DNA methylation in relation to paternal body mass index.**

Amplicon	FCB_Gender	Estimate	Standard error	p value
<i>MEST</i>	Female (n = 53)	0.003	0.002	0.296
	Male (n = 58)	0.002	0.003	0.409
<i>SNRPN</i>	Female (n = 51)	-0.001	0.001	0.276
	Male (n = 58)	-0.001	0.001	0.375
<i>NNAT</i>	Female (n = 53)	0.003	0.002	0.111
	Male (n = 59)	-0.003	0.002	0.104
<i>PEG10</i>	Female (n = 54)	-0.001	0.001	0.305
	Male (n = 59)	0.001	0.001	0.308
<i>H19-IG DMR</i>	Female (n = 52)	-0.004	0.003	0.159
	Male (n = 58)	0.002	0.003	0.447
<b><i>IGF2 DMR0</i></b>	<b>Female (n = 52)</b>	<b>-0.003</b>	<b>0.001</b>	<b>0.034</b>
	Male (n = 58)	0.000	0.002	0.809
<b><i>MEG3-IG DMR</i></b>	Female (n = 48)	0.000	0.001	0.568
	<b>Male (n = 55)</b>	<b>0.002</b>	<b>0.001</b>	<b>0.020</b>
<i>HIF3A</i>	Female (n = 54)	-0.001	0.001	0.554
	<b>Male (n = 58)</b>	<b>0.004</b>	<b>0.002</b>	<b>0.041</b>

<https://doi.org/10.1371/journal.pone.0218615.t003>



**Fig 1. Correlation of paternal BMI with cord blood DNA methylation.** Scatter plots showing the correlation between *MEG3*-IG DMR, *HIF3A*, *IGF2*, and *NNAT* methylation and the paternal BMI in male fetal cord blood (upper panel) and female fetal cord blood (lower panel) samples. Each dot in the plot represents the average methylation of several targeted CpGs in an individual cord blood sample after measurement by bisulphite pyrosequencing. Regression line suggests direction of correlation with paternal BMI. RE on each plot indicates the regression estimate.

<https://doi.org/10.1371/journal.pone.0218615.g001>

The correlation of father's body mass index ( $\text{kg}/\text{m}^2$ ) with DNA methylation (%) of eight analyzed amplicons in next-generation fetal cord blood samples. The model included birth weight and gestational week of the child, paternal and maternal age, maternal BMI, and gender of the child including the interaction between paternal BMI and child's gender to allow for a varying effect of paternal BMI between the FCB genders.

The correlation of mother's body mass index ( $\text{kg}/\text{m}^2$ ) with DNA methylation (%) of eight analyzed amplicons in next-generation fetal cord blood samples. The model included birth weight and gestational week of the child, paternal and maternal age, paternal BMI, and gender of the child including the interaction between maternal BMI and child's gender to allow for a varying effect of maternal BMI between the FCB genders. None of the analyzed amplicons showed association between cord blood methylation and maternal BMI.

## Discussion

In this study, we measured whether an increased BMI can lead to methylation changes in male sperm, which might subsequently alter the DNA methylome of the next-generation. We focused on studying potential epigenetic inheritance at imprinted genes whose DNA methylation is stably maintained through the genome-wide reprogramming phase occurring after fertilization. We quantified methylation changes in a large cohort of >290 sperm samples and in 113 fetal cord bloods. It is generally the methylation density of several contiguous CpGs in a cis-regulatory region rather than methylation at individual CpG site that turns a gene on or off

Table 4. Multivariable regression analysis using linear models: Fetal cord blood DNA methylation in relation to maternal body mass index.

