Lifestyle intervention in pregnant women with obesity impacts cord blood DNA methylation which associates with body composition in the offspring

Josefine Jönsson[1*], Kristina M. Renault[2,3*], Sonia García-Calzón[1,4], Alexander Perfilyev[1], Angela C. Estampador[5], Kirsten Nørgaard[6], Mads Vendelbo Lind[7], Allan Vaag[6], Line Hjort[8], Kim F. Michaelsen[7], Emma Malchau Carlsen[7,9], Paul W. Franks[5†] and Charlotte Ling[1†]

[1] Epigenetics and Diabetes Unit, Department of Clinical Sciences, Lund University Diabetes Centre, Lund University, Scania University Hospital, Malmö, Sweden

[2] Department of Obstetrics and Gynecology, Hvidovre Hospital, University of Copenhagen, Copenhagen, Denmark.

[3] Obstetric Clinic, Juliane Marie Centret, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark.

[4] Department of Nutrition, Food Sciences and Physiology, University of Navarra, Pamplona, Spain

[5] Genetic and Molecular Epidemiology Unit, Department of Clinical Sciences, Lund University Diabetes Centre, Lund University, Malmö, Sweden

[6] Steno Diabetes Center Copenhagen, Gentofte, Denmark

[7] Department of Nutrition, Exercise and Sports, Faculty of Science, University of Copenhagen, Frederiksberg, Denmark

[8] Departments of Obstetrics, Center for Pregnant Women with Diabetes, Rigshospitalet, Copenhagen, Denmark

[9] Department of Pediatrics, Copenhagen University Hospital Hvidovre, Hvidovre, Denmark.

*Shared first authors, [†]Shared last authors

Corresponding authors: Josefine Jönsson and Charlotte Ling, Lund University Epigenetics and Diabetes, Clinical Research Centre 91:12, Box 50332, 202 13 Malmö, Sweden. Phone: +46 40 39 12 11; e-mail: josefin.jonsson@med.lu.se and charlotte.ling@med.lu.se

Running title: Lifestyle alters DNA methylation in cord blood

ABSTRACT

Maternal obesity may lead to epigenetic alterations in the offspring and might thereby contribute to disease later in life. We investigated whether a lifestyle intervention in pregnant women with obesity is associated with epigenetic variation in cord blood and body composition in the offspring. Genome-wide DNA methylation was analyzed in cord blood from 208 offspring from the TOP-study, which includes pregnant women with obesity randomized to lifestyle interventions comprised of physical activity with or without dietary advice versus controls (standard of care). DNA methylation was altered at 379 sites, annotated to 370 genes, in cord blood from offspring of mothers following a lifestyle intervention versus controls (*FDR*<5%) when using the Houseman reference-free method to correct for cell composition and 3 of these sites were significant based on Bonferroni correction. These 370 genes are overrepresented in gene ontology terms including response to fatty acids and adipose tissue development. Offspring of mothers included in a lifestyle intervention were born with more lean mass compared to controls. Methylation at 17 sites, annotated to e.g. *DISC1*, *GBX2*, *HERC2* and *HUWE1*, partially mediates the effect of the lifestyle intervention on lean mass in the offspring (FDR<5%). Moreover, 22 methylation sites were associated with offspring BMI z-scores during the first 3 years of life ($p<0.05$). Overall, lifestyle interventions in pregnant women with obesity are associated with epigenetic changes in offspring, potentially influencing the offspring's lean mass and early growth.

Obesity and type 2 diabetes are on the rise worldwide, as is the prevalence of obesity in pregnant women (1). Obesity during pregnancy increases the risk of adverse health outcomes in the offspring including macrosomia and childhood obesity (2), which might be explained by a metabolically adverse intrauterine environment. The prevalence of childhood obesity, which is associated with an increased risk of adulthood obesity (3), metabolic syndrome (4) and early death (5), more than doubled between 1980 and 2015 (6). Greater increase in weight and height during infancy is associated with greater lean mass and lower risk of the metabolic syndrome in adulthood (7, 8). Hence, greater lean mass during infancy might protect against future metabolic disease.

As gestational weight gain (GWG) affects the health of the mother and offspring, the Institute of Medicine recommends women with pre-pregnancy BMI>30kg/m^2 to limit their GWG to 5-9kg (1). We have reported that GWG can be reduced by lifestyle interventions (9) and was positively associated with fat mass in infants born to mothers with obesity (10, 11), and with carbohydrate intake in late pregnancy (12). Subsequently, lifestyle interventions might improve the cardiometabolic profile of pregnant mothers with obesity and their offspring.

Epigenetic alterations, such as DNA methylation, may occur following intrauterine perturbations caused by obesity and excess GWG (13, 14). DNA methylation regulates gene expression, X-chromosome inactivation, imprinting and cell differentiation (13). Intrauterine epigenetic alterations may therefore affect future health outcomes in the offspring (15). For example, early maternal exposures such as gestational diabetes, pre-gestational obesity and famine were linked to dysregulated gene function in early life by altered DNA methylation (16-19), and dietary interventions during pregnancy impact the offspring epigenome (20). However, to our knowledge, it remains unknown whether interventions in pregnant women

with obesity affect the epigenetic pattern in cord blood, and whether this is associated with body composition and growth in their offspring.

Our aim was to investigate whether a lifestyle intervention including physical activity with and without advice on a low-energy Mediterranean-style diet in pregnant women with obesity from the Treatment of Obese Pregnant women (TOP)-study (9) is associated with DNA methylation alterations in offspring cord blood. We then tested whether specific epigenetic marks in cord blood are associated with body composition in the offspring at birth and growth during the first three years of life.

RESEARCH DESIGN AND METHODS

Design and clinical data of the TOP-study

The TOP-study was approved by the Ethics Committee for the Capital Region of Denmark (January 2009, H-D-2008-119) and registered at ClinicalTrials.gov (NCT01345149). Before enrolment written informed consent was obtained from all participants.

The TOP-study is a randomized controlled trial of 425 pregnant women with obesity including two lifestyle intervention groups: PA+D, physical activity assessed with pedometer and dietary advice; and PA, physical activity assessed with pedometer; and C, a control arm receiving standard of care (**Figure 1**). The primary endpoint was to assess the impact of these lifestyle interventions on GWG (9). All participants, including controls, had a consultation with a dietitian whom recommended a low-energy and low-fat Mediterranean-style diet of 1200-1675 kcal, based on Danish national recommendations, and they were encouraged to limit GWG to ≤5kg. Participants in PA+D had regular contact/visits (every 2 weeks) with an experienced dietitian giving dietary advice and measuring weight. Participants in both PA+D

and PA were encouraged to obtain 11,000 steps daily. We based our physical activity recommendation on our previous study of step counts among pregnant women and added 50% to increase physical activity (21). If this was not achievable, they were asked to set their own goal.

Due to participants having miscarriages, withdrawing from the study and moving from the region, 389 women completed the study (**Figure 1**). Maternal age, pre-pregnancy BMI, maternal educational level, and previous childbirths (parity) were recorded at enrolment (week 11-14). Smoking during pregnancy was acquired through medical records, GWG was determined by subtracting self-reported pre-pregnancy weight with weight measured during week 36-37, and energy intake was attained from self-administered validated Food Frequency Questionnaires at weeks 11-14 and 36-38. Detailed information on enrolment, conduction of the trial and clinical measurements are reported elsewhere (9, 12).

