

Young men with low birthweight exhibit decreased plasticity of genome-wide muscle DNA methylation by high-fat overfeeding

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Abstract

Aims/hypothesis The association between low birthweight (LBW) and risk of developing type 2 diabetes may involve epigenetic mechanisms, with skeletal muscle being a prime target tissue. Differential DNA methylation patterns have been observed in single genes in muscle tissue from type 2 diabetic and LBW individuals, and we recently showed multiple DNA methylation changes during short-term high-fat overfeeding in muscle of healthy people. In a randomised crossover study, we analysed genome-wide DNA promoter methylation in skeletal muscle of 17 young LBW men and 23 matched normal

birthweight (NBW) men after a control and a 5 day high-fat overfeeding diet.

Methods DNA methylation was measured using Illumina's Infinium BeadArray covering 27,578 CpG sites representing 14,475 different genes.

Results After correction for multiple comparisons, DNA methylation levels were found to be similar in the LBW and NBW groups during the control diet. Whereas widespread DNA methylation changes were observed in the NBW group in response to high-fat overfeeding, only a few methylation changes were seen in the LBW group (χ^2 , $p < 0.001$).

Conclusions/interpretation Our results indicate lower DNA methylation plasticity in skeletal muscle from LBW vs NBW men, potentially contributing to understanding the link between LBW and increased risk of type 2 diabetes.

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Keywords DNA methylation · Epigenetics · High-fat overfeeding · Insulin resistance · Low birthweight · Skeletal muscle · Type 2 diabetes

Abbreviations

LBW Low birthweight
NBW Normal birthweight
pp Percentage points

Introduction

Epigenetics has been proposed as a link between low birthweight (LBW) and increased risk of metabolic disease and type 2 diabetes [1]. The involvement of tissue-specific DNA methylation in the pathogenesis of type 2 diabetes is supported by several studies [2–4]. Maternal diet and/or the intrauterine environment may influence DNA methylation in the offspring, possibly affecting phenotypes and disease risk

[5], as exemplified by our finding of increased DNA methylation of *PPARGC1A* in skeletal muscle of LBW individuals [6].

Fat overload may induce and/or prolong the presence of methyl groups attached to DNA [2, 7], and we have shown that high-fat overfeeding introduced widespread DNA methylation changes in young men [7]. In our study of *PPARGC1A*, we found that LBW individuals, in contrast with normal birthweight (NBW) controls, did not exhibit methylation changes in skeletal muscle during overfeeding [6].

Extending our previous epigenetic findings [6, 7], we investigated whether genome-wide DNA methylation levels are different in skeletal muscle of LBW individuals compared with NBW controls, and whether a high-fat overfeeding challenge reveals a differential DNA methylation response in LBW individuals.

Methods

Study design and participants The data are part of a study investigating the metabolic effects of high-fat overfeeding in healthy LBW and NBW individuals, as previously described [6, 8]. Briefly, 20 LBW (birthweight ≤ 10 th percentile) and 26 matched NBW men born at term were recruited. All participants received a 5 day high-fat overfeeding diet and a control diet in a randomised order separated by 6–8 weeks. The study was designed to ensure identical conditions during interventions and laboratory procedures. The protocol conformed to the Declaration of Helsinki and was approved by the ethics committee of Copenhagen County. All participants gave informed consent.

Detailed clinical descriptions have previously been published [6, 8]. Muscle biopsies and blood samples were taken after an overnight fast following both diets. Biopsies were successfully obtained from the vastus lateralis muscle of 17 LBW and 23 NBW participants; among these, there were 14 paired LBW and 21 paired NBW samples, of which eight LBW and 11 NBW men received the control diet first.

DNA methylation profiling Genomic DNA was extracted using the DNeasy Kit (Qiagen, Valencia, CA, USA), and 600 ng DNA was bisulphite-treated with the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA). DNA methylation was assessed at 27,578 CpG sites close to transcription start in 14,475 genes using Illumina's Infinium 27 k BeadArray (Illumina, San Diego, CA, USA) employing previously described procedures [7]. Each array had an even distribution of LBW and NBW participants and of control and overfeeding samples. The batch effect of the dataset was examined using a principal components analysis plot. Since no batch effect was found, and as data processing beyond the BeadStudio software was not recommended by Illumina, no

further normalisation was performed. Technical validation of the BeadArray data was performed for six CpG sites/genes using pyrosequencing of bisulphite-treated DNA in the PyroMark Q96ID instrument (Qiagen) with primers designed using PyroMark Assay Design 2.0. The data were analysed using PyroMark software v.2.5.7.