Amplicon	FCB_Gender	Estimate	Standard error	p value
<i>MEST</i>	Female (n = 53)	0.002	0.002	0.426
	Male (n = 58)	-0.001	0.002	0.696
<i>SNRPN</i>	Female (n = 51)	0.000	0.001	0.890
	Male (n = 58)	-0.001	0.001	0.504
<i>NNAT</i>	Female (n = 53)	0.000	0.001	0.943
	Male (n = 59)	-0.001	0.002	0.707
<i>PEG10</i>	Female (n = 54)	0.000	0.001	0.544
	Male (n = 59)	0.001	0.001	0.537
<i>H19-IG DMR</i>	Female (n = 52)	0.000	0.002	0.901
	Male (n = 54)	-0.002	0.002	0.337
<i>IGF2 DMR0</i>	Female (n = 52)	-0.001	0.001	0.331
	Male (n = 58)	0.001	0.001	0.377
<i>MEG3-IG DMR</i>	Female (n = 48)	0.000	0.001	0.644
	Male (n = 55)	0.000	0.001	0.644
<i>HIF3A</i>	Female (n = 54)	0.000	0.001	0.825
	Male (n = 58)	0.000	0.001	0.978

<https://doi.org/10.1371/journal.pone.0218615.t004>

[25, 26]. In line with this, the designed pyrosequencing assays for all the genes systematically analyzed the methylation status of several CpGs across many thousand DNA molecules per samples. After adjusting for potential confounding factors, our analysis revealed the paternally imprinted region *MEG3-IG DMR* as one of the genes whose methylation increased (p-value = 0.043) in sperm due to an increase in the BMI levels of the donor. A similar effect was also observed in male offspring fathered by obese males where increased paternal BMI was associated with an increased methylation at *MEG3-IG DMR* (p-value = 0.020). The *MEG3-IG DMR* is positioned at the intergenic region between the maternally expressed gene 3 (*MEG3*) and delta-like 1 homolog (*DLK1*). The intergenic DMR serves as the imprinting control region for the *DLK1-MEG3* cluster and regulates gene expression and imprinting during development [27]. Recently, it has been shown that microRNAs in the *DLK1-MEG3* gene cluster are dramatically downregulated due to increased methylation at the *MEG3-DMR* in pancreatic islets of Type 2 diabetes mellitus patients [28]. The long non-coding RNA *MEG3* is also downregulated during adipogenesis and gene knockdown was shown to promote adipogenic differentiation [29].

Furthermore, we identified that DNA methylation levels of the obesity-related gene *HIF3A* showed a positive correlation with father's BMI in the cord blood of male children (p-value = 0.041). Previously, a large epigenome-wide association study of DNA methylation in adult whole blood and adipose tissue (unlike sperm and cord blood in the present study) identified a significant correlation between BMI and methylation at three CpG sites in the first intron of *HIF3A* [4]. Several studies replicated the aforementioned association at the *HIF3A* locus across different tissues including umbilical cord DNA of newborns [30–33]. While one study reported that the association between the blood DNA methylation of *HIF3A* gene and donor's BMI was stronger in young-aged population subset ( $\leq 66$  years) [34], another report did not find any correlation between the two [35]. The result of our present study contradicts with the associations observed between parental BMI and offspring *HIF3A* methylation in a study by Richmond *et al*, 2016. [36]. Here, the authors measured methylation using the Infinium HM450 BeadChip array on ~1000 samples and examined associations at the individual CpG level, however they did not implement any gender-specific testing in the offspring [36].

Furthermore, we measured methylation in cord blood DNA of children born after IVF/ICSI, which can be an additional confounding factor in our study. It is important to emphasize that previous reports, in addition to the current study, only tested for associations between *HIF3A* methylation and BMI levels, which do not indicate the causality of *HIF3A* methylation alterations to obesity. Similar to the previous studies in human blood [34–36] and in sperm [19], the data presented support the idea that DNA methylation (of *HIF3A* and other genes) was an effect rather than a cause of increased BMI.

