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Preconceptional and in utero exposure of sheep to a real-life environmental chemical mixture disrupts key markers of energy metabolism in male offspring

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Abstract

Over recent decades, an extensive array of anthropogenic chemicals have entered the environment and have been implicated in the increased incidence of an array of diseases, including metabolic syndrome. The ubiquitous presence of these environmental chemicals (ECs) necessitates the use of real-life exposure models to the assess cumulative risk burden to metabolic health. Sheep that graze on biosolids-treated pastures are exposed to a real-life mixture of ECs such as phthalates, per- and polyfluoroalkyl substances, heavy metals, pharmaceuticals, pesticides, and metabolites thereof, and this EC exposure can result in metabolic disorders in their offspring. Using this model, we evaluated the effects of gestational exposure to a complex EC mixture on plasma triglyceride (TG) concentrations and metabolic and epigenetic regulatory genes in tissues key to energy regulation and storage, including the hypothalamus, liver, and adipose depots of 11-month-old male offspring. Our results demonstrated a binary effect of EC exposure on gene expression particularly in the hypothalamus. Principal component analysis revealed two subsets (B-S1 [$n = 6$] and B-S2 [$n = 4$]) within the biosolids group (B, $n = 10$), relative to the controls (C, $n = 11$). Changes in body weight, TG levels, and in gene expression in the hypothalamus, and visceral and subcutaneous fat were apparent between biosolid and control and the two subgroups of biosolids animals. These findings demonstrate that gestational exposure to an EC mixture results in differential regulation of metabolic processes in adult male offspring. Binary effects on hypothalamic gene expression and altered expression of lipid metabolism genes in visceral and subcutaneous fat, coupled with phenotypic outcomes, point to differences in individual susceptibility to EC exposure that could predispose vulnerable individuals to later metabolic dysfunction.

KEYWORDS
biosolids, environmental-chemicals, in utero exposure, metabolism-related markers

1 | INTRODUCTION

The world is facing a significant increase in metabolic disorders, affecting over 1 billion people with conditions like fatty liver, type 2 diabetes, hypertension, hyperlipidemia, and obesity[1] costing around $2 trillion to the global economy.[2,3] This problem is expected to worsen, with a predicted 3.3 billion obese and overweight individuals worldwide by 2030.[3] While aging, lifestyle, and genetics play a role, environmental factors, particularly widespread exposure to man-made chemicals that are found ubiquitously within the environment, are thought to be contributory factors.[4] These environmental chemicals (ECs) can disrupt hormone action and lead to changes in energy balance and lipid metabolism at molecular and phenotypic levels.[4–9] Exposure to ECs during pregnancy is of particular concern, as it can have lasting effects on metabolic health across generations.[10]

Human exposure to ECs happens throughout life and involves various chemicals from detergents, pesticides, plastics, cosmetics, hydrocarbons, flame retardants, heavy metals, and more.[3] Some specific ECs such as dichlorodiphenyl dichloroethylene, bisphenol A (BPA), phthalates, parabens, polychlorinated biphenyls (PCBs), and heavy metals have been linked to metabolic problems and disrupted energy metabolism.[4,9,11–16]

The majority of research on the metabolic health consequences of EC exposure has been performed on rodents using short-term exposure to single or limited mixtures of chemicals. For example, data from murine studies have demonstrated that prenatal (F0) exposure to phthalates predisposes F1 males to obesity[17] and disrupts metabolic pathways in the liver.[18] Prenatal exposure of mice to PCB-153 is associated with abnormal glucose metabolism and lipid accumulation in the liver and viscera.[19] While useful, the results of such studies cannot be generalized to human real-life EC exposure, which is chronic, low-level exposure to an EC mixture. A more translationally relevant animal model is provided by sheep grazing on pastures treated with biosolids. Biosolids are a byproduct of wastewater treatment and are utilized as an agricultural fertilizer in many countries.[20] Biosolids contain a broad spectrum of ECs including heavy metals, brominated flame retardants, PCBs, per- and polyfluoroalkyl substances, pharmaceuticals, polyfluorinated hydrocarbons, personal care products, antibiotics, dioxins and metabolites thereof, to name but a few, and reflect the human exposome.[21–26] While individual EC concentrations are typically low, long-term gestational exposure of sheep to biosolids has been reported to adversely affect key metabolic organs such as the liver and the thyroid gland,[27,28] making biosolid grazed sheep model a valuable tool for studying the impact of real-life EC exposure. The translational relevance of the model also benefits from the sheep's greater longevity compared with most laboratory animals and additional shared physiological characteristics with humans including patterns of postnatal development, regulatory processes controlling the generation of ovarian cycles and the pathways used for steroid metabolism.[29–32] The biosolids grazed sheep model, therefore, provides a novel model to address the deficit in our knowledge concerning the metabolic consequences of exposure to real-life low-level mixtures of ECs.[33]

The regulation of energy homeostasis, a key component of metabolic control, involves various body systems and tissues including the liver, which supports metabolism, immunity, and vitamin storage; adipose tissue, an energy reservoir and the hypothalamus, which coordinates information from multiple metabolic organs/tissues including adipose tissue, the stomach, large and small intestine and other peripheral metabolic organs such as the liver and pancreas.[34,35] In the hypothalamus, the arcuate nucleus (ARC), which contains key populations of orexigenic (Agouti-related protein [AgRP] and Neuropeptide Y [NPY]) and anorexigenic (proopiomelanocortin [POMC] and cocaine- and amphetamine-regulated transcript) neurones that respond to peripheral signals like leptin, peptide YY, Ghrelin, and oestradiol, communicates with other regulatory hypothalamic nuclei including the paraventricular nucleus (PVN), dorsomedial hypothalamus, lateral hypothalamus, and ventromedial hypothalamus.[34,35]

This study aimed to evaluate the organizational effects of gestational exposure to biosolids, a source of a complex real-life mixture of ECs, on key regulatory systems that control metabolic phenotype in male offspring during their adult life.

To achieve this, we investigated the impact of gestational exposure to biosolids on offspring body weight (BW), plasma triglyceride (TG) levels, and the expression of key genes, with established roles in the regulation of metabolism, in the hypothalamus, liver, and adipose tissues (subcutaneous, visceral, and pericardial depots). The hypothalamic genes studied were ESR1 and LEPR in the medial preoptic area (mPOA), anterior hypothalamic area (AHA), and PVN[36–38] and ESR1, LEPR, NPY, and POMC in the rostral and caudal ARC.[36,39] The panel of markers assessed in the liver and adipose tissue were ZNF423 (a major determinant of preadipocyte commitment[40]), LPL (important in the distribution of fatty acids and lipoproteins outside of the liver[41]), PPARg (regulator of genes involved in lipid metabolism, obesity-induced inflammation, metabolic syndrome[42]), ESR1, PPARGC1A (transcriptional coactivator that regulates energy production genes[43]), DLK1 (inhibitor of adipogenesis and a preadipocyte marker[44,45]) and LEPTIN (adipose signal, shown to be sensitive to EC exposure.[8,46,47] Since the developmental impact of EC exposure via biosolids can be mediated by epigenetic modifications (especially DNA methylation),[48–50] the expression of epigenetic regulatory markers *DNMT1*, *DNMT3a*, and *DNMT3*b were also evaluated in key metabolism-related tissues.

Because findings from this model have shown that male, but not female, F1 offspring have a lower BW, across the first year of life compared with their respective controls,[51] this study focused on adult male offspring.

2 | MATERIALS AND METHODS

2.1 | Experimental design

All animal work was conducted at the University of Glasgow Cochno Farm and Research Centre, in accordance with the Home Office Animal (Scientific Procedures) Act 1986 under license PF10145DF.