Gene expression analysis Gene expression of *DNMT1*, *DNMT3A* and *DNMT3B* was determined by quantitative PCR using gene-specific primer/probe pairs and the ABI 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the same procedure as described previously [7]. *DNMT* gene expression data are presented in the electronic supplementary material (ESM [ESM text: Skeletal muscle gene expression of DNA methyltransferases and ESM Tables 1 and 2]).

Statistical methods Statistics were performed using R v.2.9.0 (www.r-project.org) [7]. Differences between methylation percentages are absolute percentage points (pp). Significance levels between diet intervention and birthweight groups were evaluated using paired and unpaired Student's *t* tests, respectively ($p < 0.05$). Deviations from expected distributions were estimated with χ^2 tests. Wilcoxon's rank sum tests were used to compare the absolute, individual diet-induced methylation response in LBW compared with NBW men, and unpaired Student's *t* tests were used to compare the average, diet-induced methylation response in the birthweight groups on all 27,578 sites simultaneously (delta analyses). The Benjamini–Hochberg procedure was applied to correct for multiple testing where $q < 0.10$ was considered statistically significant. Self-organising maps were employed to investigate the diet-order impact using MeV software v.4.8 (www.tm4.org). The dataset is available at NCBI Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo), accession number GSE40798.

Results

Clinical characteristics of the study participants are shown in Table 1. The biopsies used for the array analyses were the same as used in our previous paper on *PPARGC1A* DNA methylation [6]; genome-wide DNA methylation data of the NBW group were published in this journal in 2012 [7]. Besides lower birthweight and height of the LBW participants, there were no differences between the groups. The NBW men increased their fasting blood glucose levels after overfeeding. Both groups exhibited reduced NEFA levels after overfeeding. Finally, LBW men showed a tendency ($p = 0.07$) towards decreased insulin-stimulated glucose uptake after overfeeding.

Table 1 Clinical and metabolic characteristics of the NBW ($n=23$) and LBW ($n=17$)^a groups after the control and the 5 day high-fat overfeeding diets

Characteristic	NBW	LBW	<i>p</i> value
Birthweight (g)	3,903±206	2,677±272	<0.0001
Age (years)	24.6±1.1	24.1±0.5	0.12
Height (m)	1.83±0.1	1.77±0.1	0.01
Weight (kg)			
Control	78.6±9.6	77.3±11.5	0.70
High-fat overfeeding	78.9±10	76.9±11.9	0.58
<i>p</i> value	0.28	0.58	
Fat mass (kg)			
Control	14.4±6.2	17.3±9.5	0.25
High-fat overfeeding	14.2±6.3	15.6±9.1	0.58
<i>p</i> value	0.92	0.61	
FFM (kg)			
Control	61.3±6.1	58.0±5.1	0.11
High-fat overfeeding	58.2±14.0	58.1±5.4	0.99
<i>p</i> value	0.81	0.33	
BMI (kg/m ²)			
Control	23.4±2.4	24.6±3.9	0.23
High-fat overfeeding	23.2±2.5	24.7±4.0	0.32
<i>p</i> value	0.93	0.59	
Fasting plasma glucose (mmol/l)			
Control	4.63±0.47	4.86±0.66	0.20
High-fat overfeeding	5.04±0.39	5.03±0.54	0.94
<i>p</i> value	0.003	0.52	
Fasting serum insulin (pmol/l)			
Control	31.0±15.1	40.3±16.0	0.07
High-fat overfeeding	41.2±27.4	44.6±18.6	0.67
<i>p</i> value	0.16	0.33	
Fasting plasma NEFA (mmol/l)			
Control	348±134	411±201	0.24
High-fat overfeeding	211±80	196±93	0.59
<i>p</i> value	0.0002	0.0004	
Basal fat oxidation (mg kg ⁻¹ FFM ⁻¹ min ⁻¹)			
Control	0.98±0.39	1.06±0.51	0.60
High-fat overfeeding	1.04±0.65	1.14±0.32	0.55
<i>p</i> value	0.78	0.87	
Insulin-stimulated glucose uptake (mg kg ⁻¹ FFM ⁻¹ min ⁻¹)			
Control	13.1±3.7	12.9±2.1	0.85
High-fat overfeeding	12.7±4.4	11.5±2.8	0.35
<i>p</i> value	0.68	0.07	

Data are means ± SD

^aLBW ($n=16$) in unpaired comparisons between NBW and LBW men during overfeeding

p values for paired comparisons between control vs high-fat overfeeding are based on NBW ($n=21$) and LBW ($n=14$)

FFM, Fat-free mass

Only 404 CpG sites in 393 (3%) genes showed differential methylation between the groups ($p<0.05$) during the control diet, as opposed to 9% of the genes during overfeeding; both non-significant after correction for multiple comparisons ($q>0.10$).