Dual-energy X-ray absorptiometry scans were performed within 48h of birth to assess offspring body composition. Offspring measurements of interest for this study were: lean mass at birth and growth, where BMI z-scores at birth and at 9, 18 and 36 months of age were used (**Figure 1**). Data collection and body composition assessment in the offspring is further described (10, 22).

Cord blood was collected from the umbilical vein of the clamped umbilical cord at birth. Samples were immediately frozen (-80°C) and stored in a biobank at Copenhagen University Hospital Hvidovre. Whole cord blood samples were available for 232 participants (**Figure 1**).

DNA methylation analyses

Cord blood DNA was extracted using QiaAmp 96 DNA Blood Kit. DNA concentration and purity were determined using NanoDrop (NanoDrop Technologies). Eight samples had too low DNA concentrations. Bisulfite conversion was performed using the EZ 96-DNA methylation kit (Zymo Research Corporation, Irvine, CA, USA) according to the manufacturer's instructions. Six samples failed bisulfite conversion. DNA methylation was measured using Illumina Infinium HumanMethylation450 BeadChip (Illumina, San Diego, CA, USA), covering 485,577 sites (23). Illumina iScan was used to image the Infinium HumanMethylation450K BeadChips. Three samples were removed due to missing data regarding GWG (a covariate in the methylation model). Preprocessing was performed using R (24)(version 3.5.1), lumi (25) and methylumi (26) packages from Bioconductor. β-values were calculated as β=intensity of the Methylated allele (M)/(intensity of the Unmethylated allele (U)+intensity of the Methylated allele (M)+100). 1739 probes with mean detection *p*-value>0.01, 64 rs probes, 3089 ch-probes targeting non-CpG sites, 80 Y-chromosome probes, 26,772 cross-reactive probes and 436 polymorphic probes with MAF>0.1 were filtered out (27). Methylation data was obtained for 453,397 probes. For further analyses, M-values were used, calculated following the formula M=log2(β/(1-β)) (28). Background correction and quantile normalization were performed. Beta-Mixture Quantile (BMIQ) normalization method (29) was applied. ComBat was applied to correct for batch effects (30, 31). Principal component analysis (PCA) before and after applying ComBat ensures that between array batch effects were removed. Seven participants were further excluded as these samples clustered to the wrong sex in the PCA. Overall, DNA methylation data was available for 208 participants (**Figure 1** and **Table 1**). Additional gene annotation was performed using hg38, GENCODE version 22.

Statistical analyses

We performed a power calculation using R package pwrEWAS (https://bioconductor.org/packages/devel/bioc/html/pwrEWAS.html). To find 10% difference in methylation between two groups (ratio 1:2) with 75% power, we needed 230 participants. Based on this power calculation, the fact that GWG, the primary endpoint of the TOP-study, did not differ between the two lifestyle intervention groups and the modest number of samples with cord blood in each lifestyle intervention group, we decided to combine the two lifestyle intervention groups. We subsequently investigated the impact of PA+D together with PA (lifestyle intervention group, n=135) versus C (control group, n=73) on all investigated parameters (**Figure 1**).

To test if cord blood DNA methylation is associated with lifestyle intervention assignment, a linear regression model adjusted for maternal age (years), pre-pregnancy BMI (kg/m^2), GWG (kg), gestational age (GA, weeks) and offspring sex was run. Adjustment for cell composition was done using the Houseman reference-free method (32). We also used the reference-based method to adjust for cell composition (33). We additionally performed linear regression including the same variables as above and calculated principal components (PCs) of the residuals from this model. The top 5 PCs were then used as covariates to correct for possible inflation, technical variation and cell type composition.

Linear regression was used to assess whether cord blood DNA methylation (at sites significantly different between lifestyle intervention and control groups) is associated with lean mass at birth. Variables with a $p<0.25$ in univariate analyses were incorporated in the final regression models (34), which included the following covariates; maternal smoking during pregnancy, GA and offspring sex as well as factors with putative biological impact on DNA methylation (GWG and pre-pregnancy BMI). In this linear regression model, lean mass

was the dependent variable and DNA methylation of respective site was the independent variable.

To assess if methylation in cord blood (at sites significantly different between the lifestyle intervention and control groups) is associated with growth in the offspring (at birth, and at 9, 18 and 36 months of age), linear mixed-models (LMMs) for repeated measurements were performed with random intercepts and different fixed slopes of BMI z-scores for lifestyle intervention and control groups. BMI z-scores, which is weight relative to height and adjusted for age and sex of the child were calculated according to World Health Organization, using the *anthro* package in R (https://CRAN.R-project.org/package=anthro). Variables with $p<0.25$ in univariate analyses were incorporated in final models (34). Models were adjusted for maternal education level, maternal smoking during pregnancy, GA, and parity, as well as factors with a likely biological impact on methylation (GWG, pre-pregnancy BMI, breastfeeding exclusively and partially (weeks) and offspring age at measurement). Association signals for specific methylation sites were considered significant ($p<0.05$) if the direction of effect in the LMMs was consistent with at least three of the four time points in regular linear regression models.

Spearman correlations between CRP levels in pregnant mothers and cord blood DNA methylation of significant sites were performed. Grubbs's test was used to detect outliers.

Statistical analyses were performed using the software R (24)(version 3.6.1) and RStudio (https://www.rstudio.com). Data are presented as mean±standard deviation, unless stated otherwise. Normalized methylation β-values were used for Spearman correlations and the analyses related to offspring body composition. Unless stated otherwise, models were

corrected for multiple testing using FDR (Benjamini–Hochberg) where FDR<5% ($q<0.05$) was considered significant.

Gene Ontology (GO) analysis

To analyze possible biological functions of differential DNA methylation found in cord blood, we performed GO mapping using Generic GO Term Mapper (35) and GO analysis using the gometh function in the missMethyl package (36). For GO mapping we used Process Ontology in Homo sapiens, GOA slim, the list of the annotated genes and $p<0.01$. For GO analysis, we entered a list of the significantly associated methylation sites, and removed redundant GO terms using REViGO (37). We allowed for 50% similarity between different GO terms, used Homo sapiens database and SimRel as the semantic similarity measure.

Causal mediation analysis

We performed a nonparametric causal mediation analysis, using the mediation R package and default settings (38), to investigate whether DNA methylation of any of the identified 25 sites in cord blood found to be associated with lean mass are part of a pathway though which the lifestyle intervention exerts its effects on lean mass. The effect is estimated for each association between treatment and outcome in participants with different methylation levels. DNA methylation of each respective site was designated as the mediator and lean mass as outcome. The models were adjusted for GWG, maternal BMI, GA and offspring sex.

DNA methylation in muscle and adipose tissue

Sites showing differential cord blood methylation from the lifestyle intervention versus controls were also investigated in blood, muscle and adipose tissue from participants in the Monozygotic Twin cohort. Infinium DNA methylation data from blood, adipose tissue and

muscle of the Monozygotic Twin cohort has been published (39, 40). We used Spearman correlations to test whether methylation in blood correlates with methylation in muscle or adipose tissue for sites showing differential DNA methylation in cord blood from the TOP-study.