We also observed a negative correlation (p-value = 0.034) between *IGF2* methylation in female cord blood and paternal BMI. A similar association between *IGF2* hypomethylation and paternal obesity was previously reported in neonates enrolled in the Newborn Epigenetics Study cohort [37]. As shown in Fig 1, DNA methylation at the *MEG3* IGDMMR, *HIF3A*, *IGF2*, and *NNAT* exhibited a correlation with paternal BMI however in opposite directions when comparing male and female cord bloods. The gender-specific effects observed are in line with several previously published studies [16]. In rodents, Huypens *et al.* observed gender-specific effects on body-weight gains and insulin levels in mice fathered by males fed a high-fat diet [13]. Similar gender-related differences were reported in other studies investigating the influences of parental obesity or nutrition on the offspring [9, 16, 38]. Here, we would like to emphasize that none of the reported p-values in the present study (both in sperm and in cord blood) survived adjustment for multiple testing, exhibiting only nominal significance to male BMI. This indicates that the correlations displayed are weak which might be due to the low power of the study. Consistent with earlier studies [37, 39], the observed paternal BMI effects in FCB were of small effect size. Most likely it is the sum of multiple small methylation changes, which transmits the disease susceptibility associated with adverse parental factors to the next-generation.

Cord blood DNA methylation levels in none of our studied genes exhibited a significant correlation with maternal BMI values, which contrasts with another study [40]. Here, the authors systematically meta-analyzed the correlations between pre-conceptional maternal BMI and DNA methylation sites (450K) in the cord blood of the offspring. After adjusting for cell proportions, the number of genome-wide significant CpGs was reported to be only 104, however with a very small effect size/methylation variation [40]. Most importantly, none of the analyzed imprinted regions from our study harbored a differentially methylated CpG with genome-wide significance, thus possibly indicating that maternal BMI is not influencing methylation at the studied imprinted genes in cord blood DNA. As spermatogonial stem cells divide constantly to self-renew and differentiate, one might speculate that sperm cells are more prone to epigenetic errors during cell division in contrast to oocytes, which are arrested at an early stage of the first meiotic division. It is noteworthy to mention that the cord blood DNA methylation measurements performed in this study and in all previous studies represent the average methylation of both parental alleles. Paternal BMI effects on the paternal allele may be covered up by variation of maternal allele methylation or vice versa. Ideally, one would like to separately delineate paternal versus maternal allele methylation in somatic tissues of the offspring.

Male infertility and low-quality spermogram parameters are the clinical factors frequently associated with methylation alterations at imprinted genes in sperm [41, 42], which is one limitation associated with our study cohort. Nevertheless, we have corrected for ART success and analyzed methylation in a large number of samples, which allowed us to delineate minor methylation changes linked to obesity in sperm. A recent report by Soubry *et al.* identified methylation abnormalities at several imprinted genes in sperm of 23 overweight/obese fertile males when compared to 44 fertile controls [17]. In their study, *MEG3*-IG DMR was similarly reported to have an increased methylation in the sperm of obese males, which is in line with our findings. Furthermore, this confirms that the identified DNA methylation changes at

*MEG3*-IG DMR are linked to obesity since they also occur in fertile males. Our study additionally provides evidence for a gender-specific positive correlation in *MEG3*-IG DMR between male cord blood methylation and father's BMI indicating possible inheritance of methylation signatures. Several studies have reported an association between methylation changes at imprinted genes in the fetus and exposure to maternal smoking during development [43]. It is important to note that we did not include parental smoking as a confounding factor in our multivariate regression analysis as we do not have reliable information regarding smoking habits in our studied cohort.

## Conclusions

In this study, we focused on epigenetic changes in sperm in relation to donor's BMI and whether these changes can be transmitted to the offspring. We identified subtle methylation alterations correlating with donor's BMI in male sperm at the paternally imprinted *MEG3*-IG DMR locus. A gender-specific correlation between the DNA methylation of *MEG3*-IG DMR, *HIF3A*, and *IGF2* DMR0 in the cord blood of the offspring and paternal BMI was observed. Our results indicate that obesity in males can influence DNA methylation programming in sperm and also affect the epigenome of the next-generation. These findings are in line with earlier research in humans [17, 19, 37, 39]. Epigenetic inheritance might help explain the escalating incidence of obesity worldwide, which cannot be attributed to genetic factors on their own. Nevertheless, it remains to be shown whether those changes can influence the risk of developing obesity in the offspring during adulthood. A longitudinal follow-up of our studied cohort is needed to help answer this question.

