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Maternal undernutrition during periconceptional period affects whole-genome ovine muscle methylation in adult offspring

This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1873321 since 2023-05-17T09:47:30Z

Published version:

DOI:10.1093/jas/skac180

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(Article begins on next page)

11 May 2024

Short Communication: Maternal undernutrition during peri-conceptional period affects whole genome ovine muscle methylation in adult offspring

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Acknowledgement

The authors acknowledge the support of the following projects: SCALA-MEDI, Improving sustainability and quality of Sheep and Chicken production by leveraging the Adaptation potential of LocAl breeds in the MEDIterranean area, funded by the European Union's Horizon 2020 Partnership for Research and Innovation in the Mediterranean Area Programme (PRIMA) Grant 2012; LEO, Livestock Environment Opendata, 16.2 – PSRN 2014-2020 financed through Fondo Europeo Agricolo per lo Sviluppo Rurale (FEASR); NOVALSELPROV, InNOVAzioni nelle PROduzioni casearie OVine di sicilia e sardegna, PON project n. ARS01_00580.

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Lay Summary

The formation of the epigenetic pattern of an organism is highly sensitive to environmental factors, especially during early mammalian development, when epigenetic reprogramming of the whole genome takes place. In utero adverse conditions experienced during early pregnancy, such as maternal undernutrition, may induce long-lasting epigenetic changes to the resulting offspring. This study investigated the CpG methylation variations in muscle tissue of adult offspring induced by differences in the diet of their mothers during pregnancy. Our data show that undernutrition during pregnancy leads to epigenetic alterations in the muscle of the offspring, with a potential impact on animal health and productivity.

Teaser Text

Alteration of the maternal diet of sheep impacts the CpG methylation of genes related to muscular tissue development in muscle tissue of adult offspring.

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Abstract

Experimental and epidemiological studies suggest that maternal nutritional status during early pregnancy, including the period around the time of conception, may induce long-lasting epigenetic changes in the offspring. However, this remains largely unexplored in livestock. Therefore, the objective of this study was to evaluate if modification of the maternal diet of sheep (CTR: control; UND: 50% undernutrition) during the peri-conceptional period (42 days in total: -14/+28 from mating), would impact CpG methylation in muscle tissue (*Longissimus dorsi*) of adult offspring (11.5 months old). Reduced Representation Bisulfite Sequencing (RRBS), identified 262 (Edge-R, FDR<0.05) and 686 (Logistic Regression, FDR <0.001) differentially methylated regions (DMRs) between the UND and CTR groups. Gene ontology (GO) analysis identified genes related to development, functions of the muscular system and steroid hormone receptor activity within the DMRs. The data reported here show that nutritional stress during early pregnancy leads to epigenetic modifications in the muscle of the resulting offspring, with possible implications for cardiac dysfunction, muscle physiology and meat production.

Keywords: diet; epigenetics; methylation; muscle; undernutrition

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List of Abbreviations

CTR, control; UND, undernutrition; RRBS, Reduced Representation Bisulfite Sequencing; DMRs, differentially methylated regions; 5-mC, 5-methylcytosine; FDR, False Discovery Rate; GO, Gene Ontology; LR, logistic regression; *SLC2A4*, *Solute Carrier Family 2 Member 4; GLUT4, Glucose Transporter Type 4; ERK1, extracellular signal-regulated kinase 1; ERK2, extracellular signal-regulated kinase 2; PPARGC1α, peroxisome proliferator–activated receptor-γ coactivator-1α; PPARδ, peroxisome proliferator–activated receptor-δ; ABCC8, ATP Binding Cassette Subfamily C Member 8; SUR1, sulfonylurea receptor 1; IRS1, insulin receptor substrate 1; IRS2, insulin receptor substrate 2; FTO, fat mass and obesity-associated protein; ATP10B, ATPase phospholipid transporting 10B; ATP11A, ATPase phospholipid transporting 11A; ATP13A5, ATPase phospholipid transporting 13A5; PTP1N, protein tyrosine phosphatase non- receptor type 1; PBMCs, peripheral blood mononuclear cells.*

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Introduction

In utero fetal programming is well documented and supported by an extensive epidemiological and experimental literature. Maternal undernutrition, specifically during early gestation, can result in permanent changes in the physiology and metabolism of the offspring, which in turn lead to an increased risk of cardiovascular and metabolic disorders in adulthood (Ravelli et al., 1998; Roseboom et al., 2000; Yan et al., 2013).