Data and Resource Availability

DNA methylation data from cord blood of the TOPS study (accession number LUDC2020.08.14) are deposited in the LUDC repository (https://www.ludc.lu.se/resources/repository) and are available upon request.

RESULTS

Impact of a lifestyle intervention during pregnancy on DNA methylation in cord blood

To assess if a lifestyle intervention in pregnant mothers with obesity had an effect on the methylome in cord blood, we analyzed DNA methylation in participants of the TOP-study. Baseline characteristics of pregnant women with obesity included in the lifestyle intervention and control groups of the TOP-study, paternal BMI, as well as for their offspring at birth, are shown in **Table 1**. At enrolment, there was no difference in energy intake between the lifestyle intervention and controls (**Table 1**). Mothers in the lifestyle intervention group had a trend towards lower energy intake versus controls at weeks 36-38 supporting good adherence to the dietary intervention (**Supplemental Figure S1**). During week 17 of pregnancy, daily step counts were 8623±2615 for participants in the lifestyle intervention. As wearing the pedometer was part of the lifestyle intervention, no step counts were available for controls. Offspring were similar regarding weight, length and GA at birth and no detectable difference between the groups regarding breastfeeding (**Table 1**) or BMI z-scores at birth or 9 or 36 months of age (**Table 2**).

We next examined if cord blood DNA methylation at individual sites differed between lifestyle intervention and control groups. DNA methylation at 379 sites (*q*<0.05) as seen in **Figure 2A** representing the distribution of methylation sites across the genome, annotated to 370 unique genes, was different between lifestyle intervention and control groups when adjustment for cell composition was done using the Houseman reference-free method (32) and Benjamini–Hochberg FDR analysis was used to correct for multiple testing (**Supplemental Table S1**). 3 of these sites were significant based on Bonferroni correction. None of these 379 significant sites had been associated with DNA methylation signatures related to cell composition in cord blood and methylation of 376 of the 379 sites was associated with the lifestyle intervention when adjustment for cell composition was done using the reference-based method (**Supplemental Table S1,** $p=5.7x10^{-7}$- $3x10^{-2}$) (33). Moreover, methylation of 377 out of 379 sites was associated with the lifestyle intervention after adjusting for the first top 5 PCs of the residuals (**Supplemental Table S1,** $p=3.1x10^{-9}$- $4.7x10^{-2}$). All these 377 sites had FDR<5% when we performed Benjamini–Hochberg FDR analysis on 379 sites. The lifestyle intervention was associated with methylation of these sites also when adjusting for fewer covariates and when adjusting for smoking (*p*<0.05, **Supplemental Table S1**), suggesting that these covariates did not substantially influence the association. Moreover, since GWG has been associated with DNA methylation in cord blood (14), we tested if GWG was associated with methylation of the 379 sites. However, no methylation sites were associated with GWG (*q*<0.05). We also performed a model where we adjusted for maternal age, maternal BMI, smoking, gestational age as well as offspring sex and then 377 of 379 sites remained significant (**Supplemental Table S1,** $p=1.3x10^{-8}$- $2.4x10^{-4}$).

To understand the biological role of the 370 genes, we used GO Term mapper and found that ~60% of the genes with differently methylated sites are involved in metabolic processes (**Supplemental Table S2**). Moreover, performing GO and REViGO analyses we found 15 biological processes (p<0.01). These include response to fatty acids, adipose tissue development, and negative regulation of insulin secretion involved in cellular response to glucose stimulus (**Figure 2B** and **Supplemental Table S3**) (35, 37).

Using the mQTL database (http://www.mqtldb.org), we found that cord blood methylation of 110 of our 379 sites has been associated with SNPs, so called mQTLs (**Supplemental Table S4**). These include cg21753618, which is among the 3 sites significant based on Bonferroni correction. Several of these sites also appear as mQTLs in peripheral blood in children and their mothers during, childhood, adolescence, pregnancy and middle age (**Supplemental Table S5** and **S6**). Among these mQTLs, 18 SNPs were associated with disease traits in the genome-wide association studies (GWAS) Catalogue (**Supplemental Table S4**). Moreover, we found that methylation at 56 of the 110 mQTLs are associated with type 2 diabetes, obesity, maternal stress and sperm viability in previous epigenome-wide association studies (https://bigd.big.ac.cn/ewas/datahub/index) (**Supplemental Table S7**).
We then tested if SNPs that map to any of the 370 genes included in **Supplemental Table S1** have been associated with birthweight, childhood obesity, obesity, adiposity or type 2 diabetes in published GWAS (41). Sixteen genes annotated to 15 sites (**Supplemental Table S8**) were linked to SNPs associated with these traits in GWAS: three SNPs were associated with adiposity (*MAP2K5*, *MEIS1* and *IPO9*) (42, 43)(downloaded 19/12/2019), four with obesity (*MAP2K5*, *PCDH9*, *SCNN1A* and *TCF4*) (EFO_0001073, downloaded 19/12/2019), four genes (*ACSL1*, *HMGA2, RPSAP52* and *SLC9B2*) have SNPs associated with type 2 diabetes (EFO_0001360, downloaded 19/12/2019) and seven genes (*TENM4*, *HMGA2*,

MAP3K10, *RB1*, *KLHL29*, *LRIG1* and *PMFBP1*) have SNPs associated with birthweight (EFO_0004344, downloaded 23/06/2020)(41). None of the discovered genes have SNPs associated with childhood obesity (41, 44)(downloaded 22/06/2020).

We further examined if our 379 sites were overrepresented within other epigenetic marks such as histone modifications representing active (H3K4me1 and H3K27ac) or inactive (H3K27me3) chromatin. We intersected the position of 379 sites with chromatin immunoprecipitation-sequencing data of histone modifications in blood mononuclear cells from the Roadmap Epigenomics Consortium (45). A permutation distribution test using 10,000 permutations showed more significant methylation sites overlapping with H3K4me1 (p=0.030) but not H3K27me3 (p=0.356) and H3K27ac (p=0.535), compared with what would have been expected by chance if all sites on the array were analyzed, indicating an enrichment of enhancers elements in sites differentially methylated in the lifestyle intervention.

We have previously shown that C-reactive protein (CRP) levels were lower in pregnant mothers in lifestyle intervention versus controls (46). Therefore, we tested whether CRP levels in pregnant mothers correlated with cord blood methylation of our 379 sites. CRP levels correlated with methylation of 2 sites; cg17389519 which is annotated to *PTF1A* encoding a transcription factor involved in pancreas and neural tissue development (47) and cg27394563 annotated to *SART3* (**Supplemental Figure S2A-B**).

Impact of a lifestyle intervention during pregnancy on offspring lean mass

We proceeded to study the body composition of the offspring (**Table 1** and **2**). Offspring to mothers included in the lifestyle intervention group were born with 59g (95%CI: 11;108, *p*=0.017) and 0.88 percentage points (95%CI: 0.24;1.53, *p*=0.008) more abdominal lean mass versus controls (**Figure 3** and **Table 2**). We also observed a trend that offspring of mothers included in the lifestyle intervention group were born with 127g (95%CI: -5;258, *p*=0.058) and 1.36 percentage points (95%CI: -0.05;2.77, *p*=0.059) more lean mass versus controls (**Table 2**). At birth, the offspring were similar in size (**Tables 1** and **2**), indicating that it is the body composition that differs between the groups. We found that offspring of both groups were similar in size at 9 and 36 months of age, however, at 18 months children from the lifestyle intervention group were larger in size (**Table 1** and **2**).

