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A high fat, high sugar diet induces hepatic Peroxisome proliferator-activated receptor gamma coactivator 1-alpha promoter hypermethylation in male Wistar rats

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A R T I C L E I N F O

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Peroxisome proliferator-activated receptor gamma
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A B S T R A C T

Previously we reported that a high fat, high sugar (HFHS) diet induced adiposity, hyperinsulinaemia, hyperleptinaemia, hypertriglyceridaemia and increased liver mass in male Wistar rats. In the present study, the mechanisms underlying the increased liver mass were further elucidated by assessing hepatic lipid accumulation and the expression and methylation status of key metabolic genes using histology, quantitative real-time PCR and pyrosequencing, respectively. The HFHS diet induced hepatic steatosis, increased hepatic triglycerides (1.8-fold, $p < 0.001$), and increased the expression of sterol regulatory element-binding transcription factor 1 (*Srebf1*) (2.0-fold, $p < 0.001$) and peroxisome proliferator-activated receptor gamma (*Pparg*) (1.7-fold, $p = 0.017$) in the liver. The expression of peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*Pgc1a*) was decreased (2.6-fold, $p < 0.010$), which was accompanied by hypermethylation ($p = 0.018$) of a conserved CpG site in the promoter of *Pgc1a* in HFHS fed rats compared to controls. *In silico* analysis identified putative binding sites for CCAAT/enhancer-binding protein beta (C/EBPß) and hepatocyte nuclear factor 1 (HNF1) within proximity to the hypermethylated CpG. As *Pgc1a* is a co-activator of several transcription factors regulating multiple metabolic pathways, hypermethylation of this conserved CpG site in the promoter of *Pgc1a* may be one possible mechanism contributing to the development of hepatic steatosis in response to a HFHS diet. However, further work is required to confirm the role of *Pgc1a* in steatosis.

1. Introduction

The consumption of high fat, high sugar (HFHS) diets contribute to the development of hepatic steatosis, an established risk factor for type 2 diabetes and cardiovascular disease [1,2]. Hypertriglyceridemia leads to hepatic lipid accumulation [3,4], which disrupts lipid homeostasis by decreasing fatty acid oxidation and/or increasing lipid uptake or production [5]. The liver plays a critical role in maintaining metabolic homeostasis, with the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α), playing a key role in regulating lipid and metabolic homeostasis [6,7]. Studies in rodents have shown that overexpression of PGC-1α induced fatty acid oxidation and decreased hepatic triglycerides [8], while lower expression of PGC-1α was associated with steatosis [9]. Other transcription factors such as sterol regulatory element-binding transcription factor 1 (SREBF1) and peroxisome proliferator-activated receptor gamma (PPARG), have also been shown to play an important role in hepatic lipid homeostasis as they are major regulators of *de novo* lipogenesis and lipid uptake and storage [10,11]. In murine overexpression models, SREBF1 upregulates lipid metabolic enzymes such as stearoyl-CoA desaturase (SCD), fatty acid synthase (FAS) and others to stimulate *de novo* lipogenesis [12] while murine knockout models of

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PPARG found reduced hepatic steatosis and reduced expression of SCD, FAS, as well the lipid transporter cluster differentiating 36 (CD36) [13], indicating that there may be overlap between their hepatic functions. However, the mechanisms linking HFHS feeding with hepatic steatosis remain to be fully elucidated.

Epigenetic modifications may contribute to the development of hepatic steatosis, by mediating the effect of environmental exposures such as HFHS feeding on gene expression. DNA methylation is the most widely studied and best characterised epigenetic mechanism and occurs when a methyl group is added to cytosine residues. Although it has been established that DNA methylation may affect gene expression through multiple mechanisms [14], promoter methylation is usually associated with transcriptional repression due to chromatin remodelling that inhibits transcription factor binding [15,16]. Dysregulated DNA methylation patterns have been reported in liver biopsies from patients with fatty liver diseases [17,18]. Furthermore, studies have reported that altered methylation of *PGC1A* and *PPARG* in humans are associated with the development and progression of non-alcoholic fatty liver disease (NAFLD) [17,19].

Previously, we reported that HFHS feeding induced adiposity, hyperinsulinaemia, hyperleptinaemia, hypertriglyceridaemia and increased liver weight in male Wistar rats [20]. The present study aimed to investigate whether the HFHS diet induced lipid accumulation and altered the expression and methylation status of key metabolic genes in the liver. We show that the HFHS diet induced hepatic steatosis and decreased the expression of *Pgc1a,* which was accompanied by hypermethylation of an evolutionarily conserved CpG site within its promoter. Our study, for this first time, reports hypermethylation of this conserved CpG site in the promoter of *Pgc1a* and propose that methylation of this CpG may be a possible mechanism underlying the development of hepatic steatosis in response to a HFHS diet.

2. Materials and methods

2.1. Animals

Details of the animal study have been described previously [20]. Briefly, three-week-old male Wistar rats were randomly allocated into groups and fed either a HFHS diet consisting of a patty made of 40% fat, 15% protein and 44% carbohydrate by energy (Table S1) and a jelly cube supplemented with 7.5% sucrose and 7.5% fructose (n = 10), or a standard rodent (STD) diet consisting of 11% fat, 15% protein and 74% carbohydrate by energy and a jelly cube (n = 10) for three months (Rodent diet, Epol, Atlas Animal Feed, South Africa). Food and water were given *ad libitum.* Liver tissue was harvested for histological, gene expression and DNA methylation analysis. Ethical approval for this study was granted by the Ethics Committee for Research on Animals (ECRA) of the South African Medical Research Council (SAMRC, Tygerberg, South Africa) (ECRA no. 11/03/B) and the Ethics Committee for Animal Research at Stellenbosch University (ACU-2020-14614). All animal experiments were conducted in accordance with the ARRIVE guidelines for research using animals.