The epigenome constitutes the interface between the external environment and the genome, and suboptimal maternal nutrition may have long term consequences for gene expression in the resulting offspring. The early developmental window, especially the peri-conceptional period, seems to be particularly sensitive to nutrient deficiencies (Van Soom and Fazeli, 2015; Sun et al., 2016). In this period extensive epigenetic reorganization of the genome occurs (Reik et al., 2001). Shortly after fertilization the mammalian genome undergoes extensive demethylation, then in preimplantation embryos lineage-based methylation differences are established (Santos et al., 2002). As a result, different germ layers display distinct genome-wide DNA methylation pattern, which affect gene expression in the resulting tissues (Slieker et al., 2015). Therefore, the time around conception is crucial for the establishment of the adult epigenotype, and nutritional induced epigenetic alterations can be maintained throughout life, influencing tissue specific gene expression patterns (Lee, 2015).

Maternal undernutrition is frequently reported in livestock, due to seasonal and environmental variation of food quality and availability (Dunlap et al., 2015). Skeletal muscle has a low priority for nutrient allocation during fetal development (Redmer et al., 2004) and is vulnerable to reduced nutrient availability compared with other organs such as the brain or liver (Zhu et al., 2006; Yan et al., 2013). Skeletal muscle is therefore a good candidate tissue for assessing whether undernutrition induces long-lasting epigenetic changes.

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There are several reports of altered methylation of the genome in muscle tissues of fetuses resulting from maternal undernutrition (Lan et al., 2013; Yan et al., 2013; Lie et al., 2014; Peñagaricano et al., 2014), while there are limited data regarding the epigenetic status after birth. Experimental evidence in sheep has shown that maternal undernutrition during the peri-conceptional period leads to poor metabolic outcomes in the resulting offspring due to altered regulation of the glucose-insulin axis (Yan et al., 2013; Dunlap et al., 2015; Oliver et al., 2020). Similarly in pigs, maternal undernutrition during pregnancy decreases *GLUT4* expression in the muscle of adult offspring, which is most likely due to the increased methylation of the muscle *GLUT4* promoter (Wang et al., 2016).

We explored the effect of reduced diet (50% of the standard daily food ration) imposed on sheep during peri-conceptional period on the epigenetic programming of the skeletal muscle of adult offspring. Genome wide methylation status of adult muscle tissues was explored using Reduced Representation Bisulfite Sequencing (RRBS) of adult offspring that had experienced peri-conceptional maternal undernourishment (UND) and normal feeding (CTR).

Material and methods

All experimental procedures involving animals were conducted in accordance with Animal Protection Regulations of Italy, (DPR 27/1/1992) in conformity with European Community regulation 86/609. All animal experiments were performed with the approval of the local ethical committee (CEISA-comitato etico interistituzionale per la sperimentazione animale N° PROT.UNCHD12-222/2014). All chemicals were obtained from Sigma Aldrich unless otherwise stated.

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Animal nutrition and breeding management

A commercial flock provided 12 multiparous Sarda breed sheep that were randomly assigned to two groups of 6 ewes and administered one of the 2 diets during the peri-conceptional period, from 14 days before mating (oocyte maturation) until 28 days after mating (early organogenesis), 42 days in total which corresponds to Carnegie stage 19 (Butler and Juurlink, 1987). Control (CTR) ewes were fed ad libitum with a balanced diet satisfying nutritional requirements of pregnant ewes (300gr/animal/day). Undernourished (UND) ewes were fed a restricted diet (50% of the requirements: 150gr/animal/day) of the same diet. The nutritional stress imposed on the animals was sufficient to induce a gross caloric restriction without affecting the health and wellbeing of the animals. All animals had a full clinical assessment each week (mucosal score, temperature, heart rate and respiration). To optimize the pregnancy rate, estrus was synchronized by treating all ewes with intravaginal progesterone sponges (25-mg Chronogest; Intervet Ireland Ltd, Dublin) for 14 days. At the time of sponge removal, a ram of proven fertility was used to mate the two nutritional treatment groups, temporarily kept in a common paddock. From the time of mating onwards, ewes were group housed in pens under natural day length conditions with ad libitum access to water. At day 28 of gestation all ewes were returned to an ad libitum diet, to meet daily energy and nutritional needs and ensure that all animals had regained sufficient fat reserves to maintain the pregnancy for the 149.7 days average gestation period for the Sarda sheep breed and for the onset and maintenance of lactation. Following parturition and birth-weight recording, all animals derived from a singleton pregnancy; 6 CTR (4 males and 2 females) and 6 UND (3 males and 3 females) were managed under standard farm conditions until 11.5 months of age. A non-parametric one-way ANOVA (Kruskal-Wallis test) was used to analyse weight data using Graph Pad Prism software. Data are presented as mean ± SEM.