Associations between DNA methylation in cord blood and offspring lean mass and growth

Offspring in the lifestyle intervention group had more lean mass at birth and differential cord blood methylation at 379 sites versus controls (**Table 2** and **Supplemental Table S1**); thus, we further tested whether there were associations between methylation of these 379 sites and lean mass (%) at birth in the offspring. Cord blood methylation of 25 sites was associated with lean mass ($q<0.05$, **Supplemental Table S9**). For the majority of these sites (80%), cord blood methylation levels were higher in the lifestyle intervention group and positively associated with greater lean mass.

We proceeded to assess whether cord blood methylation of the 379 sites was associated with growth over time in the offspring using LMMs and BMI z-scores at birth and 9, 18 and 36 months of age. We found that methylation of 22 sites was associated with BMI z-scores ($p<0.05$). Next, we performed linear regression models to test if methylation at the 379 sites was associated with BMI z-scores at each timepoint. The direction of effects for the LMMs were consistent with that for the linear regression models at all timepoints but for two sites,

for these two sites the direction altered at one timepoint (**Supplemental Table S10**). Included within the genes annotated to the 22 sites are *ACSL1,* which is involved in fatty acid beta-oxidation and harboring a SNP associated with type 2 diabetes, and *TCF4,* encoding a transcription factor involved in Wnt signaling and harboring a SNP associated with obesity (EFO_0001073, downloaded 19/12/2019), in GWAS (41) (**Supplemental Table S8**).

Causal mediation analysis

We next used a causal mediation analysis (38) to investigate whether DNA methylation of any of the 25 sites in cord blood found to be associated with lean mass are part of a pathway through which the lifestyle intervention exerts its effects on offspring lean mass. The mediation analysis breaks down the total effect of treatment (lifestyle intervention) on outcome (lean mass) into two parts, first, the indirect effect acting via the mediator of interest (DNA methylation) and second, the direct effect acting directly or via a mediator other than what is under study. The analyses showed that 1) the lifestyle intervention has an overall effect of β=1.35 (95%CI: -0.092;2.741) on lean mass and that 2) that effect may operate via an indirect path (indirect effect), possibly through methylation, with a significant average causal mediator effect for 17 methylation sites (q<0.05, **Table 3**); 3) consequently, the total effect of the lifestyle intervention on lean mass, 32.0-61.8% are suggested to act via these 17 methylation sites. According to these results, we may call methylation of these sites partial mediators.

Cross-tissue methylation of sites associated with lean mass or growth

We finally examined whether DNA methylation in blood of the 46 unique sites (one methylation site cg11594420, overlap) associated with lean mass or growth in the offspring mirrors the methylation pattern in two other tissues of importance for obesity and type 2

diabetes, skeletal muscle and adipose tissue. We used available methylation array data from blood, muscle and adipose tissue taken from the same individuals (**Supplemental Table S11**) (39, 40). Among these sites, the methylation pattern in blood correlated positively with methylation of four sites in adipose tissue, and two sites in muscle (p<0.05, **Supplemental Table S12**). Five correlations were nominal and one significant after FDR. These findings suggest that methylation of a few sites may have a biological role in tissues of relevance for obesity and type 2 diabetes.

DISCUSSION

This is to our knowledge the first genome-wide epigenetic analysis in cord blood of pregnant women with obesity randomized to a lifestyle intervention including physical activity, with or without a hypocaloric Mediterranean-style diet, versus controls receiving standard of care. There are four key findings: First, DNA methylation at individual sites in cord blood differed between lifestyle intervention and controls. These sites were annotated to genes overrepresented in relevant GO terms e.g., response to fatty acids and adipose tissue development. Second, we found that genes linked to SNPs associated with birthweight, obesity, adiposity and type 2 diabetes by GWAS also have been annotated to sites which have altered DNA methylation in our study. Additionally, SNPs previously associated with DNA methylation in cord blood of our identified sites were linked to disease traits in the GWAS catalogue. Third, offspring to mothers included in the lifestyle intervention were born with more lean mass. Finally, methylation at 17 sites partially mediates the effect of the lifestyle intervention on lean mass in the offspring. Together, these data provide evidence that the presented lifestyle intervention altered the epigenome of genes linked to metabolism and metabolic disease in offspring cord blood from pregnant mothers with obesity.

Previous studies have shown that tissues from people with obesity have different methylation profiles versus lean people (13, 16, 48); however, DNA methylation can be changed by lifestyle (40, 49). Obesity during pregnancy increases the risk of metabolic disease in offspring (2), and obesity in pregnant mothers is associated with epigenetic alterations in cord blood (17). We demonstrate that exercise and healthy diets during pregnancy can change cord blood DNA methylation and that these epigenetic changes took place on genes involved in metabolic processes. It is possible that a healthier lifestyle during pregnancy and the consequential epigenetic changes help enhance the offspring's health later in life. The epigenetic mechanisms linked to exercise and healthy diets in our study seem to be different compared with those previously associated with maternal BMI (17) and gestational diabetes (16, 19).

We demonstrate that offspring to mothers in the lifestyle intervention have higher abdominal and a trend towards higher total lean mass versus controls. This result indicates a positive effect of the lifestyle intervention on the offspring as they were born with more metabolically active tissue, which might protect against future metabolic diseases. This is supported by studies showing that the body composition of newborns being born small-for-gestational-age (SGA) differ more in terms of less lean mass than differences in fat mass, compared to appropriate-for-gestational-age newborns (50), and SGA increases the risk of metabolic disease later in life (51). Thereby, suggesting that negative effects of being born SGA could be due to decreased lean mass. Increased muscle mass and higher metabolic activity on the other hand may have beneficial effects on insulin sensitivity and protect from obesity and type 2 diabetes (7). We found associations between cord blood epigenetics and lean mass in the offspring at birth. For the majority of these sites, methylation levels were higher in cord blood of the lifestyle intervention group and positively associated with greater lean mass, and

methylation at several sites seem to partially mediate the lifestyle effect on lean mass. Interestingly, the lifestyle intervention group had decreased methylation of *SETD3*, which encodes a methyltransferase. Hypomethylation of *SETD3*, correlates with increased expression and in turn, increased muscle mass (52), which might in part explain the greater lean mass we see in the offspring of the lifestyle intervention group.

Finally, we found that blood-based methylation of sites associated with lean mass or growth in the offspring mirrors methylation patterns in muscle and adipose tissue, tissues of importance for metabolic disease (39, 40). These include sites annotated to *TCF4* and *SYT9* encoding transcription factor 4 and synaptotagmin 9, respectively, which have been implicated in diabetes related traits (53, 54). Although these results are based on nominally significant *p*-values they give a possible indication that methylation of some identified sites may also have a biological role in tissues of importance for obesity and type 2 diabetes.