Among 11 candidate genes relevant to intrauterine growth restriction (four imprinted/seven non-imprinted) and 38 type 2 diabetes candidate genes (ESM [text: Significant DNA methylation changes of candidate genes relevant to intrauterine growth restriction and type 2 diabetes]) [5, 7, 9], we found significant differences ($p<0.05$, uncorrected) between the NBW and LBW men in two genes for intrauterine growth restriction (*IGF2R*, *TNF*) and in three genes for type 2 diabetes (*CDKN2B*, *KCNJ11*, *KCNQ1*) (ESM [Table 3]).

As previously reported, DNA methylation changed in 4,316 (30% of all) genes in response to overfeeding in NBW individuals ($p<0.05$, non-significant after multiple testing corrections) [7]. However, looking only at those who received the control diet first, this number increased to 11,457 sites in 8,710 (60%) genes ($p<0.05$), of which 7,909 sites in 6,508 (45%) genes were significant after multiple testing corrections ($q<0.05$) [7]. We found no significant effect on DNA methylations of diet order in the LBW group, as determined by an unsupervised clustering analysis with self-organising maps (χ^2 test, $p=0.53$). Interestingly, there were no pronounced effects of high-fat overfeeding in the entire LBW group, with only 846 sites in 830 (6%) genes changing methylations ($p<0.05$, none with $q<0.10$) (Fig. 1a). A similar number of 1,107 (8%) genes changed in the group receiving the control diet first among the LBW men (none with $q<0.10$). Accordingly, the proportion of genes with altered DNA methylation following overfeeding was significantly higher in NBW compared with LBW men (χ^2 , $p<0.001$) (Fig. 1a). This was also evident by unpaired analyses of the diet-induced DNA methylation response in NBW vs LBW men performed on all 27,578 sites simultaneously. Thus, delta analyses based on average overall DNA methylation changes in each birthweight group were highly significantly different in the full cohort ($p<0.001$) as well as in the control diet first groups ($p<0.001$). Both positive and negative DNA methylation changes were significantly larger in NBW compared with LBW subgroups (Fig. 1b, c). To this end, delta analyses based on numerical, individual changes revealed significantly larger changes in the NBW compared with the LBW men in the entire cohort ($p<0.001$) and in the control diet first groups ($p<0.001$).

We have previously validated the BeadArray data in the NBW participants after overfeeding in 12 selected CpG sites/genes [7]. Additionally, we applied pyrosequencing to validate the DNA methylation responses in LBW men in *GABRA3*, *UGT2B7*, *FOLH1*, *FUT1*, *NDUFS2* and *FAP* (ESM [text: Technical validation of DNA methylation BeadArray data with pyrosequencing and ESM Table 4]), and indeed these validations supported the BeadArray data.