EasyCare ewes were divided into two groups approximately 1 month before mating: the first group was grazed on pastures treated with inorganic fertilizer (control [C]), and the second group (B) was grazed on pastures fertilized with biosolids, at conventional rates (4 tonnes/ha, twice per annum, April, and September), which supplied equivalent levels of nitrogen to the pasture as the inorganic fertilizer. Ewes were mated via artificial insemination, with semen from four rams that had not been grazed on biosolid-treated pastures, ensuring there was an equal number of females mated to each male. Consequently, the offspring originated from four distinct genetic backgrounds or sire families. Both groups of pregnant ewes were maintained on their respective pastures until approximately 2 weeks before parturition when they were housed indoors and fed a standard ration; however, forage fed to B ewes was harvested from biosolids-treated pastures. After parturition, all C and B ewes and their lambs were maintained outdoors and grazed on C pastures. Lambs remained with their mothers until weaning, after which male lambs were maintained separately from females. At 11 months of age, 11 C and 10 B ram lambs were selected randomly (balanced across the four sires, with only one lamb from each dam), euthanized using an intravenous barbiturate overdose (140 mg/kg Dolethal, Vetroquinol, UK) and tissue samples collected (as detailed below). Before euthanasia, animals were weighed, and blood samples were collected by jugular venipuncture for assessment of plasma TG levels.

2.2 | Harvest and processing of metabolism-related tissues

At postmortem, the hypothalamus was removed and halved, one half was frozen on dry ice, and the other half was fixed in 10% neutral buffered formalin. The frozen samples were used for RNA extraction and molecular analysis. Samples of subcutaneous fat, visceral fat, pericardial fat, and liver ($20 \times 20 \times 5$ mm^3) were collected and snap-frozen in liquid nitrogen. Samples were stored at −80°C until analysis. Specific hypothalamic areas were isolated for RNA extraction as described previously.[52] Briefly, the hemi-hypothalamic block was cut into 2 mm coronal slices, and relative to visible anatomical landmarks (the anterior commissure, the mammillary body, etc.), 2 mm diameter punches representative of specific hypothalamic areas collected into TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). For this study, punches from the mPOA, AHA, PVN, rostral ARC, and caudal ARC were studied. These hypothalamic regions play pivotal roles in regulating energy metabolism.[38,53]

2.3 | Assessment of expression levels of mRNAs in metabolism-related tissues

Liver and hypothalamic samples were homogenized in TRIzol® and mRNA was extracted using a Qiagen RNeasy® RNA extraction Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, with one additional step for the tissue homogenates from fat, which were first centrifuged at 12000 ×*g* for 10 min at 4°C and the clear red part of the lysate was used for RNA extraction as described for the liver/hypothalamus.

For all tissues, the RNA concentration was measured and the quality was assessed by examination of the optical density 260/280 ratio (NanoDrop, Wilmington, DE, USA). To confirm RNA integrity, samples were electrophoresed on a 1% agarose gel, revealing distinct 18S and 28S bands. For each sample, 500 µg mRNA was reverse transcribed to cDNA using QuantiTect Reverse Transcription Kits (Qiagen, Hilden, Germany). The expression level of the following genes was determined: Hypothalamus: *LEPR*, *ESR1* (mPOA, AHA, PVN), *LEPR*, *ESR1*, *NPY*, *POMC* (rostral and caudal ARC), subcutaneous fat, visceral fat, and pericardial fat; and liver: *Leptin*, *ESR1*, *ZNF423*, *PGC1a*, *LPL*, *PPARg*, and *DLK1*. Expression of mRNAs for the methylation genes *DNMT1*, *DNMT3a*, and *DNMT3b* mRNAs was assessed in all tissues. The primer sequences for all the genes examined are shown in Table 1. Quantitative real-time polymerase chain reaction (PCR) was performed using Brilliant II SYBR Master Mix (Agilent Technologies, USA) on a Stratagene 3000 machine. In each real-time PCR reaction, the melting curve of the PCR product was checked to ensure primer specificity. REST© software[54] was used to analyze the real-time PCR data. Relative expression (log2) for each transcript was calculated following normalization to the endogenous reference gene, *ACTb* (the most stable one among the three tested housekeeping genes).

2.4 | Assessment of plasma TG concentrations

Plasma TG concentrations were measured in duplicate using a TG Colorimetric Assay Kit (Cayman Chemical, USA) according to the manufacturer's instructions. The sensitivity of the assay was 3.13 ng/mL with the intraassay coefficient of variation averaging 1.34%.

2.5 | Statistical analyses

All data in the text are presented as means ± SEM unless indicated otherwise. Each dataset was tested for homogeneity of variance and normality using Levene's and Shapiro–Wilk tests, respectively. Gene expression, BW, and TG levels were compared using Student's *t*-test on MetaboAnalyst 5.0 web tool (https://www.metaboanalyst.ca). A two-way analysis of variance and Tukey-honestly significant difference (HSD) in R (version 4.2.3) through R Studio (version 2023.03.0+386) for treatment and genotype was also applied to analyze the effects of sire and biosolids exposure as explanatory variables. $p < .05$ was considered statistically significant, and for each evaluated marker that changed significantly relative to C group, the percentage of changes is also included in the text. Because of the small sample sizes, to characterize the magnitude of differences and assess trends, BW and TG data were also analyzed by Cohen's effect size analysis, and Cohen's *d* values (.2, .5, and .8 considered as small, medium, and large effect sizes, respectively)[55,56] are included. Due to the importance of the distribution of data, the results are

TABLE 1 Primers used for real-time RT-PCR analysis.

Gene	Forward primer	Reverse primer	Seq. Ac. Num.[a]	Ann. Temp.[b]
Actb	CTGAGCGCAAGTACTCCGTGT	GCATTTGCGGTGGACGAT	NM_001009784.3	62
LEPR	AGTGTGCTTCCTGGGTCTTC	GTAGTAAAGGTAAGAAGGGCG	NM_001009763.1	63
ESR1	CTGCCAAGGAGACCCGCTACTG	TTTATCAATTGTGCACTGGTTGGTGG	XM_027972563.2	63
Leptin	AGGTGGGAAATGTGTTGATGG	ATGGATGGTTAGCAAGCCC	XM_027968780.2	63
NPY	CTCCAAGCCTGACAACCCTG	TTTCCCGTATCTCTGCCTGG	NM_001009452.1	63
POMC	ACCTCACCACGGAAAGTAACC	CTGCTGCTACCATTCCGACG	NM_001009266.1	62
ZNF423	CCAGAAGAAGATGCGGGATG	TTGAGGTTGTAGAGGGTGGG	XM_027977817.1	62
PPARGC1a	ACCAAACCCACAGAGAACCG	AGTTGTGGGTGGAGTTAGGC	XM_004009738.4	62
LPL	TGGAGTGACGGAATCTGTGG	AGACACTGGATAATGCTGCTG	NM_001009394.1	63
PPARg	TGGTTGACACAGAGATGCCG	TGGAGAAGTCAACGGTGGTG	NM_001100921.1	62
DLK1	AGTGTGTGACCTTTCCCG	CCTGGCAATCCTTTCCCGAG	XM_027957404.1	62
DNMT1	CAGCTCTCGTACATCCA	AATCTCGCGTAGTCTTG	NM_001009473.1	63
DNMT3a	CCAGCCAAAAAGCCCCGAAA	TGTTCCAAGGTGACGTTGAGGCTC	XM_015094252.3	63
DNMT3b	AGAGTTTGGAATAGGAGATCTTGTGTGGG	CTGCTGGAATCTCGGAGAACTTGC	XM_042230024.1	63

[a]Sequence accession number on NCBI sequence bank.
[b]Primers annealing temperature (°C).

shown as box plots. The box represents the interquartile range (IQR) containing the central 50% of the data, whereas the first quartile (25th percentile) and third quartile (75th percentile) mark the boundaries of the box. The yellow dot represents the mean, and the horizontal line represents the median of the data for each group. The whiskers extend to the minimum and maximum values within 1.5 times the IQR in each group.

For the clustering analysis of the expression of different evaluated markers, a heatmap of Pearson distances (complete clustering method) was plotted. The data were further analyzed with principal component analysis (PCA) and orthogonal projections to latent structure discriminant analysis (OPLS-DA, MetaboAnalyst 5.0, http://www.metaboanalyst.ca). Five principal components (PCs) were gained from the comparison between C and B groups in terms of the expression of various markers. Clustering analysis indicated that the B animals could be statistically subdivided into two subgroups (referred from now on as B-S1 and B-S2 and discussed in detail in the Results section). Correlation analysis was conducted using the Pearson correlation method, and coexpression of various markers within and between tissues were analyzed.