Strengths and Limitations

A strength of this study is the randomized design, the high rate of completers in the TOP-study and the relatively homogenous study population in terms of pre-pregnancy BMI and ethnicity, which should reduce the risk of bias. A pedometer intervention is an inexpensive method for increasing daily physical activity and can easily be implemented into daily life. Other physical activity interventions often include attendance to classes which can be difficult to implement. It is also a strength that we used several different methods to adjust for cell composition and technical variation, which showed that DNA methylation of the majority of the identified sites was associated with the lifestyle intervention independent of the method used. It is important to adjust the DNA methylation data for cell type

composition. For discovery of our significant DNA methylation results, we used the reference-free method developed by Houseman et al. for deconvolve heterogeneous cell mixtures (32). We then used the reference-based (33) and PCA-based methods to validate our results. These different methods (e.g. reference-based vs. reference-free) have their pros and cons. A reference-based method might provide robust estimations. However, it is usually based on very few samples with limited clinical conditions. For umbilical cord blood there is an available reference of 26 samples (33), which might not be able to cover the variance in our dataset as it is 8 times larger. In the publication by Houseman et al (32), they show via a simulation study and several real data analyses that their method can perform as well as or better than methods that make explicit use of reference datasets. They also discuss that this reference-free method may adjust for detailed cell type differences that may be unavailable even in existing reference datasets. Additionally, the algorithm estimates the number of cell types, meaning it should also consider the nucleated red blood cells that cord blood contains. On the other hand, two methods were used to correct for multiple testing, Benjamini-Hochberg and Bonferroni. Bonferroni is too conservative for EWAS since DNA methylation values at nearby probes are known to be correlated and many sites on the array are non-variable (PMID: 31088362). The alternative approach, Benjamini-Hochberg adjustment, is potentially a more powerful method identifying the top associated sites with the phenotype of interest.

A limitation is that the two lifestyle intervention groups were merged. This was however necessary to have sufficient statistical power due to the modest number of samples with cord blood in each group. However, we have shown that lifestyle interventions in the TOP-study were effective and could reduce GWG (9), suggesting that both interventions successfully achieved the main primary endpoint, thus reducing potential bias when merging them. It should also be noted that the maternal energy intake is self-reported and may infer a type of

reporting bias. Nevertheless, the maternal energy intake at late gestation was reduced in the lifestyle intervention group versus controls, indicating a successful intervention. Previous dietary analyses of participants in the TOP-study showed that participants having dietary and physical activity intervention changed their dietary composition in a healthier direction (12). Participants in the group only performing physical activity demonstrated a trend towards dietary changes in the same direction (12). This supports the approach of merging the intervention groups.

As in other intervention studies of pregnant women with obesity, it might be a challenge that the intervention intensity was too low, and a high proportion of the participants were non-compliant to the recommended diet and physical activity intervention. Few women achieved the target of the physical activity intervention, possibly indicating that this target was set high for this group and should be revised for future studies.

Of note, the methylation array covers ~2% of sites in the human genome and it is therefore possible that methylation of additional sites may be associated with the intervention. Future studies are needed to fully understand the biology behind the associations presented in this paper and their possible health effects.

In summary, this study demonstrates that a lifestyle intervention in pregnant women with obesity is associated with the cord blood epigenome in offspring. We also provide evidence that epigenetic markers in cord blood associate with lean mass and growth in offspring. These results underline that the intrauterine environment in humans might have the ability to program the epigenome, which in turn may affect metabolism and growth later in life.

ARTICLE INFORMATION

Acknowledgements. The authors thank Marlena Maziarz, for your guidance and expertise in biostatistics and Silja Schrader, for your help with power calculations. We also thank the Danish Diabetes Academy via the Novo Nordisk Foundation for support throughout this project, and the National Bioinformatics Infrastructure Sweden, SciLifeLab, for the Bioinformatics Long-term support with Nikolay Oskolkov.

Funding. Sygekassernes Helsefond, Brødrene Hartmann Fonden, Hvidovre Hospitals Forskningsfond and The Danish Council for Strategic Research supported the TOP-study. The work performed by A.C.E. and P.W.F. was supported by grants from the EFSD, Swedish Research Council, Swedish Heart Lung Foundation and the European Research Council (CoG-2015_681742_NASCENT) and Novo Nordisk Foundation. The work performed by J.J., S.G-C., A.P. and C.L. was supported by grants from the Novo Nordisk foundation, Swedish Research Council, Region Skåne (ALF), ERC-Co Grant (PAINTBOX, No 725840), H2020-Marie Skłodowska-Curie grant agreement No 706081 (EpiHope), Hjärt-Lungfonden, Exodiab, Swedish Foundation for Strategic Research for IRC15-0067 and Swedish Diabetes Foundation. All researchers from LUDC were supported by a research center grant from the Swedish Strategic Science Foundation.

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. J.J. analyzed data, performed the statistical analyses and drafted and revised the manuscript. K.M.R., A.V., P.F.W. and C.L. designed and planned the current study and participated in drafting the manuscript. K.M.R and K.N. designed and planned the TOP-study. K.M.R. and E.M.C. conducted the TOP-study and collected data. S.G-C. participated in analyzing data and drafting the manuscript. A.P. performed DNA methylation analysis. A.C.E. contributed to designing the study and performed statistical analyses. M.V.L. interpreted data and gave statistical support. M.V.L., K.M.F. and E.M.C. collected data on the

offspring at follow-up after delivery. L.H. contributed to planning the epigenetic part of the study. E.M.C. analyzed the DXA scans. P.W.F. and C.L. supervised the analyses.

All authors reviewed and provided critical comments on the manuscript.

J.J. and K.M.R. are the guarantors of this work and, as such, had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