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Muscle tissue collection

Muscle tissue (50mg) from *Longissimus dorsi* were surgically sampled, between the 12th and 13th rib, under general anesthesia (acethyl-promazin IM followed by Thyopental Sodium) from all CTR and UND lambs at around 11.5 month of age and stored at -80°C for subsequent molecular analysis.

Reduced Representational Bisulfite (RRBS) Library preparation and sequencing

Genomic DNA from each muscle sample (n=12; 6 CTR and 6 UND), was isolated using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions. DNA concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). One µg of DNA was digested with MspI (New England Biolabs, Ipswich, MA, United States) by overnight incubation at 37°C, following the manufacturer's instruction. Libraries were generated using a TruSeq DNA PCR-Free Library Preparation Kit (Illumina, San Diego, CA, United States). After adapter ligation, samples were converted using an EpiTect Bisulfite Kit (Qiagen, Venlo, Netherlands) and finally PCR amplified with KAPA HiFi Uracil+ kit (Kapa Biosystems, Potters Bar, United Kingdom). RRBS libraries were sequenced on an Illumina Hiseq 3000 (San Diego, CA, United States) to generate 150-base paired-end reads.

Bioinformatic Analysis

The preliminary quality control of raw sequence reads was carried out with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Illumina raw sequences were then filtered with Trimmomatic software to remove adapters and low-quality bases at the ends of reads, using a sliding window approach (Bolger et al., 2014). Data are available in the Sequence Reads Archive (SRA), BioProject accession number, PRJNA757927. Bismark

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software v.0.17.0 was used to align each read to a bisulfite-converted sheep genome (Oar_v4.0) with option -N 1. Methylation calls were extracted using the Bismark methylation_extractor function. Seqmonk software (version 1.47.1) (http://www.bioinformatics.babraham.ac.uk/projects/seqmonk/) was used for the visualization and analysis of the Bismark output. Only cytosines present in all 12 animals (6 CTR and 6 UND) with a coverage of 10X were imported and analyzed by splitting the reference genome into 200 bp sliding windows with a 100 bp offset. Methylation percentage was calculated for each window if at least 3 cytosines were detected. DMRs were identified by comparing CTR vs UND groups. DMRs were calculated using both EdgeR (FDR < 0.05) and a logistic regression (LR) filter in R (FDR < 0.001), with differences of methylation level between CTR and UND groups above 10%. LR uses a binomial distribution and is a simple but yet efficient method for estimation of differential methylation (Huh et al., 2019). EdgeR uses empirical Bayesian methods to model gene specific biological variation between biological replicates through the negative binomial dispersion parameter. EdgeR linear models fit the total read count (methylated + un-methylated) along loci. At each locus, the ratio of methylated reads is modelled indirectly as an over-dispersed binomial-like distribution. It has been demonstrated as an efficient solution for methylation studies even when only a few biological replicates are available (Chen et al., 2017). To compute shared DMRs, and DMR associated genes across comparisons, commonality analysis was run and visualized through Venn diagrams, using the Venn Diagrams software (http://bioinformatics.psb.ugent.be/webtools/Venn/). DMR associated genes were classified according to classical Gene Ontology (GO) categories, using the Cytoscape plug-in ClueGO which integrates GO and enhances biological interpretation of large lists of *genes* (Bindea et al., 2009). In ClueGO, the *P*-value was calculated with the Fisher Exact Test corrected using the Bonferroni step down method.