## Supporting information

**S1 Table. PCR and sequencing primers used for bisulphite pyrosequencing.** S1 Table shows the forward and reverse primers for polymerase chain reaction and bisulphite pyrosequencing for all the eight amplicons analyzed in this study. Primers indicated by a star are biotinylated at the 5' end and the chromosomal location is based on Ensembl release 89.

(DOC)

**S1 File. DNA methylation correlations without adjusting to confounding factors.** S1 File shows the methylation statistics when DNA methylation is correlated with BMI without adjusting to any confounding factors.

(XLS)

**S2 File. DNA methylation correlation between sperm and cord blood.** S2 File shows the spearman's rank correlation of DNA methylation levels in sperm and corresponding cord blood samples.

(XLS)

## Acknowledgments

We thank all the couples and obstetric clinics participating in the study.

## Author Contributions

**Conceptualization:** Thomas Haaf, Nady El Hajj.

**Formal analysis:** Marcus Dittrich.

**Funding acquisition:** Thomas Haaf, Nady El Hajj.

**Investigation:** Ramya Potabattula.

**Methodology:** Ramya Potabattula.

**Resources:** Martin Schorsch, Thomas Hahn.

**Supervision:** Nady El Hajj.

**Validation:** Ramya Potabattula.

**Writing – original draft:** Ramya Potabattula, Thomas Haaf, Nady El Hajj.

**Writing – review & editing:** Ramya Potabattula, Thomas Haaf, Nady El Hajj.

## References

1. van Vliet-Ostaptchouk JV, Snieder H, Lagou V. Gene-Lifestyle Interactions in Obesity. *Current nutrition reports*. 2012; 1:184–96. <https://doi.org/10.1007/s13668-012-0022-2> PMID: 24392269; PubMed Central PMCID: PMC3873060.
2. Elks CE, den Hoed M, Zhao JH, Sharp SJ, Wareham NJ, Loos RJ, et al. Variability in the heritability of body mass index: a systematic review and meta-regression. *Frontiers in endocrinology*. 2012; 3:29. <https://doi.org/10.3389/fendo.2012.00029> PMID: 22645519; PubMed Central PMCID: PMC3355836.
3. Locke AE, Kahali B, Berndt SI, Justice AE, Pers TH, Day FR, et al. Genetic studies of body mass index yield new insights for obesity biology. *Nature*. 2015; 518(7538):197–206. <https://doi.org/10.1038/nature14177> PMID: 25673413; PubMed Central PMCID: PMC4382211.
4. Dick KJ, Nelson CP, Tsaprouni L, Sandling JK, Aissi D, Wahl S, et al. DNA methylation and body-mass index: a genome-wide analysis. *Lancet*. 2014; 383(9933):1990–8. [https://doi.org/10.1016/S0140-6736\(13\)62674-4](https://doi.org/10.1016/S0140-6736(13)62674-4) PMID: 24630777.
5. Kuhnen P, Handke D, Waterland RA, Hennig BJ, Silver M, Fulford AJ, et al. Interindividual Variation in DNA Methylation at a Putative POMC Metastable Epiallele Is Associated with Obesity. *Cell Metab*. 2016; 24(3):502–9. <https://doi.org/10.1016/j.cmet.2016.08.001> PMID: 27568547.
6. Daxinger L, Whitelaw E. Understanding transgenerational epigenetic inheritance via the gametes in mammals. *Nat Rev Genet*. 2012; 13(3):153–62. <https://doi.org/10.1038/nrg3188> PMID: 22290458.
7. Rando OJ, Verstrepen KJ. Timescales of genetic and epigenetic inheritance. *Cell*. 2007; 128(4):655–68. <https://doi.org/10.1016/j.cell.2007.01.023> PMID: 17320504.
8. El Hajj N, Schneider E, Lehnen H, Haaf T. Epigenetics and life-long consequences of an adverse nutritional and diabetic intrauterine environment. *Reproduction*. 2014; 148(6):R111–20. <https://doi.org/10.1530/REP-14-0334> PMID: 25187623; PubMed Central PMCID: PMC4241689.
9. Chen YP, Xiao XM, Li J, Reichetzeder C, Wang ZN, Hochar B. Paternal body mass index (BMI) is associated with offspring intrauterine growth in a gender dependent manner. *PloS one*. 2012; 7(5):e36329. <https://doi.org/10.1371/journal.pone.0036329> PMID: 22570703; PubMed Central PMCID: PMC3343080.
10. Freeman E, Fletcher R, Collins CE, Morgan PJ, Burrows T, Callister R. Preventing and treating childhood obesity: time to target fathers. *International journal of obesity*. 2012; 36(1):12–5. <https://doi.org/10.1038/ijo.2011.198> PMID: 22005717.
11. Kaati G, Bygren LO, Edvinsson S. Cardiovascular and diabetes mortality determined by nutrition during parents' and grandparents' slow growth period. *European journal of human genetics:EJHG*. 2002; 10(11):682–8. <https://doi.org/10.1038/sj.ejhg.5200859> PMID: 12404098.
12. Kaati G, Bygren LO, Pembrey M, Sjöström M. Transgenerational response to nutrition, early life circumstances and longevity. *European journal of human genetics:EJHG*. 2007; 15(7):784–90. <https://doi.org/10.1038/sj.ejhg.5201832> PMID: 17457370.
13. Huypens P, Sass S, Wu M, Dyckhoff D, Tschop M, Theis F, et al. Epigenetic germline inheritance of diet-induced obesity and insulin resistance. *Nature genetics*. 2016; 48(5):497–9. <https://doi.org/10.1038/ng.3527> PMID: 26974008.
14. Carone BR, Fauquier L, Habib N, Shea JM, Hart CE, Li R, et al. Paternally induced transgenerational environmental reprogramming of metabolic gene expression in mammals. *Cell*. 2010; 143(7):1084–96. <https://doi.org/10.1016/j.cell.2010.12.008> PMID: 21183072; PubMed Central PMCID: PMC3039484.
15. Sharma U, Rando OJ. Metabolic Inputs into the Epigenome. *Cell Metab*. 2017; 25(3):544–58. <https://doi.org/10.1016/j.cmet.2017.02.003> PMID: 28273477.