REFERENCES

1. Rasmussen KM, Yaktine AL, eds. Weight Gain During Pregnancy: Reexamining the Guidelines. Washington (DC): National Academies Press (US); 2009.
2. Gu S, An X, Fang L, Zhang X, Zhang C, Wang J, et al. Risk factors and long-term health consequences of macrosomia: a prospective study in Jiangsu Province, China. J Biomed Res. 2012;26(4):235-40.
3. Simmonds M, Llewellyn A, Owen CG, Woolacott N. Predicting adult obesity from childhood obesity: a systematic review and meta-analysis. Obes Rev. 2016;17(2):95-107.
4. Ervin RB. Prevalence of metabolic syndrome among adults 20 years of age and over, by sex, age, race and ethnicity, and body mass index: United States, 2003-2006. Natl Health Stat Report. 2009(13):1-7.
5. Franks PW, Hanson RL, Knowler WC, Sievers ML, Bennett PH, Looker HC. Childhood obesity, other cardiovascular risk factors, and premature death. N Engl J Med. 2010;362(6):485-93.
6. Global Burden of Disease Study 2015 (GBD 2015) Obesity and Overweight Prevalence 1980-2015 [Internet]. 2017.
7. Kim G, Lee SE, Jun JE, Lee YB, Ahn J, Bae JC, et al. Increase in relative skeletal muscle mass over time and its inverse association with metabolic syndrome development: a 7-year retrospective cohort study. Cardiovasc Diabetol. 2018;17(1):23.
8. Bann D, Wills A, Cooper R, Hardy R, Aihie Sayer A, Adams J, et al. Birth weight and growth from infancy to late adolescence in relation to fat and lean mass in early old age: findings from the MRC National Survey of Health and Development. Int J Obes (Lond). 2014;38(1):69-75.
9. Renault KM, Norgaard K, Nilas L, Carlsen EM, Cortes D, Pryds O, et al. The Treatment of Obese Pregnant Women (TOP) study: a randomized controlled trial of the effect of physical activity intervention assessed by pedometer with or without dietary intervention in obese pregnant women. Am J Obstet Gynecol. 2014;210(2):134.e1-9.
10. Carlsen EM, Renault KM, Norgaard K, Nilas L, Jensen JE, Hyldstrup L, et al. Newborn regional body composition is influenced by maternal obesity, gestational weight gain and the birthweight standard score. Acta Paediatr. 2014;103(9):939-45.
11. Renault KM, Carlsen EM, Norgaard K, Nilas L, Pryds O, Secher NJ, et al. Intake of carbohydrates during pregnancy in obese women is associated with fat mass in the newborn offspring. Am J Clin Nutr. 2015;102(6):1475-81.
12. Renault KM, Carlsen EM, Norgaard K, Nilas L, Pryds O, Secher NJ, et al. Intake of Sweets, Snacks and Soft Drinks Predicts Weight Gain in Obese Pregnant Women: Detailed Analysis of the Results of a Randomised Controlled Trial. PLoS One. 2015;10(7):e0133041.
13. Ling C, Ronn T. Epigenetics in Human Obesity and Type 2 Diabetes. Cell Metab. 2019;29(5):1028-44.
14. Morales E, Groom A, Lawlor DA, Relton CL. DNA methylation signatures in cord blood associated with maternal gestational weight gain: results from the ALSPAC cohort. BMC Res Notes. 2014;7:278.
15. Jorgensen SW, Brons C, Bluck L, Hjort L, Faerch K, Thankamony A, et al. Metabolic response to 36 hours of fasting in young men born small vs appropriate for gestational age. Diabetologia. 2015;58(1):178-87.
16. Hjort L, Martino D, Grunnet LG, Naeem H, Maksimovic J, Olsson AH, et al. Gestational diabetes and maternal obesity are associated with epigenome-wide methylation changes in children. JCI Insight. 2018;3(17):e122572.
17. 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.
18. Heijmans BT, Tobi EW, Stein AD, Putter H, Blauw GJ, Susser ES, et al. Persistent epigenetic differences associated with prenatal exposure to famine in humans. Proc Natl Acad Sci U S A. 2008;105(44):17046-9.
19. Howe CG, Cox B, Fore R, Jungius J, Kvist T, Lent S, et al. Maternal Gestational Diabetes Mellitus and Newborn DNA Methylation: Findings From the Pregnancy and Childhood Epigenetics Consortium. Diabetes Care. 2020;43(1):98-105.
20. Geraghty AA, Sexton-Oates A, O'Brien EC, Alberdi G, Fransquet P, Saffery R, et al. A Low Glycaemic Index Diet in Pregnancy Induces DNA Methylation Variation in Blood of Newborns: Results from the ROLO Randomised Controlled Trial. Nutrients. 2018;10(4).
21. Renault K, Norgaard K, Andreasen KR, Secher NJ, Nilas L. Physical activity during pregnancy in obese and normal-weight women as assessed by pedometer. Acta Obstet Gynecol Scand. 2010;89(7):956-61.
22. Ejlerskov KT, Christensen LB, Ritz C, Jensen SM, Molgaard C, Michaelsen KF. The impact of early growth patterns and infant feeding on body composition at 3 years of age. Br J Nutr. 2015;114(2):316-27.
23. Bibikova M, Barnes B, Tsan C, Ho V, Klotzle B, Le JM, et al. High density DNA methylation array with single CpG site resolution. Genomics. 2011;98(4):288-95.
24. R Core Team. R: A language and environment for statistical computing. 3.6.1 ed. R Foundation for Statistical Computing, Vienna, Austria. 2019.
25. Du P, Kibbe WA, Lin SM. lumi: a pipeline for processing Illumina microarray. Bioinformatics. 2008;24(13):1547-8.
26. Davis S DP, Bilke S, Triche, Jr. T, Bootwalla M. methylumi: Handle Illumina methylation data. R package version 2.28.0. 2018.
27. McCartney DL, Walker RM, Morris SW, McIntosh AM, Porteous DJ, Evans KL. Identification of polymorphic and off-target probe binding sites on the Illumina Infinium MethylationEPIC BeadChip. Genom Data. 2016;9:22-4.
28. Du P, Zhang X, Huang CC, Jafari N, Kibbe WA, Hou L, et al. Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis. BMC Bioinformatics. 2010;11:587.
29. Teschendorff AE, Marabita F, Lechner M, Bartlett T, Tegner J, Gomez-Cabrero D, et al. A beta-mixture quantile normalization method for correcting probe design bias in Illumina Infinium 450 k DNA methylation data. Bioinformatics. 2013;29(2):189-96.
30. Johnson WE, Li C, Rabinovic A. Adjusting batch effects in microarray expression data using empirical Bayes methods. Biostatistics. 2007;8(1):118-27.
31. Leek JT, Johnson WE, Parker HS, Jaffe AE, Storey JD. The sva package for removing batch effects and other unwanted variation in high-throughput experiments. Bioinformatics (Oxford, England). 2012;28(6):882-3.
32. Houseman EA, Molitor J, Marsit CJ. Reference-free cell mixture adjustments in analysis of DNA methylation data. Bioinformatics. 2014;30(10):1431-9.
33. Gervin K, Salas LA, Bakulski KM, van Zelm MC, Koestler DC, Wiencke JK, et al. Systematic evaluation and validation of reference and library selection methods for deconvolution of cord blood DNA methylation data. Clinical Epigenetics. 2019;11(1):125.
34. Bursac Z, Gauss CH, Williams DK, Hosmer DW. Purposeful selection of variables in logistic regression. Source Code for Biology and Medicine. 2008;3(1):17.
35. Boyle EI, Weng S, Gollub J, Jin H, Botstein D, Cherry JM, et al. GO::TermFinder--open source software for accessing Gene Ontology information and finding significantly enriched Gene Ontology terms associated with a list of genes. Bioinformatics. 2004;20(18):3710-5.