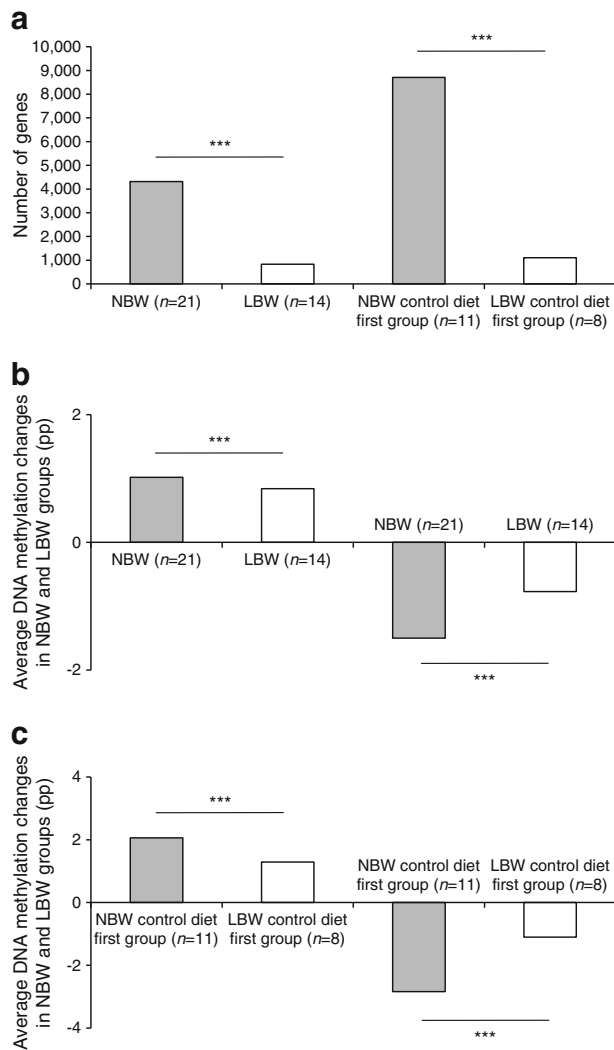


Fig. 1 DNA methylation response to high-fat overfeeding in NBW (grey bars) and LBW (white bars) men. **(a)** Number of genes that show a DNA methylation difference with $p < 0.05$ after the high-fat overfeeding diet compared with the control diet in the NBW and LBW groups. The total number of genes on the array was 14,475. **(b, c)** Average DNA methylation increase and decrease (pp) in response to high-fat overfeeding in the NBW and LBW groups in both the full cohort **(b)** and the control diet first groups **(c)**. Combined analyses of both positive and negative DNA methylation changes in LBW vs NBW groups were highly significant in the full cohort and in the control diet first groups ($***p < 0.001$)

Discussion

Our results indicate that LBW and NBW individuals exhibit similar DNA methylation patterns in skeletal muscle on a control diet. Interestingly, when exposed to 5 days' high-fat overfeeding, the LBW group displayed an impairment of their capability to change DNA methylations compared with the NBW controls [7].

We are, to the best of our knowledge, the first to perform genome-wide examination of DNA methylations in a primary insulin-sensitive tissue of LBW individuals at increased risk of developing type 2 diabetes.

We found no differences between the groups at baseline nor during overfeeding after correction for multiple testing, as well as no major differences in skeletal muscle DNA methylation among selected candidate genes associated with intrauterine growth restriction [5] or type 2 diabetes susceptibility [7, 9]. Importantly, with the applied BeadArray platform that only covers 0.1% of the CpG sites in the genome and predominantly includes CpG sites in promoter regions and CpG islands, we cannot rule out that significant differences in DNA methylations at other intra- and intergenic locations might have been overlooked.

Although the statistical power to detect diet-induced methylation changes appears to have been slightly lower in the LBW group, our additional delta analyses of all array CpG sites signifies that diet-induced differences are truly less abundant in LBW individuals. Furthermore, the present epigenome-wide data are in line with our previous study of *PPARGC1A* [6], where DNA methylation changed only in NBW individuals after overfeeding.

Our finding that the lower DNA methylation response included changes in both positive and negative directions underscores that decreased plasticity and not unidirectional preferential changes characterise LBW individuals (Fig. 1b, c). It appears reasonable to speculate that short-term changes to DNA methylations represent a normal systemic response to lifestyle and metabolic factors including both overfeeding and physical activity [3]. In support of this, a recent study reported that obesity and weight loss induced by bariatric surgery exhibited dynamic effects on skeletal muscle DNA methylation [10]. We hypothesise that the decreased plasticity of muscle DNA methylation changes in LBW individuals may adversely affect protective gene functions of various pathways, including inflammation [7]. This may increase their susceptibility to metabolic disease following fat overloading, especially if sustained over longer time periods. Theoretically, this could have paradigm changing consequences in our attempts to understand fetal programming of metabolic diseases by epigenetic mechanisms [6].

The increase in DNA methyltransferases among NBW controls exposed first to the control diet [7] was not observed among the LBW group (ESM [Tables 1 and 2]), which to some extent supports the possibility that differential overfeeding responses between LBW and NBW individuals may be caused by differential de novo DNA methylations. However, direct measurements of the activities of these enzymes are needed to substantiate this idea.

In conclusion, no major absolute skeletal muscle DNA methylation differences in LBW vs NBW controls at baseline or after 5 days' high-fat overfeeding were observed using a random array approach. We did, however, discover

indications of decreased plasticity of muscle DNA methylation dynamics in LBW men after overfeeding, which may contribute to the risk of developing insulin resistance and type 2 diabetes in those born with LBW.

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Duality of interest SCJ and PP are shareholder and employee at Novo Nordisk A/S; AV is a shareholder and lecturer at Novo Nordisk A/S; CB and JB-J are shareholders at Novo Nordisk A/S. Other authors declare that there is no duality of interest in connection with their involvement in this study.

Contribution statement SCJ and LG acquired, analysed and interpreted the data, and drafted the manuscript. CB designed the study and analysed and interpreted the data. JB-J, RR-M, MFF, CL and PP analysed and interpreted the data. AFF, EL and VC acquired the data. AV designed the study and interpreted the data, and is responsible for the integrity of the work as a whole. All authors critically revised and approved the final version of the manuscript.

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