Differences in BW, TG levels, and gene expression patterns in the different metabolism-related tissues between C and B, C and B-S1, C and BS-2, and BS-1 and BS-2 were analyzed using Student's *t*-test.

3 | RESULTS

3.1 | Body weight and blood triglyceride level

Overall comparison of C and B groups showed a trend ($p \approx .08$) for the BW of B rams (41.7 ± 1.44 kg) to be lower (9.6%) than C rams (46.1 ± 1.93 kg) at 11 months of age (Figure 1A). Mean blood TG concentration at 11 months of age did not differ significantly between C (17.9 ± 1.31 mg/dL) and B rams (18.6 ± 1.82 mg/dL) (Figure 1B).

3.2 | Expression of metabolic and epigenetic gene markers in the hypothalamus

Maternal biosolids exposure was associated with differences in gene expression in the hypothalami of adult male offspring (Figure 2). Relative to C rams, B rams had significantly higher levels of expression of *LEPR* mRNA in the caudal ARC and PVN (33.5% and 26%, respectively) but not in the rostral ARC. An opposing picture was seen with regard to *NPY* and *ESR1*, which had significantly lower expression in the rostral ARC (14% and 34%, respectively) but was not affected in the caudal ARC or PVN of B compared with C rams. There were no differences in *POMC* expression in the ARC or any of these metabolic markers in the mPOA and AHA of B compared with C males (Figure S1). *DNMT1* (7%) and *DNMT3a* (5%) but not *DNMT3b* were expressed at a significantly lower level in the rostral ARC of B compared with C rams (Figure 2). Biosolid treatment had no impact on these three *DNMTs* in the other hypothalamic regions studied (Figures 2 and S1). There was a significant negative correlation between mRNA levels of *NPY* in the rostral ARC and *LEPR* in the caudal ARC ($r = -.46$). Within the rostral ARC, the level of *ESR1* mRNA expression was positively correlated with *DNMT1* ($r = .82$, $p = 4.79\text{E-}06$), *NPY* ($r = .71$, $p = 2.855 \times 10^{-4}$), and *DNMT3a* ($r = .67$, $p = 9.1351\text{E-}04$).

3.3 | Expression of metabolic and epigenetic genes in adipose tissue

mRNA expression of lipogenic markers—*ZNF423* and *LPL*—was lower (4.4% and 4.6%, respectively) in visceral but not the subcutaneous

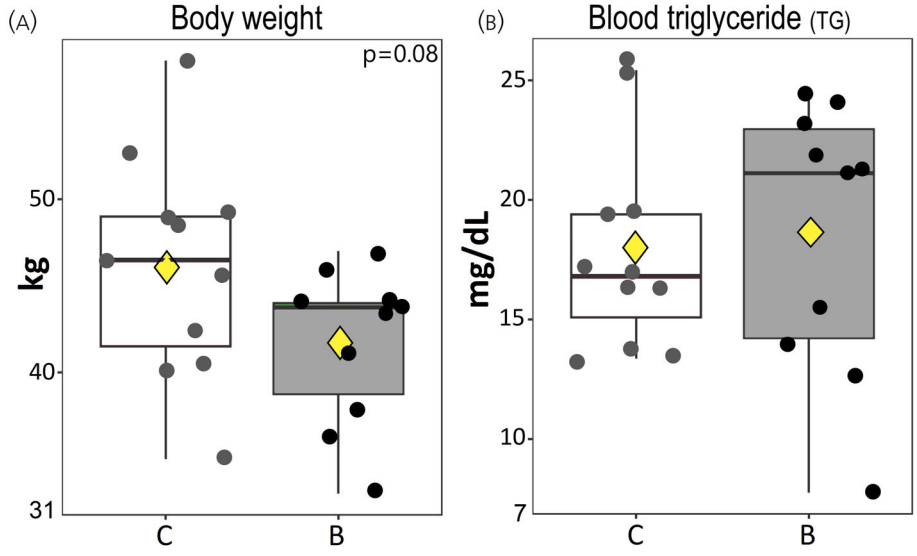

FIGURE 1 (A) At 11 months of age, there was a trend (Student's *t*-test, $p = .08$) for biosolid (B) rams ($n = 10$) to weigh less than control (C) rams ($n = 11$). Each dot represents an individual ram, the yellow dot represents the mean, and the horizontal line represents the median of the data for each group. The upper and lower whiskers represent maximum and minimum BW in each group, respectively. (B) The mean blood triglyceride (TG) concentrations in the C and B groups did not differ (Student's *t*-test). The yellow dot represents the mean, and the horizontal line represents the median of the data for each group. The upper and lower whiskers represent maximum and minimum amounts in each group, respectively.

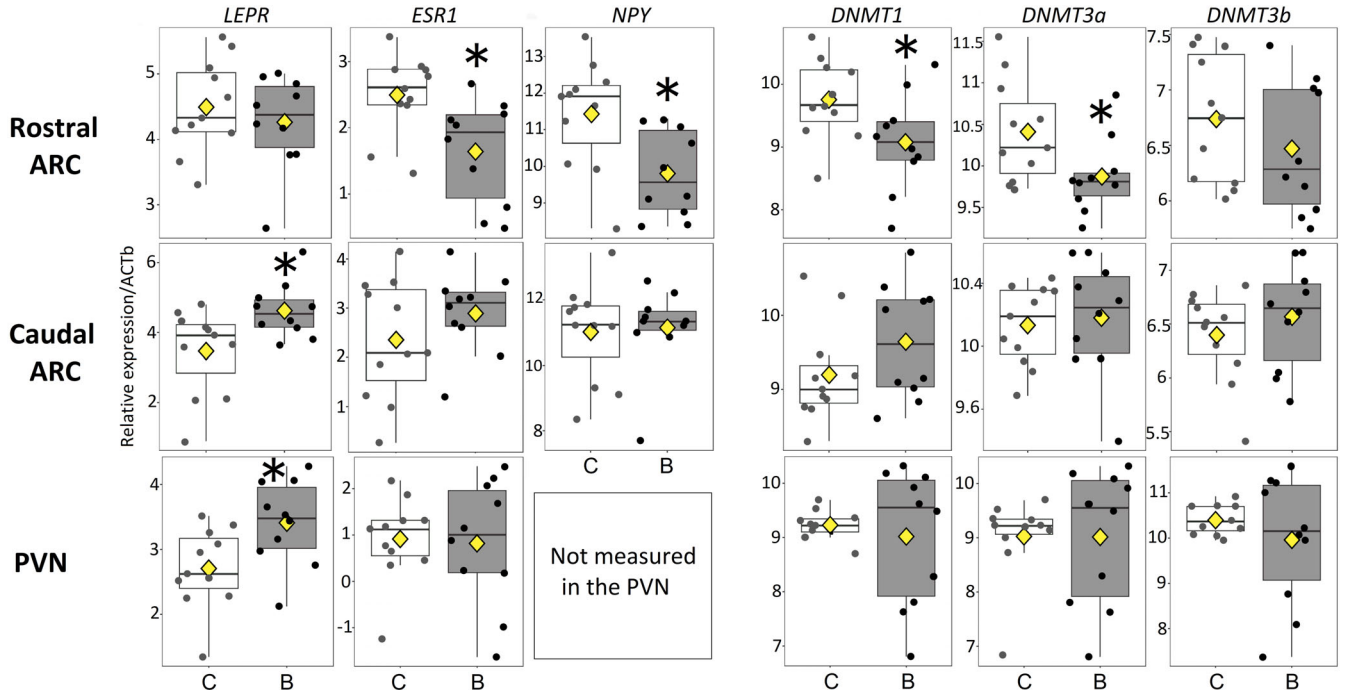

FIGURE 2 Differentially expressed markers in various hypothalamic nuclei of control (C, $n = 11$) vs Biosolid (B, $n = 10$) rams. B males possessed higher *LEPR* mRNA in the PVN, significant lower transcript levels of *ESR1*, *DNMT1*, *DNMT3a*, and *NPY* and a trend for *POMC* ($p = .1$) to be expressed at lower level in the rostral ARC and higher level of *LEPR* in the caudal ARC (Student's *t*-test, $^*p < .05$, the yellow dot represents the mean, and the horizontal line represents the median of the data for each group. The upper and lower whiskers represent maximum and minimum amounts in each group, respectively).