36. Phipson B, Maksimovic J, Oshlack A. missMethyl: an R package for analyzing data from Illumina's HumanMethylation450 platform. Bioinformatics. 2016;32(2):286-8.
37. Supek F, Bosnjak M, Skunca N, Smuc T. REVIGO summarizes and visualizes long lists of gene ontology terms. PLoS One. 2011;6(7):e21800.
38. Tingley D, Yamamoto T, Hirose K, Keele L, Imai K. mediation: R Package for Causal Mediation Analysis. 2014. 2014;59(5):38.
39. Nilsson E, Jansson PA, Perfilyev A, Volkov P, Pedersen M, Svensson MK, et al. Altered DNA methylation and differential expression of genes influencing metabolism and inflammation in adipose tissue from subjects with type 2 diabetes. Diabetes. 2014;63(9):2962-76.
40. Nitert MD, Dayeh T, Volkov P, Elgzyri T, Hall E, Nilsson E, et al. Impact of an exercise intervention on DNA methylation in skeletal muscle from first-degree relatives of patients with type 2 diabetes. Diabetes. 2012;61(12):3322-32.
41. Buniello A, MacArthur JAL, Cerezo M, Harris LW, Hayhurst J, Malangone C, et al. The NHGRI-EBI GWAS Catalog of published genome-wide association studies, targeted arrays and summary statistics 2019. Nucleic Acids Res. 2019;47(D1):D1005-d12.
42. Graff M, Scott RA, Justice AE, Young KL, Feitosa MF, Barata L, et al. Genome-wide physical activity interactions in adiposity - A meta-analysis of 200,452 adults. PLoS Genet. 2017;13(4):e1006528.
43. Karlsson T, Rask-Andersen M, Pan G, Hoglund J, Wadelius C, Ek WE, et al. Contribution of genetics to visceral adiposity and its relation to cardiovascular and metabolic disease. Nat Med. 2019;25(9):1390-5.
44. Bradfield JP, Vogelezang S, Felix JF, Chesi A, Helgeland Ø, Horikoshi M, et al. A trans-ancestral meta-analysis of genome-wide association studies reveals loci associated with childhood obesity. Hum Mol Genet. 2019;28(19):3327-38.
45. Kundaje A, Meuleman W, Ernst J, Bilenky M, Yen A, Heravi-Moussavi A, et al. Integrative analysis of 111 reference human epigenomes. Nature. 2015;518(7539):317-30.
46. Renault KM, Carlsen EM, Haedersdal S, Nilas L, Secher NJ, Eugen-Olsen J, et al. Impact of lifestyle intervention for obese women during pregnancy on maternal metabolic and inflammatory markers. Int J Obes (Lond). 2017;41(4):598-605.
47. Jin K, Xiang M. Transcription factor Ptf1a in development, diseases and reprogramming. Cell Mol Life Sci. 2019;76(5):921-40.
48. Davegårdh C, Broholm C, Perfilyev A, Henriksen T, García-Calzón S, Peijs L, et al. Abnormal epigenetic changes during differentiation of human skeletal muscle stem cells from obese subjects. BMC Med. 2017;15(1):39-.
49. Perfilyev A, Dahlman I, Gillberg L, Rosqvist F, Iggman D, Volkov P, et al. Impact of polyunsaturated and saturated fat overfeeding on the DNA-methylation pattern in human adipose tissue: a randomized controlled trial. Am J Clin Nutr. 2017;105(4):991-1000.
50. Hediger ML, Overpeck MD, Kuczmarski RJ, McGlynn A, Maurer KR, Davis WW. Muscularity and fatness of infants and young children born small- or large-for-gestational-age. Pediatrics. 1998;102(5):E60.
51. Hattersley AT, Tooke JE. The fetal insulin hypothesis: an alternative explanation of the association of low birthweight with diabetes and vascular disease. Lancet. 1999;353(9166):1789-92.
52. Seaborne RA, Strauss J, Cocks M, Shepherd S, O'Brien TD, van Someren KA, et al. Human Skeletal Muscle Possesses an Epigenetic Memory of Hypertrophy. Sci Rep. 2018;8(1):1898.
53. Wei H, Qu H, Wang H, Ji B, Ding Y, Liu D, et al. 1,25-Dihydroxyvitamin-D3 prevents the development of diabetic cardiomyopathy in type 1 diabetic rats by enhancing

autophagy via inhibiting the beta-catenin/TCF4/GSK-3beta/mTOR pathway. J Steroid Biochem Mol Biol. 2017;168:71-90.
54. Shim YJ, Kim JE, Hwang SK, Choi BS, Choi BH, Cho EM, et al. Identification of Candidate Gene Variants in Korean MODY Families by Whole-Exome Sequencing. Horm Res Paediatr. 2015;83(4):242-51.

TABLES

Table 1. Parental and offspring baseline characteristics according to the lifestyle intervention and control groups, for subjects with available cord blood of the TOP-study.

	Lifestyle intervention	Control	*p*-value
Maternal characteristics	n = 135	n = 73	
Maternal age at enrolment (years)*	30.90 (4.30)	31.40 (4.74)	0.440
Pre-pregnancy BMI (kg/m^2)*	34.19 (4.00)	34.36 (3.98)	0.763
Maternal educational level, n (%)†			0.970
1. Grammar school 10 years	15 (11.1)	6 (8.2)	
2. Secondary school 12 years	16 (11.9)	9 (12.3)	
3. Vocational training school	13 (9.6)	6 (8.2)	
4. Further education 1-2 years	26 (19.3)	12 (16.4)	
5. Tertiary education 3-4 years (Bachelor level)	46 (34.1)	29 (39.7)	
6. Advanced education (post-graduate)	18 (13.3)	10 (13.7)	
7. NA	1 (0.7)	1 (1.4)	
Smoking during pregnancy (yes/no), n (%)†	10 (7.4)	3 (4.1)	0.524
Parity (single/multi), n (%)†	75 (55.6)	39 (53.4)	0.882
Energy intake at enrolment, week 11-14 (kJ)‡§	8019 (2784)	7540 (3246)	0.587
Paternal characteristics	n = 115	n = 65	
BMI (kg/m^2) at enrolment, week 11-14*	27.39 (4.51)	27.01 (4.52)	0.585
Offspring characteristics	n = 135	n = 73	
Sex, n (%)†			0.862
Male	69 (51.1)	39 (53.4)	
Female	66 (48.9)	34 (46.6)	
Gestational age (weeks)*	40.17 (1.23)	40.01 (1.31)	0.393
Weight (g), birth*	3724.01 (481.92)	3677.36 (512.96)	0.515

Weight (kg), 9 months*‖	9.61 (1.03)	9.38 (1.15)	0.299
Weight (kg), 18 months*¶	11.86 (11.83)	11.26 (10.27)	0.014
Weight (kg), 36 months*#	15.30 (18.64)	14.71 (12.97)	0.141
Length (cm), birth*,**	52.50 (2.17)	52.48 (2.24)	0.958
Length (cm), 9 months*,‖	73.14 (2.32)	72.99 (1.97)	0.740
Length (cm), 18 months*,††	82.75 (2.87)	82.55 (2.48)	0.724
Height (cm), 36 months*,‡‡	96.42 (4.21)	95.95 (3.07)	0.599
Breastfeeding, exclusively (weeks)*,§§	10.98 (9.41)	8.38 (10.07)	0.163
Breastfeeding, partially (weeks)*,§§	16.30 (11.05)	14.88 (10.71)	0.501

BMI, body mass index; *IQR*, interquartile range; *SD*, standard deviation.

* Mean (SD), two-sided Student *t*-test
† Frequencies, chi-square test
‡ Median (IQR), one-sided Mann-Whitney U test
§ Lifestyle intervention n = 133, Control n = 68
‖ Lifestyle intervention n = 60, Control n = 39
¶ Lifestyle intervention n = 58, Control n = 36
# Lifestyle intervention n = 51, Control n = 29
** Lifestyle intervention n = 129, Control n = 71
†† Lifestyle intervention n = 57, Control n = 36
‡‡ Lifestyle intervention n = 51, Control n = 28
§§ Lifestyle intervention = 77, Control n = 42

Table 2. Estimated differences from linear regression models in offspring lean mass and BMI z-scores, and their associated 95% confidence intervals (Est (95% CI)) when comparing lifestyle intervention (n = 92) versus control (n = 47) groups, for subjects with available cord blood of the TOP-study.