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Reverse transcription quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from muscle tissues using TRIzol (Invitrogen, Carlsbad, CA) and cleaned up with a NucleoSpin miRNA kit (Macherey-Nagel, Dueren, Germany), following the protocol which gives small and large RNA in one fraction (total RNA). RNA was reverse transcribed into cDNA in a total reaction volume of 18 µl. Nine µl of RNA was added of 0.5 µl of random hexamers, 0.5µl oligodT, 0.5µl dNTPs and incubated at 65°C for 5 minutes, then placed at 4°C. Four µl RT buffer (5 x), 1µl of DTT (0.1 M), 0.5µl RNase inhibitor and 1µl of SuperScript II Reverse Transcriptase (Thermo Fisher, Waltham, MA USA) were added. Reverse transcription was carried out at 25˚C for 5 minutes, 42˚C for 1 hour and 70˚C for 15 minutes. The expression level of *PPARGC1α PPARδ*, *PTP1N* and *IRS2* was assessed by Reverse transcription quantitative real-time PCR (RT-qPCR), using the expression of *GADPH*, *ACTB* genes as reference. Primers were designed from specific exon-exon junctions to avoid amplifying genomic DNA (Supplementary Table S1). RT-qPCR was performed with three technical replicates, using 5 µl of the Power SYBR Green Master Mix (Applied Biosystems, Carlsbad, California, USA) and 0.5 µl of forward and reverse primers (final concentration 400nM) and 4 µl of diluted cDNA (1:20 Vol.), with QuantStudio 6 Flex Real-Time PCR Systems (Applied Biosystems, Carlsbad, California, USA). Relative expression of genes between CTR and UND groups and *P*-Value (t-test) were calculated using the PCR R package (Ahmed et al., 2018).

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Results

Offspring weight

Lamb birth weights were similar between maternal dietary groups (CTR: 3.5±0.32Kg; UND: 3.6±0.28Kg) and were appropriate, given the size of the ewe and plan of nutrition during pregnancy. At around 1 yr of age, at the time of muscle tissue collection, there was no difference in body weight between the two groups of sheep (11.5 months; CTR: 44.20±2.93 Kg; UND: 40.63±2.10 Kg).

Muscle Methylation

The average number of sequence reads per muscle sample was 20.4 M (ranging from 12.9 M to 31.3 M) with a mapping efficiency of about 60%. RRBS had an average level of cytosine methylation in the CpG context of 59.2% across all muscle samples in both CTR and UND animals (Supplementary Table S2). A total of 112,852 methylated regions (MRs) were detected across all samples using SeqMonk software. All these regions were evaluated using two different statistical methods to identify methylation variation between the two groups greater than 10% (Figure 1). Pairwise comparison of muscle methylation between experimental groups identified 262 and 686 DMRs in CTR vs UND calculated using Edge-R (FDR<0.05) and LR (FDR<0.001), respectively; annotation of which identified 99 and 253 associated genes for Edge-R and LR, respectively.

Both statistical approaches identified genes that were hyper and hypo methylated between CTR and UND groups. We investigated their function by GO analysis (Table1). The GO classes showed enrichment for genes which had functions that were predominantly related to muscle development, growth, and maintenance, including actin cytoskeleton organization, myoblast proliferation, muscle cell formation and contraction, regulation of carbohydrate

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metabolic process, steroid hormone activity and negative regulation of *ERK1* and *ERK2* cascade (Table1).

RT-qPCR evaluation of PPARGC1α, PPARδ, PTP1N and IRS2 expression in muscle

The expression level of, *PPARGC1α*, *PPARδ*, and *PTP1N* was lower in the UND vs CTL group while *IRS2* increased in UND animals. *PPARGC1α* showed the highest variation in gene expression (Supplementary Figure 1).

Interestingly, the expression of these genes was inversely correlated with the methylation level of DMRs in which they were found (Supplementary Table S3). *PPARGC1α, PPARδ* DMRs had higher methylation and *IRS2* lower methylation in UND animals compared to CTL. Conversely, *PTPN1* had both a higher methylation level and a slightly higher expression in UND animals (Supplementary Table S3).

Discussion

To date, diet induced effects on the development of muscle have been assessed using morphological parameters, such as size, weight, fibre number and composition (Fahey et al., 2005; Quigley et al., 2005; Sharples et al., 2016), however, the molecular mechanisms controlling differences remain largely unexplored. This study found that the 50% diet restriction imposed on sheep around the time of conception lead to epigenetic alterations in the muscle of the adult offspring. This is consistent with other reports that have shown that reduced nutrition during early fetal life is associated with abnormal methylation status in fetal muscle tissues (Lan et al., 2013; Namous et al., 2018).