(Figure 3) and pericardial (data not shown) fat of B relative to C rams. In contrast, expression of *DLK1* mRNA was significantly higher (14%) in the subcutaneous but not in visceral (Figure 3) and pericardial fat (Figure S2) of B compared with C rams. The levels of expression of other metabolic (*PGC1a*, *PPARg*, *ESR1*, and *LEPTIN*) and methylation genes (*DNMT1*, *DNMT3a*, and *DNMT3b*) did not differ between C and B groups in any of the adipose tissue depots examined (Figures S2–S4).

3.4 | Expression of metabolic and epigenetic genes in liver

No significant differences were seen in the mRNA levels of any of the tested lipid metabolism and methylation markers (*ZNF423*, *PGC1a*, *LPL*, *PPARg*, *DLK1*, *ESR1*, and *LEPR*) in the liver of C and B animals (Figure S5).

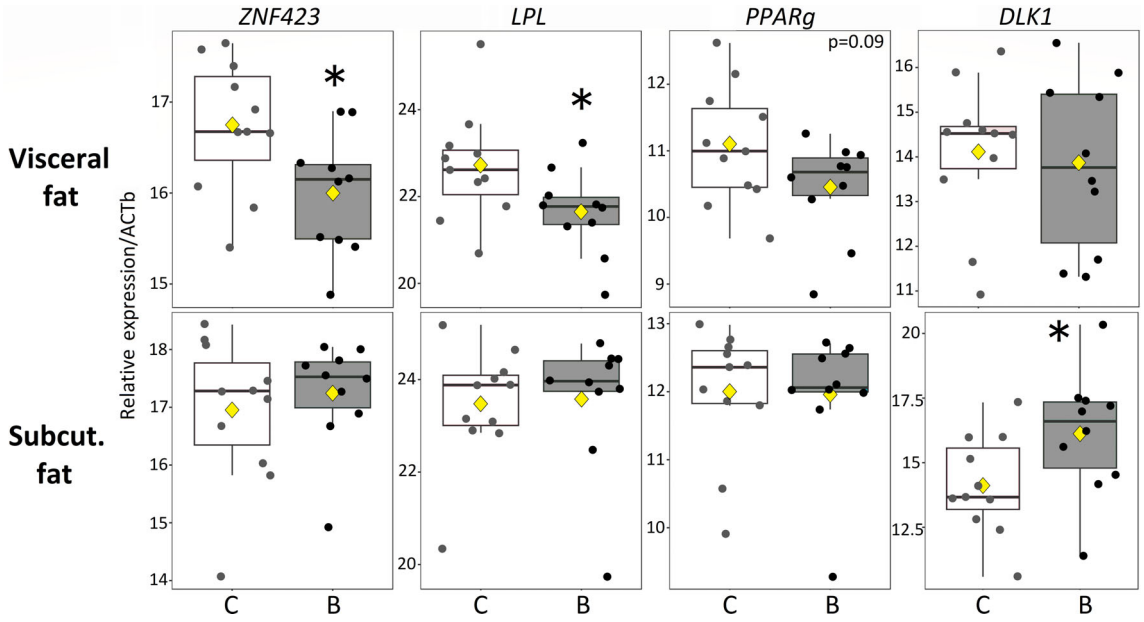

FIGURE 3 Molecular analysis revealed downregulation of the lipogenic markers, ZNF423, LPL, and PPARg in the visceral fat of B rams and higher expression of anti-lipogenic marker- DLK1- in the subcutaneous (subcut.) fat of B rams compared to C rams. No changes were observed in the gene expression profile of pericardial fat (Student's *t*-test, * $p < .05$, the yellow dot represents the mean, and the horizontal line represents the median of the data for each group. The upper and lower whiskers represent maximum and minimum amounts in each group, respectively).

3.5 | Variation in the effects of Biosolids exposure on gene expression

To assess the overall effects of EC exposure on gene expression, data obtained from all tissues from each animal were analyzed by generation of a heatmap (Figure 4A). This form of visualization indicated that although C and B animals shared some similarities in terms of the expression of the markers evaluated in the hypothalamus, liver, subcutaneous, visceral, and pericardial adipose tissues, a number of B rams separated into different clusters compared with the C rams. The Pearson's correlation heatmap of the expression of all tested markers, OPLS-DA chart, table of values of the Pearson's correlation coefficient, and the related *p* values are shown in Figure S6B,C and Table S1A,B, respectively.

PCA analysis indicated that there were overlapping population profiles between C- and B-treated rams. Of note, six B rams had a different overall pattern of gene expression and phenotypical features compared with the C group (Figure 4B). This clustering was driven by expression levels of *POMC* in rostral and caudal ARC, blood TG level, and subcutaneous fat *DLK1* mRNA expression (Figure S6A). B animals were, therefore, subdivided into two groups; the six that were phenotypically distinct from the C animals (B-S1, $n = 6$) vs. the rest of the B rams (B-S2, $n = 4$).

Comparative statistical analysis of the data from the B-S1 rams, relative to the C rams, indicated differences in the expression of multiple genes between the two groups (Table 2). Across the different tissues tested, except for *LEPR* that was expressed at a significantly higher level (32.4%) in the caudal ARC, eight of the tested markers—*ESR1* ($p < .05$, 46.5%), *NPY* ($p < .005$, 19.7%), *POMC* ($p = .053$, 34.9%), *DNMT1* ($p < .01$, 10.8%), and *DNMT3a* ($p < .01$, 6.4%) in the rostral ARC, *DNMT3b* ($p = .056$, 28.8%) in the AHA (Figure 5A), and *ZNF423* ($p = .067$, 3.7%) and *LPL* ($p = .087$, 3.4%) in the visceral fat samples (Figure 5B)—were expressed at lower levels in B-S1 compared with C rams. There was a positive correlation between the mRNA expression levels of *NPY* with *DNMT1* ($r = .68$, $p = .002$), *ESR1* with *DNMT1* ($r = .9$, $p = 8.61E\text{-}07$) and *DNMT3a* ($r = .699$, $p = .001$) in the rostral ARC of B-S1 rams. B-S1 rams had lower mean BW relative to C rams ($p = .055$, Cohen's $d = .97$) (Figure 5C). The OPLS-DA chart, Pearson's correlation, gene expression heatmaps, Pearson's correlation coefficient table, and the related *p* values when comparing B-S1 to C rams are shown in Figure S7A–C and Table S2A,B. The B-S1 and C groups did not differ in the TG levels.

Analysis of the data from the B-S2 and C rams (Table 2, Figure 6) demonstrated that the expression levels of *LEPR* (40.9%) in the PVN, *POMC* (16.5%), *ESR1* (45.9%), and *LEPR* (35%) in the caudal ARC ($p < .05$) (Figure 6A), and *DLK1* ($p = .09$; trend, 12.4%) in the subcutaneous fat (Figure 6B) were all significantly higher following maternal biosolids exposure. Moreover, B-S2 rams had significantly ($p < .01$, 26%) higher blood TG levels (22.62 ± 0.67 mg/dL) with a very large effect size (Cohen's $d = 1.2$) compared with C (17.94 ± 1.31 mg/dL) rams (Figure 6C). The *ESR1* expression level was highly correlated with the expression of *DNMT1* ($r = .535$, $p = .039$) in the caudal ARC in B-S2 rams. The gene expression levels and their correlation heatmaps, PCA biplots, OPLS-DA chart, and correlation coefficient tables of the expression of the assessed markers for the B-S2 and C animals are provided in Figure S8A–E and Table S3A,B.