16. Ng SF, Lin RC, Laybutt DR, Barres R, Owens JA, Morris MJ. Chronic high-fat diet in fathers programs beta-cell dysfunction in female rat offspring. *Nature*. 2010; 467(7318):963–6. <https://doi.org/10.1038/nature09491> PMID: 20962845.
17. Soubry A, Guo L, Huang Z, Hoyo C, Romanus S, Price T, et al. Obesity-related DNA methylation at imprinted genes in human sperm: Results from the TIEGER study. *Clinical epigenetics*. 2016; 8:51. <https://doi.org/10.1186/s13148-016-0217-2> PMID: 27158277; PubMed Central PMCID: PMC4859994.
18. Atsem S, Reichenbach J, Potabattula R, Dittrich M, Nava C, Depienne C, et al. Paternal age effects on sperm FOXX1 and KCNA7 methylation and transmission into the next generation. *Hum Mol Genet*. 2016; 25(22):4996–5005. <https://doi.org/10.1093/hmg/ddw328> PMID: 28171595; PubMed Central PMCID: PMC5418740.
19. Donkin I, Verstehey S, Ingerslev LR, Qian K, Mechta M, Nordkap L, et al. Obesity and Bariatric Surgery Drive Epigenetic Variation of Spermatozoa in Humans. *Cell Metab*. 2016; 23(2):369–78. <https://doi.org/10.1016/j.cmet.2015.11.004> PMID: 26669700.
20. Reik W, Dean W, Walter J. Epigenetic reprogramming in mammalian development. *Science*. 2001; 293(5532):1089–93. <https://doi.org/10.1126/science.1063443> PMID: 11498579.
21. Reik W, Walter J. Genomic imprinting: parental influence on the genome. *Nat Rev Genet*. 2001; 2(1):21–32. <https://doi.org/10.1038/35047554> PMID: 11253064.
22. Moore T, Reik W. Genetic conflict in early development: parental imprinting in normal and abnormal growth. *Reviews of reproduction*. 1996; 1(2):73–7. PMID: 9414442.
23. Peters J. The role of genomic imprinting in biology and disease: an expanding view. *Nat Rev Genet*. 2014; 15(8):517–30. <https://doi.org/10.1038/nrg3766> PMID: 24958438.
24. Cribari-Neto F, Zeileis A. Beta Regression in R. 2010. 2010; 34(2):24. Epub 2010-04-05. <https://doi.org/10.18637/jss.v034.i02>
25. Sontag LB, Lorincz MC, Georg Luebeck E. Dynamics, stability and inheritance of somatic DNA methylation imprints. *J Theor Biol*. 2006; 242(4):890–9. <https://doi.org/10.1016/j.jtbi.2006.05.012> PMID: 16806276.
26. Weber M, Hellmann I, Stadler MB, Ramos L, Paabo S, Rebhan M, et al. Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nature genetics*. 2007; 39(4):457–66. <https://doi.org/10.1038/ng1990> PMID: 17334365.
27. Takada S, Paulsen M, Tevendale M, Tsai CE, Kelsey G, Cattanach BM, et al. Epigenetic analysis of the Dlk1-Gtl2 imprinted domain on mouse chromosome 12: implications for imprinting control from comparison with Igf2-H19. *Hum Mol Genet*. 2002; 11(1):77–86. <https://doi.org/10.1093/hmg/11.1.77> PMID: 11773001.