In the present study the methylation frequency of CpG sites of *Longissimus dorsi* muscle tissues from 11.5 month old lambs ranged between 56% and 62% with no difference in overall methylation levels between UND and CTR groups. Similar results have been reported

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for sheep *Longissimus dorsi* muscles of eight-month old lambs with about 50-55% of the total CpG sites methylated (Couldrey et al., 2014). A small increase of 5-mC content in muscle DNA in pigs receiving a methyl supplemented diet has been suggested, although the difference was not significant (Braunschweig, 2009).

In the present study, differential methylation between UND and CTR groups found several DMRs which were consistently identified using two different statistical methods. The two methods identified alterations in the methylation status of specific genes, the functions of which are mainly related to muscular development and activity. GO analysis of annotated DMRs identified "negative regulation of the ERK1 and ERK2 cascade" pathway, which has recently been associated with the switch between fast-to-slow muscular fibre types (Boyer et al., 2019). We found several genes containing DMRs that have also been identified previously in diet restriction studies. These include the peroxisome proliferator–activated receptor-γ coactivator-1α (*PPARGC1α*), which is involved in glucose and fatty acid metabolism in liver and muscle (Liang et al., 2006). A rat model of intra-uterine growth restriction showed an increase in the DNA methylation of specific CpG sites in *PPARGC1α*, and relative decrease in the transcriptional level in muscle tissues with a positive correlation with fasting insulin concentration (Xie et al., 2015). We also found increased methylation in *PPARδ*, and *PPARGC1α*, and a lower, although not significant, expression of these two genes in UND ewes. Interestingly, both PPARGC1α and PPARδ have been previously reported to regulate muscle fibre composition. PPARGC1a is a principal factor regulating muscle fibre type determination by activating genes implicated in slow fibre gene expression (Lin et al., 2002). Endurance exercise training promotes PPARδ expression by inducing an increase in the number of type I muscle fibres (Wang et al., 2004). Fibre composition within a muscle is important for the growth potential of skeletal muscle, for endurance fitness, and for adaptability to environmental stress. Maternal nutrition during pregnancy may influence type

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and amount of adult muscle fibres which are determined in utero (Zhu et al., 2004; Fahey et al. 2005, Quigley et al. 2005). Interestingly, despite the lack of muscle structural analysis, in this work, PPARGC1α and PPARδ both showed changes in methylation level and expression in lambs born to ewes that had undergone diet restriction.

PPARGC1and PPARδ also impact aerobic physical fitness and insulin sensitivity in humans (Stefan et al., 2007). The long-term effects of undernutrition of sheep during the peri-conceptional period has been reported to impair glucose-insulin axis function (Todd et al., 2009; Dunlap et al., 2015), affect the insulin signalling pathway (Nicholas et al., 2013) as well as glucose uptake (Dunlap et al., 2015) in skeletal muscle of adult offspring. This suggests diet affects the metabolic health of the next generation.

Maternal undernutrition is known to have a transgenerational effect, causing fetal hyperinsulinemia, increased diabetic risk and obesity in both first and second generation offspring of rats (Aerts and Assche, 2006). Transgenerational transmission requires alterations in the DNA of the germ cell line. Although a general demethylation occurs during early embryo cleavage, a proportion of genomic domains are resistant to early embryo methylation reprogramming (Li et al., 2018). In mice, the intergenerational transmission of glucose intolerance is the result of altered expression of *ATP Binding Cassette Subfamily C Member 8* (*ABCC8*) coding for the *sulfonylurea receptor 1* (SUR1) protein in β-cell islets (Jimenez-Chillaron et al., 2009). Sperm of in utero undernourished offspring has been shown to have specific DMRs in *ABCC8* that are retained in the F2 population (Radford et al., 2014). In our study, specific alterations in *ABCC8* methylation were also observed in lamb muscle tissue. In addition, methylation patterns of other key genes related to glucose metabolism including insulin receptor substrate 2 (*IRS2*), fat mass and obesity-associated protein or (*FTO*), ATPase phospholipid transporting (*ATP11A* and *ATP13A5*) and protein

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tyrosine phosphatase, non- receptor type 1 (*PTP1N*) differed between CTR and UND progeny.