PCA analysis of B-S1 and B-S2 revealed that the two B subgroups were completely separated (Figure 7A). Comparative analysis of B-S2

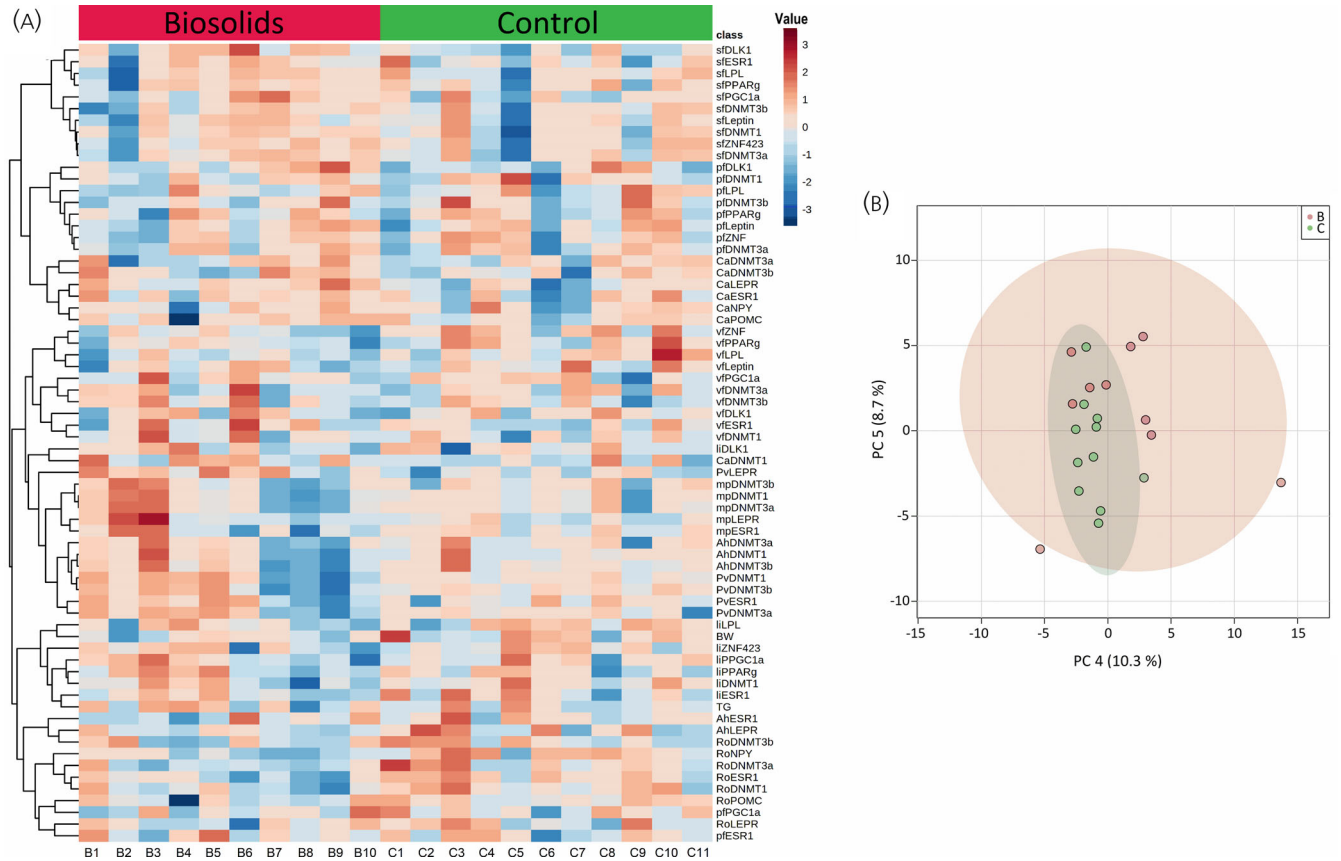

FIGURE 4 (A) The heatmap shows the hierarchical clustering of gene expression in B ($n = 10$) and C ($n = 11$) F1 rams. (B) The PCA analysis revealed that 6 of 10 F1 B rams (B-S1, $n = 6$) were not classified with the rest of B rams (B-S2, $n = 4$) and controls, instead forming a distinct group. These individuals displayed different molecular and phenotypic marker expression profiles, likely resulting from their in utero exposure to biosolids' ECs.

and B-S1 rams indicated significant differences in the patterns of gene expression for the metabolic markers in some tissues (Table 2). Specifically, B-S1 rams had lower expression levels for *NPY*, *POMC* and *DNMT1* ($p = .06$) in the rostral ARC, *ESR1* ($p = .06$) in the caudal ARC, *LEPR* ($p = .06$), *DNMT1* ($p = .07$), *DNMT3a*, and *DNMT3b* in the AHA, and *DNMT3b* in the PVN (Figure 7B). B-S2 rams had significantly higher blood TG level (22.6 ± 0.67 mg/dL, Cohen's $d = 1.38$) compared with B-S1 (15.9 ± 2.48 mg/dL) rams (Figure 7C). Heatmaps of the correlation analysis between expression levels of various markers, PCA biplots, OPLS-DA chart, and correlation coefficient tables of the expression of the assessed markers for the B-S1 and B-S2 rams are provided in the Figure S9A–D and Table S4A,B.

3.6 | Paternal genotype effects on gene expression

With regard to the comparison between B and C rams, sire-family had a significant effect on the expression of *LEPR* in the caudal ARC. *DNMT3a* and *POMC* in the rostral ARC and *LEPR* in the caudal ARC of B-S1 were affected by sire-family compared with C rams. In the comparison between B-S2 and C animals, the only marker affected by sire genotype was *LEPR* in the caudal ARC. Comparative analysis of B-S1 with B-S2 rams showed a significant sire effect on the expression of *NPY* in the rostral ARC. All results are shown in Table 3. No sire effect was observed on the expression of the rest of the evaluated markers, blood TG or BW.

4 | DISCUSSION

4.1 | Summary of findings

The current study evaluated the effects of maternal exposure to a real-life mixture of ECs, on key mediators of metabolic health of adult male offspring. While EC exposure was only received by the mothers, immediately before and during gestation, effects were seen on markers of metabolic function and energy partitioning in the tissues of their adult offspring, with the largest effects in the hypothalamus, a key regulatory center, and in visceral fat. Based on gene expression profiles and phenotypic outcomes (BW and plasma TG) of the offspring, two subgroups (B-S1 and B-S2) were statistically identifiable within the biosolids offspring (Figure 8), indicative of differences in individual susceptibility of systems involved in the regulation of energy partitioning and metabolism to real-life mixed EC exposure.

TABLE 2 Summary of the study findings.

Traits				
Groups	**Body weight**	**Blood plasma triglyceride**	**Upregulated genes and related tissues**	**Downregulated genes and related tissues**
B-S1/C	↓ $p = .055$	≈	LEPR (CaARC), $p = .048$	NPY (RoARC), $p = .003$ DNMT3a (RoARC), $p = .005$ DNMT1 (RoARC), $p = .007$ ESR1 (RoARC), $p = .015$ POMC (RoARC), $p = .053$ DNMT3b (AHA), $p = .056$ ZNF423 (visceral fat), $p = .067$ LPL (visceral fat), $p = .087$
B-S2/C	≈	↑ $p = .007$	LEPR (PVN), $p = .01$ POMC (CaARC), $p = .02$ ESR1 (CaARC), $p = .03$ LEPR (CaARC), $p = .04$ DLK1 (subcut. fat), $p = .09$	-
B-S1/B-S2	≈	↓ $p = .04$	-	NPY (RoARC), $p = .019$ DNMT3b (AHA), $p = .028$ DNMT3a (AHA), $p = .03$ DNMT3b (PVN), $p = .033$ POMC (RoARC), $p = .035$ DNMT1 (RoARC), $p = .059$ ESR1 (CaARC), $p = .064$ LEPR (AHA), $p = .064$ DNMT1 (AHA), $p = .072$

Note: C: control rams ($n = 11$), B-S1 ($n = 6$): subgroup 1 of biosolids exposed rams, B-S2 ($n = 4$): subgroup 2 of biosolids exposed rams. Statistically significant differences between groups were determined by Student's t test.
Abbreviations: CaARC, caudal ARC; RoARC, rostral ARC.