Phenotype	**Est (95% CI)**	***p*-value**
Lean mass (g), birth	126.55 (-4.52; 257.62) *	0.058 *
Lean mass (%), birth	1.36 (-0.05; 2.77) *	0.059 *
Abd. lean mass (g), birth	59.09 (10.53; 107.65) *	0.017 *
Abd. lean mass (%), birth	0.88 (0.24; 1.53) *	0.008 *
Fat mass (g), birth	51.26 (-19.93; 122.44) *	0.157 *
Fat mass (%), birth	1.35 (-0.06; 2.76) *	0.061 *
Abd. fat mass (g), birth	6.88 (-3.51; 17.26) *	0.192 *
Abd. fat mass (%), birth	0.49 (-0.58; 1.57) *	0.365 *
BMI z-score, birth[1]	0.15 (-0.14; 0.43) †	0.352 †
BMI z-score, 9mon[2]	0.31 (-0.14; 0.76) ‡	0.315 ‡
BMI z-score, 18mon[3]	0.54 (0.14; 0.93) §	0.006 §
BMI z-score, 36mon[4]	0.30 (-0.13; 0.74) §	0.169 §

[1]Lifestyle intervention n = 129, Control n = 71.

[2]Lifestyle intervention n = 60, Control n = 39.

[3]Lifestyle intervention n = 57, Control n = 36.

[4]Lifestyle intervention n = 51 Control n = 28.

*Adjusted for; maternal education level, maternal smoking during pregnancy (yes/no), GWG (in kilograms), pre-pregnancy BMI, parity (single/multi), GA (in weeks) and offspring sex.

† Adjusted for; maternal education level, maternal smoking during pregnancy (yes/no), GWG (in kilograms), pre-pregnancy BMI, parity (single/multi), GA (in weeks).

‡ Adjusted for; maternal education level, maternal smoking during pregnancy (yes/no), GWG (in kilograms), pre-pregnancy BMI, parity (single/multi), GA (in weeks), breastfeeding partially and exclusively and BMI z-score at birth.

§ Adjusted for; maternal education level, maternal smoking during pregnancy (yes/no), GWG (in kilograms), pre-pregnancy BMI, parity (single/multi), breastfeeding partially and exclusively and BMI z-score at birth.

Table 3. Causal mediation analysis on the significant associations between the lifestyle intervention and lean mass related methylation (CpG) sites as mediators and lean mass (%) as outcome (ACME *q*-value<0.05)

CpG site	Gene	ACME estimate of mediator CpG (95% CI)	ACME *q*-value	ADE estimate (95% CI)	Total effect (95% CI)	Proportion mediated by CpG (95% CI)
cg07405330	*MOBP*	0.84 (0.35; 1.39)	<0.001	0.52 (-0.84; 1.89)	1.35 (-0.09; 2.74)	0.62 (-0.73; 3.58)
cg06480224	*KIAA2012*	0.78 (0.19; 1.51)	0.013	0.57 (-0.97; 1.98)	1.35 (-0.09; 2.74)	0.58 (-1.11; 4.27)
cg11612786	*AC079135.1; GBX2*	0.64 (0.13; 1.38)	0.013	0.71 (-0.66; 2.03)	1.35 (-0.09; 2.74)	0.47 (-0.58; 2.78)
cg20982052		0.53 (0.14; 1.11)	0.013	0.82 (-0.68; 2.18)	1.35 (-0.09; 2.74)	0.39 (-0.62; 2.63)
cg00154557	*DSE*	0.50 (0.08; 1.08)	0.021	0.86 (-0.64; 2.26)	1.35 (-0.09; 2.74)	0.37 (-0.58; 2.42)
cg13002044	*TMEM178B*	0.70 (0.14; 1.42)	0.021	0.65 (-0.73; 1.98)	1.35 (-0.09; 2.74)	0.52 (-0.85; 2.87)
cg18088415	*LSM2*	0.70 (0.19; 1.41)	0.021	0.65 (-0.77; 2.07)	1.35 (-0.09; 2.74)	0.52 (-0.73; 3.42)
cg11594420	*TEX101*	0.53 (0.10; 1.18)	0.025	0.82 (-0.59; 2.15)	1.35 (-0.09; 2.74)	0.39 (-0.45; 2.84)
cg04678315		0.48 (0.08; 1.02)	0.028	0.87 (-0.56; 2.20)	1.35 (-0.09; 2.74)	0.36 (-0.64; 2.05)
cg08144675		0.70 (0.10; 1.42)	0.032	0.66 (-0.85; 2.20)	1.35 (-0.09; 2.74)	0.52 (-0.98; 3.35)
cg22454673	*HERC2*	0.63 (0.16; 1.29)	0.032	0.73 (-0.71; 2.18)	1.35 (-0.09; 2.74)	0.46 (-0.77; 3.10)
cg04058675	*HUWE1*	0.45 (0.08; 1.03)	0.033	0.90 (-0.46; 2.22)	1.35 (-0.09; 2.74)	0.34 (-0.48; 2.09)
cg03190725	*RP3-468B3.2*	0.54 (0.10; 1.10)	0.035	0.81 (-0.67; 2.19)	1.35 (-0.09; 2.74)	0.40 (-0.65; 2.58)
cg06799721	*TARS*	0.52 (0.05; 1.27)	0.036	0.84 (-0.54; 2.07)	1.35 (-0.09; 2.74)	0.38 (-0.30; 2.00)
cg15157974	*DISC1*	0.43 (0.04; 0.96)	0.040	0.92 (-0.50; 2.21)	1.35 (-0.09; 2.74)	0.32 (-0.24; 2.10)
cg26142132	*AAT*	0.45 (0.06; 0.96)	0.041	0.90 (-0.57; 2.40)	1.35 (-0.09; 2.74)	0.34 (-0.44; 2.26)
cg00354884	*ABR*	0.53 (0.03; 1.18)	0.047	0.83 (-0.62; 2.17)	1.35 (-0.09; 2.74)	0.39 (-0.45; 2.32)

Models adjusted for GWG (in kilograms), maternal BMI, GA (in weeks) and offspring sex.

Based on 139 participants, lifestyle intervention (n = 92), control (n = 47)

ACME = average causal mediator effect

ADE = average direct effect

FIGURE LEGENDS

Figure 1. Flow diagram.

Figure 2. A) A Manhattan plot, representing the distribution of methylation sites across the genome, for the association between lifestyle intervention and offspring cord blood DNA methylation, after adjustment for covariates and cell composition adjustment. The black line shows the FDR threshold for multiple testing. Methylation sites that surpassed the FDR threshold (p<4.17x10-5) are highlighted in color, red = hypermethylated, and blue = hypomethylated sites in the lifestyle intervention group versus the control group. **B)** Pathways from GO analysis after removal of redundant GO Terms using REViGO (p<0.05). The gray bars indicate the total number of genes in the pathway; the blue bars indicate the number of differently methylated (DM) genes in lifestyle intervention versus controls. Data from panel A is also presented in Supplemental Table S1 and data from panel B is also presented in Supplemental Table S3.

Figure 3. Boxplots are showing **A)** abdominal lean mass (g) and **B)** abdominal lean mass (%) in the lifestyle intervention and control groups at birth, medians (IQR). The p-values are based on linear regression models adjusted for maternal education level, maternal smoking during pregnancy (yes/no), GWG (kg), pre-pregnancy BMI (kg/m^2), parity (single/multi), GA (weeks) and offspring sex. Data is also presented in Table 2.