Whole genome bisulfite sequencing of human sperm revealed methylation differences in *PPARGC1α* and *FTO* and other family members including insulin receptor substrate 1 (*IRS1*) and ATPase phospholipid transporting 10B (*ATP10*) in patients with type 2 diabetes mellitus (Chen et al., 2019). *PTPN1* also showed variations in methylation in peripheral blood mononuclear cells (PBMCs) in patients with type 2 diabetes mellitus (Huang et al., 2017). The effect of maternal undernutrition is known to cause insulin resistance in skeletal muscle by reducing glucose uptake stimulated by insulin and promote lipid accumulation in muscle cells, which may be ascribed to methylation changes in lamb muscle (Phielix and Mensink, 2018).

In conclusion, the present study shows that maternal peri-conceptional undernourishment of sheep impacts the muscle methylome in adult offspring. Due to the limited number of samples and an unbalanced sex ratio we are not able to define gender related differences as reported in a previous work analysing the periconceptional undernutrition effect on the phenotype of adult sheep (Jaquiery et al.,2012; Tood et al., 2009). However, specific genes involved in muscle fibre structure and muscular energy metabolism are affected, suggesting that early life events have long term consequence for adult life. Interestingly, in the most differentially methylated regions, many genes responsible for muscle development were identified, suggesting that undernutrition may increase the risk of metabolic disease. The investigation of the expression of genes associated with metabolism in differentially methylated regions showed a decreased expression in UND. The follow up of muscle methylation status *vs.* metabolic profiles and structural differences of muscle in progeny of ewes with different nutritional diets are needed, to shed light on the biological mechanisms linking undernutrition, epigenomics and muscle physiology.

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Ethics approval

All experimental procedures involving animals were conducted in accordance with DPR 27/1/1992 (Animal Protection Regulations of Italy) in conformity with European Community regulation 86/609. All animal experiments were performed under the authority of the local ethical committee approval (CEISA-comitato etico interistituzionale per la sperimentazione animale N° PROT.UNCHD12-222/2014).

Data availability

The datasets presented in this study can be found in online repositories. Raw data was deposited to the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under BioProject accession number PRJNA757927.

Author contributions

Conceptualization and planned the experiments, P.L. J.L.W. and P.A.M: methodology for animal work P.T. and P.L.; methodology for molecular biology experiments E.C.; data analysis, M.D.C and B.L; data curation, E.C. B.L and P.A.M.; writing—original draft preparation E.C. and P.T.; writing—review and editing, J.L.W. A.S., P.L. and P.A.M. Funding acquisition J.L.W. All authors have read and agreed to the published version of the manuscript."

Declaration of interest

The authors declare no conflict of interest.

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Figure Legend

Fig. 1. Venn diagram of Shared Differentially Methylated Regions (DMRs) identified with Edge-R and Logistic Regression (LR) methods.

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Table 1

Gene Ontology (GO) analysis of annotated genes found to be differentially methylated between UND and CTR group using two Edge-R and LR approaches and considering the regions that were hypermethylated in UND and CTR group.

Total DMRs (Edge-R)			
GO-ID	Associated Genes Found	GO-Term	P-Value*
2000291	[MEIS2, PPARD, ZNF609]	regulation of myoblast proliferation	5.97E-04
2027	[CACNA1C, CACNA1G, DMD, DSP, IRX5]	regulation of heart rate	1.30E-03
51450	[MEIS2, PPARD, ZNF609]	myoblast proliferation	1.52E-03
31532	[EZR, NTF3, PARVB, PHACTR1, PTPN1]	actin cytoskeleton reorganization	1.94E-03
14909	[BCL2, ITGB3, PPARD, PPARGC1A]	smooth muscle cell migration	3.91E-03
70373	[DMD, EZR, PTPN1]	negative regulation of ERK1 and ERK2 cascade	3.94E-03
86003	[CACNA1C, CACNA1G, DSP]	cardiac muscle cell contraction	3.94E-03
86001	[CACNA1C, CACNA1G, DMD, DSP]	cardiac muscle cell action potential	4.00E-03