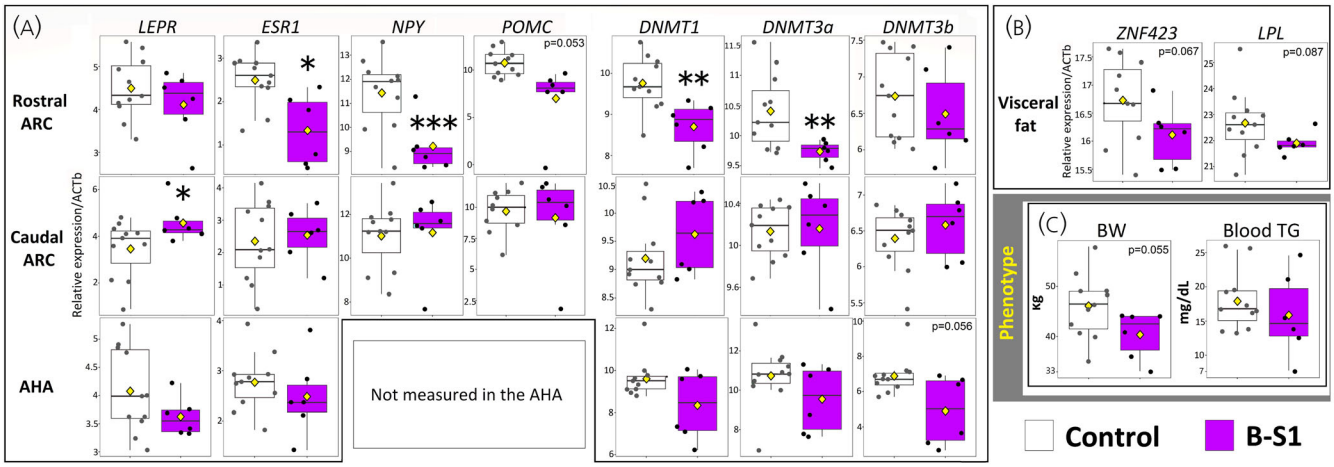

FIGURE 5 (A) In utero exposure to biosolids ECs affected three out of five evaluated hypothalamic regions in B-S1 rams. Relative to C, B-S1 ($n = 6$) rams possessed decreased transcript levels of ESR1, NPY, DNMT1, DNMT3a, and POMC in the rostral ARC, increased expression level of LEPR in the caudal ARC and lower DNMT3b mRNA level in the AHA. (B) B-S1 rams had decreased ZNF423 and LPL expression levels in the visceral fat tissue. (C) B-S1 rams had lower mean body weight (BW) compared to C rams with a very large effect size (Cohen's $d = .97$) (Student's t-test, * $p < .05$, ** $p < .01$ and *** $p < .005$, the yellow dot represents the mean, and the horizontal line represents the median of the data for each group. The upper and lower whiskers represent maximum and minimum amounts in each group, respectively).

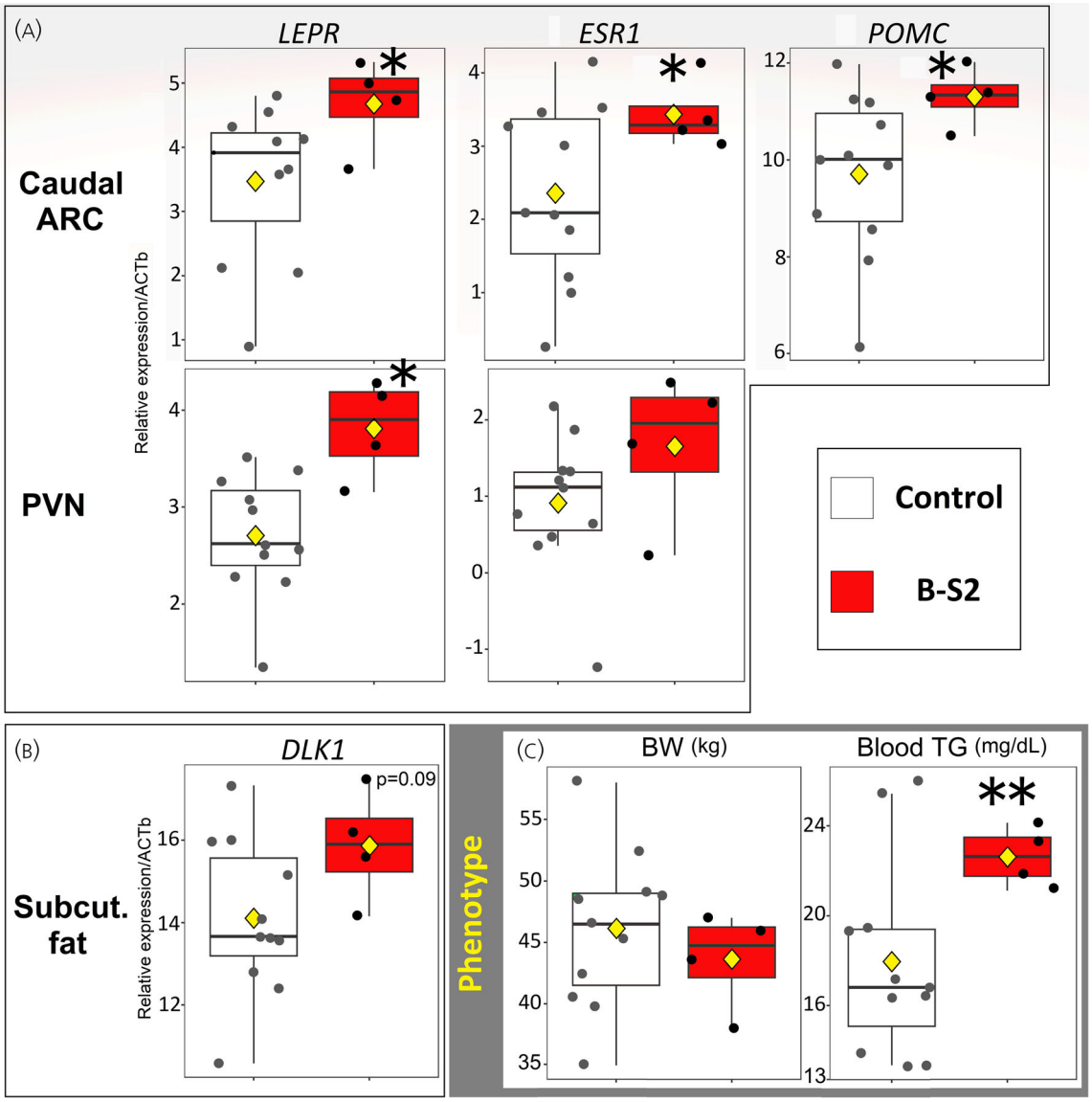

FIGURE 6 Hypothalamic caudal ARC and PVN (A) and subcutaneous (subcut.) fat (B) were the affected tissues in B-S2 compared to C rams. Moreover, B-S2 rams had significantly higher blood plasma triglyceride (TG) level (Cohen's $d = 1.2$) than C rams (C) (Student's *t*-test, * $p < .05$, ** $p < .01$, the yellow dot represents the mean, and the horizontal line represents the median of the data for each group. The upper and lower whiskers represent maximum and minimum amounts in each group, respectively).

These findings are consistent with earlier results, which have also described differential effects of gestational biosolids exposure on testicular development.[57,58]

4.2 | Impact of maternal biosolid exposure on the hypothalamus of offspring

The hypothalamus plays a critical role in the central control of energy homeostasis and food intake, and disruptions in its activity can lead to metabolic disorders.[34,59] This study demonstrated that in utero biosolids-exposed rams had higher *LEPR* expression in specific hypothalamic areas (caudal ARC and PVN), which would normally be associated with higher levels of *POMC* expression, suppressed *AgRP/NPY* activity decreased food intake increased energy expenditure[34] and decreased TG levels.[60,61] However, in one subgroup (B-S2), *POMC* (rostral ARC) expression was higher, *NPY* expression was unaffected, and TG levels were higher, relative to the controls. In contrast, in the other subgroup (B-S1), both *POMC* and *NPY* expressions were decreased (rostral ARC), with no change in TG concentrations. NPY, a neurotransmitter that stimulates appetite, is influenced by factors like food deprivation, feeding, and leptin, a hormone associated with fat storage.[34,62–65] Leptin inhibits *NPY* and food intake.[66–68] The elevated *LEPR* expression (caudal ARC) in the biosolids-exposed rams could lead to hyperleptinemia.[69,70] This could contribute to reduced *NPY* expression,[62] potentially decreasing energy intake/storage and increasing energy expenditure, and explain the lower BW observed in the B-S1 subgroup. Leptin also typically