28. Kameswaran V, Bramswig NC, McKenna LB, Penn M, Schug J, Hand NJ, et al. Epigenetic regulation of the DLK1-MEG3 microRNA cluster in human type 2 diabetic islets. *Cell Metab*. 2014; 19(1):135–45. <https://doi.org/10.1016/j.cmet.2013.11.016> PMID: 24374217; PubMed Central PMCID: PMC3932527.
29. Li Z, Jin C, Chen S, Zheng Y, Huang Y, Jia L, et al. Long non-coding RNA MEG3 inhibits adipogenesis and promotes osteogenesis of human adipose-derived mesenchymal stem cells via miR-140-5p. *Molecular and cellular biochemistry*. 2017; 433(1–2):51–60. <https://doi.org/10.1007/s11010-017-3015-z> PMID: 28382492.
30. Pan H, Lin X, Wu Y, Chen L, Teh AL, Soh SE, et al. HIF3A association with adiposity: the story begins before birth. *Epigenomics*. 2015; 7(6):937–50. <https://doi.org/10.2217/epi.15.45> PMID: 26011824; PubMed Central PMCID: PMC4863876.
31. Demerath EW, Guan W, Grove ML, Aslibekyan S, Mendelson M, Zhou YH, et al. Epigenome-wide association study (EWAS) of BMI, BMI change and waist circumference in African American adults identifies multiple replicated loci. *Hum Mol Genet*. 2015; 24(15):4464–79. <https://doi.org/10.1093/hmg/ddv161> PMID: 25935004; PubMed Central PMCID: PMC4492394.
32. Ronn T, Volkov P, Gillberg L, Kokosar M, Perfilyev A, Jacobsen AL, et al. Impact of age, BMI and HbA1c levels on the genome-wide DNA methylation and mRNA expression patterns in human adipose tissue and identification of epigenetic biomarkers in blood. *Hum Mol Genet*. 2015; 24(13):3792–813. <https://doi.org/10.1093/hmg/ddv124> PMID: 25861810.
33. Pfeiffer S, Kruger J, Maierhofer A, Bottcher Y, Kloting N, El Hajj N, et al. Hypoxia-inducible factor 3A gene expression and methylation in adipose tissue is related to adipose tissue dysfunction. *Scientific reports*. 2016; 6:27969. <https://doi.org/10.1038/srep27969> PMID: 27346320; PubMed Central PMCID: PMC4921806.
34. Mendelson MM, Marioni RE, Joehanes R, Liu C, Hedman AK, Aslibekyan S, et al. Association of Body Mass Index with DNA Methylation and Gene Expression in Blood Cells and Relations to Cardiometabolic Disease: A Mendelian Randomization Approach. *PLoS Med*. 2017; 14(1):e1002215. <https://doi.org/10.1371/journal.pmed.1002215> PMID: 28095459; PubMed Central PMCID: PMC5240936 not an employee when the work was conducted. El is a scientific advisor and consultant for Precision