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105 18	[ADCYAP1R1, ESR1, NTF3]	positive regulation of phospholipase activity	6.74 E-03
148 12	[BCL2, ITGB3, PPARD, PPARGC1A]	muscle cell migration	6.81 E-03
860 91	[CACNA1C, CACNA1G, DSP]	regulation of heart rate by cardiac conduction	7.33 E-03
518 99	[ADORA2A, BCL2, CACNA1C, CACNA1G]	membrane depolarization	7.85 E-03
149 10	[BCL2, PPARD, PPARGC1A]	regulation of smooth muscle cell migration	8.93 E-03
860 65	[CACNA1C, CACNA1G, DSP]	cell communication involved in cardiac conduction	1.09 E-02
190 027 4	[ADCYAP1R1, ESR1, NTF3]	regulation of phospholipase C activity	1.09 E-02
108 63	[ADCYAP1R1, ESR1, NTF3]	positive regulation of phospholipase C activity	1.09 E-02
370 7	[ESR1, NR2C2, PPARD]	steroid hormone receptor activity	1.12 E-02
106 76	[ADCYAP1R1, KAT2B, PPARGC1A]	positive regulation of cellular carbohydrate metabolic process	1.14 E-02
860 02	[CACNA1C, CACNA1G, DSP]	cardiac muscle cell action potential involved in contraction	1.25 E-02

Hyper-methylated UND (Edge-R)

GO-	Associated Genes Found	GOTerm	P-

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ID			Value*
14909	[BCL2, ITGB3, PPARD, PPARGC1A]	smooth muscle cell migration	7.31E-04
86001	[CACNA1C, CACNA1G, DMD, DSP]	cardiac muscle cell action potential	7.70E-04
14812	[BCL2, ITGB3, PPARD, PPARGC1A]	muscle cell migration	1.50E-03
70373	[DMD, EZR, PTPN1]	negative regulation of ERK1 and ERK2 cascade	1.51E-03
86003	[CACNA1C, CACNA1G, DSP]	cardiac muscle cell contraction	1.51E-03
86091	[CACNA1C, CACNA1G, DSP]	regulation of heart rate by cardiac conduction	1.73E-03
14910	[BCL2, PPARD, PPARGC1A]	regulation of smooth muscle cell migration	2.26E-03
10676	[ADCYAP1R1, KAT2B, PPARGC1A]	positive regulation of cellular carbohydrate metabolic process	3.25E-03
86065	[CACNA1C, CACNA1G, DSP]	cell communication involved in cardiac conduction	3.28E-03
3707	[ESR1, NR2C2, PPARD]	steroid hormone receptor activity	3.53E-03
86002	[CACNA1C, CACNA1G, DSP]	cardiac muscle cell action potential involved in contraction	4.02E-03
	Total DMRs (LR)		

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GO-ID	Associated Genes Found	GOTerm	P-Value*
2000291	[GDNF, MEIS2, PPARD, ZNF609]	regulation of myoblast proliferation	1.19E-03
51450	[GDNF, MEIS2, PPARD, ZNF609]	myoblast proliferation	4.90E-03
2000288	[GDNF, MEIS2, PPARD]	positive regulation of myoblast proliferation	9.70E-03
31532	[BAIAP2, EZR, FARP2, NTF3, PARVB, PHACTR1, PTPN1]	actin cytoskeleton reorganization	1.75E-02
7422	[ALDH3A2, DMD, GDNF, NTF3, PLXNA4, RUNX1]	peripheral nervous system development	1.84E-02
48813	[BAIAP2, CUX1, KIF1A, MAP2, NSMF, PHACTR1, SEMA4D, SHANK1]	dendrite morphogenesis	2.54E-02
86001	[CACNA1C, CACNA1G, DMD]	cardiac muscle cell action potential	4.89E-02

Hyper-methylated UND (LR)

GO-ID	Associated Genes Found	GOTerm	P-Value*
86001	[CACNA1C, CACNA1G, DMD]	cardiac muscle cell action potential	1.55E-02

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360 37	[BCL2, RUNX1, SH3RF1]	CD8-positive, alpha-beta T cell activation	2.14 E-02
508 55	[FOXP1, LYN, RUNX1]	regulation of B cell receptor signaling pathway	2.28 E-02
305 21	[FOXP1, NCOR2, PMEPA1, PPARGC1A]	androgen receptor signaling pathway	2.29 E-02
105 22	[ADCYAP1R1, BCL2, CACNA1C, DMD, LYN]	regulation of calcium ion transport into cytosol	2.33 E-02
508 54	[EZR, FOXP1, LYN, RUNX1]	regulation of antigen receptor-mediated signaling pathway	2.46 E-02
193 95	[ACOXL, ALDH3A2, PPARD, PPARGC1A, SOAT2]	fatty acid oxidation	2.55 E-02
315 32	[BAIAP2, EZR, FARP2, PHACTR1, PTPN1]	actin cytoskeleton reorganization	2.59 E-02
344 40	[ACOXL, ALDH3A2, PPARD, PPARGC1A, SOAT2]	lipid oxidation	2.76 E-02
254 8	[CCL16, CCL17, LYN]	monocyte chemotaxis	2.78 E-02
400 14	[BCL2, EZR, FTO, RAI1]	regulation of multicellular organism growth	2.88 E-02
149 09	[BCL2, ITGB3, PPARD, PPARGC1A]	smooth muscle cell migration	2.94 E-02
703 73	[DMD, EZR, LYN, PTPN1]	negative regulation of ERK1 and ERK2 cascade	2.99 E-02
742	[ALDH3A2, DMD, PLXNA4, RUNX1]	peripheral nervous system	3.53