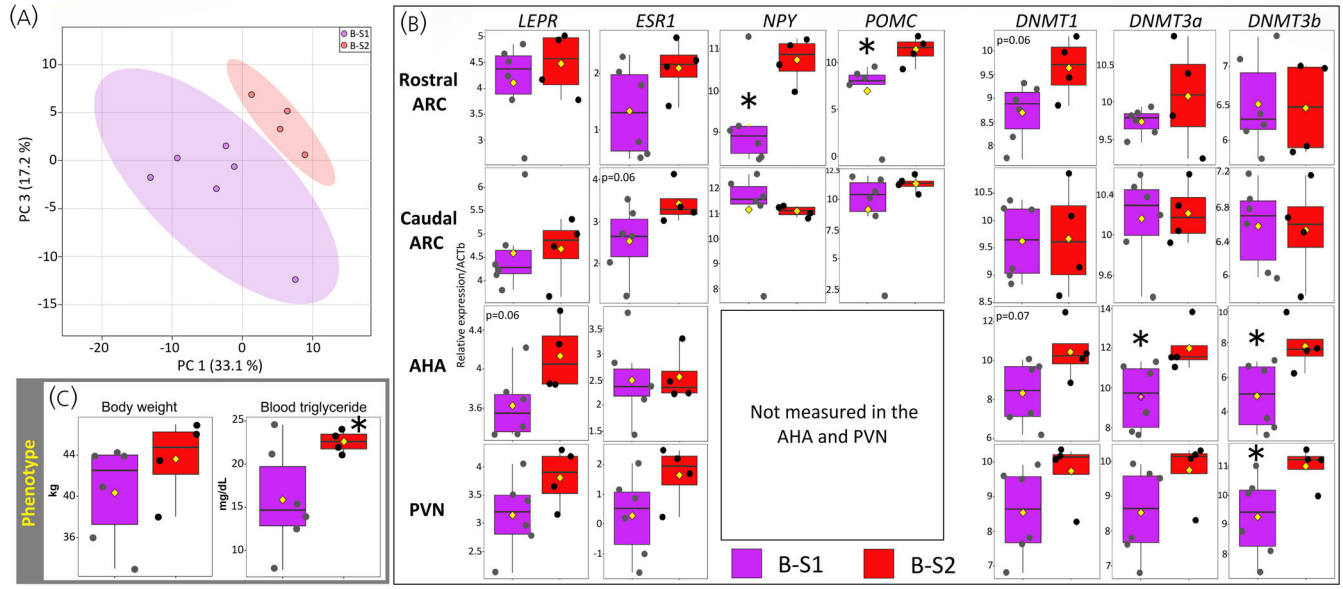

FIGURE 7 (A) B-S1 ($n = 6$) and B-S2 ($n = 4$) were placed in completely separated groups in PCA analysis. (B) Relative to B-S2, B-S1 rams had lower expression levels of NPY, POMC, and DNMT1 in the rostral ARC, LEPR, DNMT1, DNMT3a, and DNMT3b in the AHA, and DNMT3b in the PVN and ESR1 in the caudal ARC. (C) A very large magnitude increase (Cohen's $d = 1.38$) in blood triglyceride (TG) level was observed in B-S2 compared to B-S1 rams (Student's t-test, $^*p < .05$, the yellow dot represents the mean, and the horizontal line represents the median of the data for each group. The upper and lower whiskers represent maximum and minimum amounts in each group, respectively).

TABLE 3 List of the genes whose expression levels were affected by both exposure to biosolids chemical mixture and paternal (sire) genotype.

Factors groups	Genes (tissue)	Factors	Degree of freedom	*F* value	Factors effect (*p* value)
B vs. C	*LEPR* (caudal ARC)	Treatment	1	9.2114	.007
		Sire	3	3.7017	.03
B-S1 vs. C	*DNMT3a* (rostral ARC)	Treatment	1	10.9732	.006
		Sire	3	4.2371	.03
	LEPR (caudal ARC)	Treatment	1	5.7653	.03
		Sire	3	3.5068	.05
	POMC (rostral ARC)	Treatment	1	18.3714	.001
		Sire	3	5.6137	.012
B-S2 vs. C	*LEPR* (caudal ARC)	Treatment	1	5.5052	.04
		Sire	3	3.7972	.05
B-S1 vs. B-S2	*NPY* (rostral ARC)	Treatment	1	19.4644	.0069
		Sire	3	6.0418	.04

Note: B-S1: subgroup 1 of biosolids-exposed rams ($n = 6$). B-S2: subgroup 2 of biosolids-exposed rams ($n = 4$). C: control rams ($n = 11$). Statistically significant differences between different study groups were determined by two-way ANOVA and Tukey-HSD post-hoc test.
Abbreviation: ANOVA, analysis of variance.

stimulates *POMC* expression.[62,63] Higher *LEPR* and POMC expression in certain hypothalamic areas may predispose animals to altered lipid metabolism.[71,72] These findings suggest that exposure to a real-life EC mixture, through biosolids, can affect these neurotransmitter systems, which are important for the regulation of energy balance, in at least two different ways. In this regard, the results support previous studies where divergent effects have been reported following exposure to specific ECs.[73,74]

The observation of interindividual variation in the effects of B exposure on central systems involved in the regulation of food intake and energy balance and on plasma TG profiles is in line with our previous results with this model with regard to testes phenotype[57,75] and gene expression.[58] This variation is likely to reflect genetic differences between individual animals in susceptibility to particular ECs or combinations of ECs, present in biosolids, which may be more likely with this model compared to most laboratory rodent studies, as a result of