Wellness, Inc. and scientific advisor for Cellink for work unrelated to this paper. IJD has research grants from Age UK and the UK Medical Research Council.

35. Wahl S, Drong A, Lehne B, Loh M, Scott WR, Kunze S, et al. Epigenome-wide association study of body mass index, and the adverse outcomes of adiposity. *Nature*. 2017; 541(7635):81–6. <https://doi.org/10.1038/nature20784> PMID: 28002404; PubMed Central PMCID: PMC5570525.
36. Richmond RC, Sharp GC, Ward ME, Fraser A, Lyttleton O, McArdle WL, et al. DNA Methylation and BMI: Investigating Identified Methylation Sites at HIF3A in a Causal Framework. *Diabetes*. 2016; 65(5):1231–44. <https://doi.org/10.2337/db15-0996> PMID: 26861784; PubMed Central PMCID: PMC4839211.
37. Soubry A, Schildkraut JM, Murtha A, Wang F, Huang Z, Bernal A, et al. Paternal obesity is associated with IGF2 hypomethylation in newborns: results from a Newborn Epigenetics Study (NEST) cohort. *BMC medicine*. 2013; 11:29. <https://doi.org/10.1186/1741-7015-11-29> PMID: 23388414; PubMed Central PMCID: PMC3584733.
38. Bygren LO, Tinghog P, Carstensen J, Edvinsson S, Kaati G, Pembrey ME, et al. Change in paternal grandmothers' early food supply influenced cardiovascular mortality of the female grandchildren. *BMC genetics*. 2014; 15:12. <https://doi.org/10.1186/1471-2156-15-12> PMID: 24552514; PubMed Central PMCID: PMC3929550.
39. Soubry A, Murphy SK, Wang F, Huang Z, Vidal AC, Fuemmeler BF, et al. Newborns of obese parents have altered DNA methylation patterns at imprinted genes. *International journal of obesity*. 2015; 39(4):650–7. <https://doi.org/10.1038/ijo.2013.193> PMID: 24158121; PubMed Central PMCID: PMC4048324.
40. Sharp GC, Salas LA, Monnereau C, Allard C, Yousefi P, Everson TM, et al. Maternal BMI at the start of pregnancy and offspring epigenome-wide DNA methylation: findings from the pregnancy and childhood epigenetics (PACE) consortium. *Hum Mol Genet*. 2017; 26(20):4067–85. <https://doi.org/10.1093/hmg/ddx290> PMID: 29016858; PubMed Central PMCID: PMC5656174.
41. El Hajj N, Zechner U, Schneider E, Tresch A, Gromoll J, Hahn T, et al. Methylation status of imprinted genes and repetitive elements in sperm DNA from infertile males. *Sexual development: genetics, molecular biology, evolution, endocrinology, embryology, and pathology of sex determination and differentiation*. 2011; 5(2):60–9. <https://doi.org/10.1159/000323806> PMID: 21293114.
42. Poplinski A, Tuttelmann F, Kanber D, Horsthemke B, Gromoll J. Idiopathic male infertility is strongly associated with aberrant methylation of MEST and IGF2/H19 ICR1. *International journal of andrology*. 2010; 33(4):642–9. <https://doi.org/10.1111/j.1365-2605.2009.01000.x> PMID: 19878521.
43. Joubert BR, Felix JF, Yousefi P, Bakulski KM, Just AC, Breton C, et al. DNA Methylation in Newborns and Maternal Smoking in Pregnancy: Genome-wide Consortium Meta-analysis. *Am J Hum Genet*. 2016; 98(4):680–96. <https://doi.org/10.1016/j.ajhg.2016.02.019> PMID: 27040690; PubMed Central PMCID: PMC4833289.