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GO-ID	Associated Genes Found	GOTerm	P-Value*
2		development	E-02
60612	[FTO, PPARD, PPARGC1A]	adipose tissue development	4.01E-02
32623	[EZR, HDAC7, RUNX1]	interleukin-2 production	4.01E-02
14812	[BCL2, ITGB3, PPARD, PPARGC1A]	muscle cell migration	4.28E-02
71526	[FARP2, PLXNA4, SEMA4D]	semaphorin-plexin signaling pathway	4.43E-02
1901505	[SLC25A48, SLC29A3, SLC35A2]	carbohydrate derivative transmembrane transporter activity	4.60E-02
50919	[ITGB3, PLXNA4, SEMA4D]	negative chemotaxis	4.61E-02
5089	[ARHGEF10L, ARHGEF28, FARP2]	Rho guanyl-nucleotide exchange factor activity	4.75E-02
14910	[BCL2, PPARD, PPARGC1A]	regulation of smooth muscle cell migration	4.75E-02

Hyper-methylated CTR (LR)

GO-ID	Associated Genes Found	GOTerm	P-Value*
2000291	[GDNF, MEIS2, ZNF609]	regulation of myoblast proliferation	1.38E-04

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51450	[GDNF, MEIS2, ZNF609]	myoblast proliferation	2.97E-04
50885	[CAMTA1, RBFOX1, SHANK1]	neuromuscular process controlling balance	2.59E-03
48512	[ADORA2A, BTBD9, ZFHX3]	circadian behavior	2.82E-03

*Corrected with Bonferroni step down

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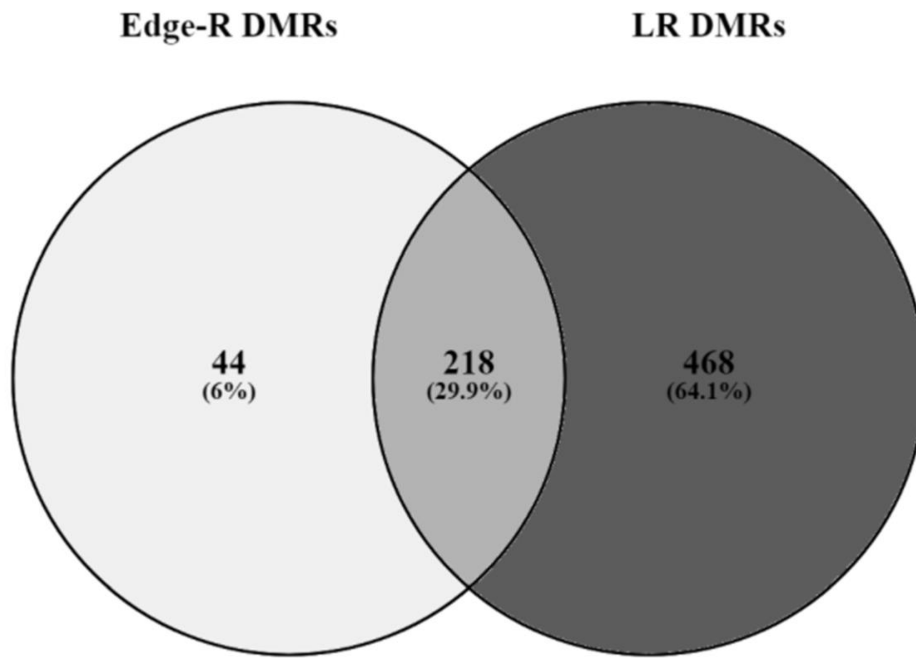
Figure 1



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