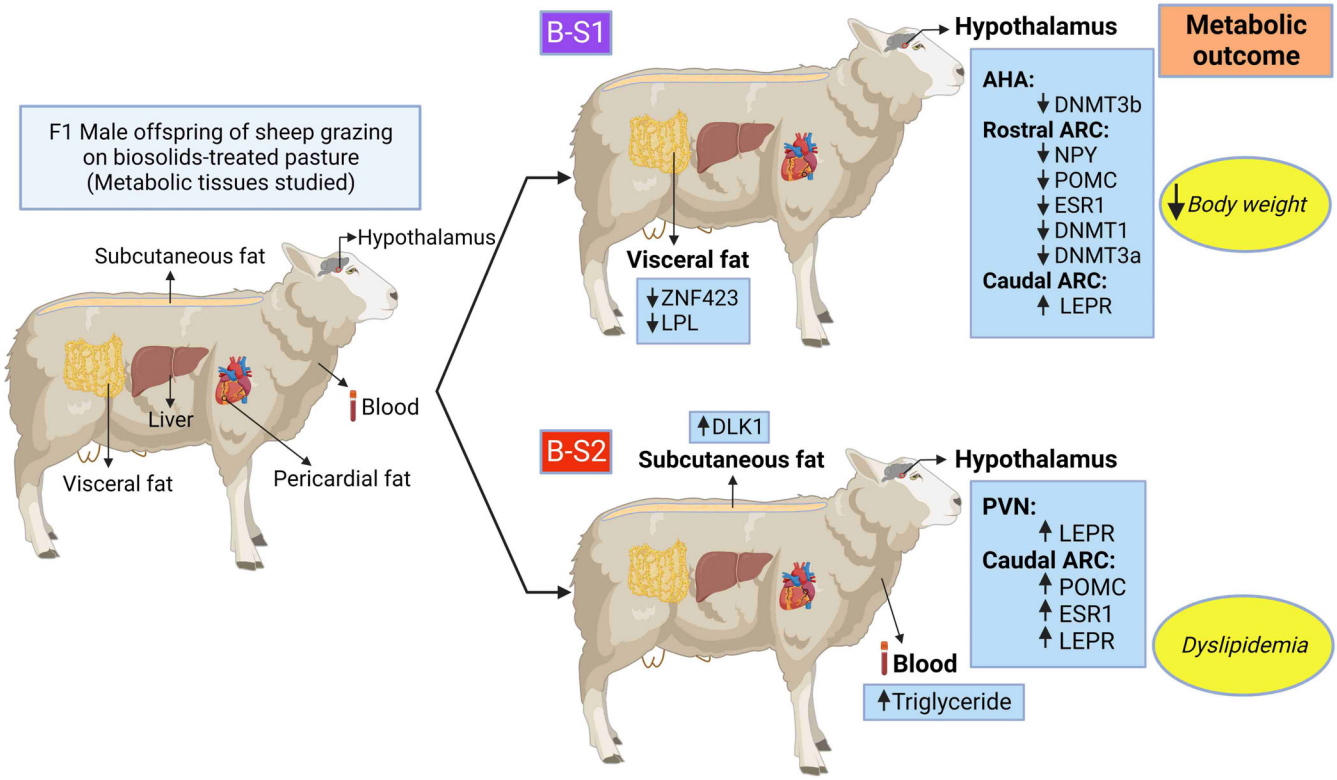

FIGURE 8 In utero exposure to biosolids environmental chemicals (ECs) resulted in two different phenotypes in the male offspring. A group of males (B-S1) with affected gene expression profiles in the hypothalamic regions, AHA, rostral and caudal ARC, and visceral fat and with lower body weight compared to control animals and other groups (B-S2) with altered gene expression in the hypothalamic PVN and caudal ARC and subcutaneous fat along with significantly higher blood triglyceride relative to control rams. The figure was created with BioRender.com.

the outbred nature of the sheep studied. While a potential complication with regard to the interpretation of the results, this variation is of translational relevance as the human population is typically outbred whereas many strains of laboratory animals are inbred to minimize genetic variance and to standardize experimental findings.

Gestational exposure to biosolids affected *ESR1* expression in specific hypothalamic nuclei, which play key roles in the regulation of food intake and metabolism.[36,76] Similar effects have been observed in male rats after gestational exposure to low doses of BPA.[77] This diversity between effects of maternal biosolids exposure across B rams suggests potential genetic influences on the effects of ECs on energy regulation systems.

ECs can have transgenerational impacts on metabolic health mediated in part by alteration in the expression of DNMTs and epigenetic changes in germ cells.[4,15,78–81] In the current study, lower expression of key methyltransferase genes (*DNMT1* and *DNMT3a*) was observed in certain areas of the hypothalamus in biosolids-exposed rams. This is the first study to demonstrate that hypothalamic expression of *DNMT3a* and *DNMT3b* can be affected by developmental exposure to a low-dose EC mixture. These changes in *DNMT3a* and *DNMT3b* expression were correlated with changes in *ESR1* and *NPY* expression. These findings suggest that in utero exposure to a real-life chemical mixture through biosolids may lead to epigenetic alterations that could influence metabolic regulation. This possibility is supported by the observations that fetal exposure to ECs can induce epigenetic alterations and perturb ESR1 expression and energy and lipid metabolism.[77,82–84]

4.3 | Impact of in utero biosolids exposure on adipose tissues and liver

Maternal exposure to ECs can affect adipose tissue which is crucial in obesity and related metabolic conditions such as type 2 diabetes and insulin resistance.[47,85] In this study, exposure to a real-life EC mixture may have activated an anti-lipogenic mechanism in the subcutaneous fat of exposed rams, possibly through increased *DLK1* expression.[86] This could be due to higher signals for adipose tissue formation.[44,87] In addition, lipogenic markers (*ZNF423* and *LPL*) were lower in the visceral fat of a subgroup of the B-exposed rams, suggestive of lower fat formation. This, combined with potentially reduced food intake, might explain this group's lower BW trend. Previous research in mice showed similar effects of EC exposure on BW and visceral fat mass. While in that study these changes were attributed to epigenetic changes in fat-related genes,[46] in the current study, no changes in epigenetic markers were observed. There were also no significant effects of biosolid exposure observed on hepatic metabolic markers in the current study. Overall, the results of the current study demonstrate

that susceptibility to developmental EC exposure via biosolids may vary within a population, and different mechanisms may be affected in subcutaneous versus visceral fat. The exact reasons for these differences should be explored in future studies.

TGs are important in lipid metabolism,[88] and high blood concentrations are associated with metabolic syndrome.[89] Interestingly, in the current study, TG concentrations were higher in one subgroup of EC-exposed rams (B-S2), whereas the overall mean TG concentrations were similar between EC-exposed and control rams. Previous studies on the effects of ECs on TGs have shown mixed results, indicating that different types of ECs and exposure scenarios can lead to varying outcomes.[90–96] However, none of these studies have specifically assessed prenatal EC mixture exposure and repeated measurements of TG concentrations may have provided a clearer understanding of the effects of in utero exposure to ECs on lipid metabolism.

4.4 | Susceptibility differences in responses to exposure

This study reveals two distinct response profiles following developmental exposure to the real-life EC mixture found in biosolids, in terms of hypothalamic and fat gene expression, BW, and blood TG concentrations. One response profile (B-S2) suggests direct effects on energy and lipid metabolism, whereas the other (B-S1) indicates effects mediated via epigenetic changes in DNMTs genes. The molecular pathways affected by EC exposure also differed between the two subsets of rams, which could lead to variations in the health consequences for the animals in the two groups. This variability in the metabolic outcomes observed underscores the importance of genetic background in understanding the diverse impacts of real-life EC exposure.

4.5 | Potential translation

Overall, these findings indicate that exposure to the complex mixture of ECs present in biosolids can affect regulatory systems required for normal energy homeostasis. Identification of two molecular metabolic phenotypes following biosolids exposure indicate that the EC mixture resulted in different effects in different subsets of the exposed male offspring. In addition to remarkable effects at the hypothalamic level, which may affect the metabolic health of EC-exposed animals, altered gene expression profiles were also seen in the subcutaneous and visceral fat which could lead to an increased risk of metabolic disorders.[46,97] Indeed, the observation of elevated TG concentrations in one of the exposed subsets could suggest that those animals are at risk of metabolic disorders.[61] These findings confirm that normal metabolic processes in all of the developmentally biosolids-exposed rams were altered by chronic exposure of their mothers to a translationally relevant cocktail of ECs. More in-depth studies will help to uncover the associated health consequences and nature of diseases, which can arise due to chronic exposure to real-life EC mixtures, as found in biosolids, as well as the mechanisms by which ECs can affect human health.

4.6 | Drawbacks and possible future studies

The ability to model and investigate the effects of real-life EC exposure is complex as the EC mixture profile can differ across time and geographical area. However, as most developed countries show contamination from a wide range of industrial/anthropogenic ECs and biosolids are derived from human and industrial waste, the biosolids model does reflect the human exposome. Given the potential variation in EC content, the chemical composition of the biosolids used and the forage derived from the biosolids-treated pasture were not determined for this study. Consequently, it is not possible to attribute biological effects to a specific chemical or group of chemicals, which is also of limited utility given the multitude of ECs humans are exposed to, in parallel, and the potential for interactions among the ECs present within the mixtures. The current dataset is derived from an ongoing program of research that seeks to determine the transgenerational phenotypic and epigenetic consequences of in utero exposure to a complex real-life mixture of ECs derived from biosolids-treated pastures on metabolic and reproductive health.

AUTHOR CONTRIBUTIONS

Mohammad Ghasemzadeh-Hasankolaei: Data curation; formal analysis; investigation; methodology; writing – original draft; writing – review and editing. **Chris S. Elcombe:** Validation; writing – review and editing. **Samantha Powls:** Investigation. **Richard G. Lea:** Conceptualization; funding acquisition; methodology; writing – review and editing. **Kevin D. Sinclair:** Conceptualization; funding acquisition; methodology; writing – review and editing. **Vasantha Padmanabhan:** Conceptualization; funding acquisition; methodology; project administration; resources; supervision; writing – original draft; writing – review and editing. **Neil P. Evans:** Conceptualization; funding acquisition; investigation; methodology; project administration; resources; supervision; writing – original draft; writing – review and editing. **Michelle Bellingham:** Conceptualization; funding acquisition; investigation; methodology; project administration; resources; supervision; writing – original draft; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Data is available on request.

ETHICS STATEMENT

All animal work was conducted in accordance with the Home Office Animal (Scientific Procedures) Act (A(SP)A), 1986 under license PF10145DF.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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