2.2. Histological analysis

Formalin-fixed liver tissue was cut into 4 μM sections using a rotary microtome (Leica RM 2125, Leica Microsystems, Nussloch, Germany), mounted on Histobond® microscope glass slides (Marienfeld, Lauda-Königshofen, Germany), allowed to dry and placed in an oven at 60 °C for an hour. The slides were stained with haematoxylin and eosin (H&E) as described by Fischer et al. Digital images were captured using a Zeiss Axioscope 5 microscope (Carl Zeiss, Hamburg, Germany) at 100 × and 400 × magnification with four fields per rat. For the detection of lipids, snap frozen liver tissue was cut into 8 μM sections using a cryostat microtome (Leica CM1860, Leica Microsystems, Nussloch, Germany), mounted on X-tra Adhesive® glass slides (Leica Microsystems, Nussloch, Germany) and stained with haematoxylin and Oil Red O [21]. Digital images were captured using a Zeiss Axioscope 5 microscope (Carl Zeiss, Hamburg, Germany) at 100 × and 400 × magnification for six rats per group and ten fields per rat. Oil Red O quantification was performed with the QuPath software package (version 0.3.2, QuPath. Edinburgh, UK) [22] using positive pixel detection. Analysis was limited to sections which returned positive pixel count.

2.3. Hepatic triglyceride quantification

Hepatic triglycerides were quantified using the BioVision Triglyceride Quantification Colorimetric/Fluorometric Kit (BioVision, Milpitas, CA, USA) according to the manufacturer's instructions. Briefly, 100 mg of snap frozen liver tissue was homogenised in 5% Triton X-100 (v/v) using the TissueLyser (Qiagen, Hilden, Germany) 5x for 1 min at 25 Hz. The lysate was heated to 95 °C, cooled to room temperature and reheated to 95 °C to solubilise triglycerides. Samples were centrifuged for 2 min at 13 500 rpm and triglycerides were quantified in the supernatant. The fluorescence was measured at Ex/Em 535/587 nm using the SpectraMax i3 plate reader (Molecular Devices, San Jose, CA, USA).

2.4. RNA isolation

Total ribonucleic acid (RNA) was isolated from approximately 30 mg of liver tissue using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA quantity and purity were measured using the Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA quality was measured using the 2100 Bioanalyzer and the RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA, United States) according to the manufacturer's instructions. RNA was DNAse-treated with the Turbo DNase kit (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions.

2.5. Rat Fatty Liver PCR array

The Rat Fatty Liver RT^2 Profiler PCR Array (Qiagen, Hilden, Germany) was used to quantify the expression of 84 genes involved in lipid, glucose, and mitochondrial regulation in the livers of rats fed a HFHS or STD diet (n = 4). Complementary DNA (cDNA) was synthesised from 1 μg of DNAse-treated RNA using the RT^2 First-strand cDNA synthesis kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Thereafter, cDNA was mixed with RT^2 qPCR ROX master mix containing SYBR™ Green (Qiagen, Hilden, Germany) and dispensed into wells of the RT^2 Profiler PCR Array plate according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was conducted on the Quantstudio 7™ Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following conditions: denaturation for 15 min at 95 °C, combined annealing and extension for 40 cycles at 60 °C for 1 min. Default baseline and cycle threshold (Ct) settings were used and the PCR array values were exported to an Excel file to create a table of Ct values, which was uploaded onto the Qiagen GeneGlobe Design and Analysis Hub web portal (https://dataanalysis.qiagen.com/pcr/arrayanalysis.php). A cut-off Ct value of 35 cycles was applied and samples with multiple melt curves were removed. Gene expression data were normalised to beta-2 microglobulin (*B2m*) and ribosomal protein lateral stalk 1 (*Rplp1*), which were identified as the best endogenous controls using Normfinder software [23], and were quantified using the ΔΔCt method [24]. PCR arrays were assessed for reproducibility, reverse transcription efficiency and genomic DNA contamination. Genes were considered to be differentially expressed if they showed at least a 1.5-fold change in expression with a p-value of less than or equal to 0.05.

2.6. TaqMan® Gene Expression Assays

Genes for TaqMan® gene expression assay validation were selected for qRT-PCR analysis (TaqMan® Gene Expression Assays, Applied Biosystems, Foster City, CA, USA) using data from the PCR arrays. A total of 1 μg RNA was reverse transcribed using the High-Capacity Reverse Transcription kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. TaqMan® Gene Expression Assays and Universal PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the transcriptional status of genes listed in Table S2. PCR was conducted on the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following conditions: denaturation for 15 min at 95 °C and, combined annealing and extension for 40 cycles at 60 °C for 1 min. Default baseline and cycle threshold (Ct) settings were used and expression levels were calculated using the standard curve method and normalised to *B2m* and *Rplp1* expression, which were identified as the best endogenous controls by Normfinder software [23].

2.7. DNA isolation

Genomic DNA was isolated from approximately 30 mg of liver tissue using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA purity and quantity were assessed using the Nanodrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and Qubit fluorometer (Life Technologies, Carlsbad, CA, USA), respectively. DNA quality was determined by electrophoresis using a 1% (w/v) agarose gel.

2.8. Pyrosequencing

The PyroMark® Q96 ID Pyrosequencing System (Qiagen, Hilden, Germany) was used to determine the methylation status of a conserved CpG site in the promoter region of *Pgc1a*. Forward, reverse, and sequencing primers for the *Pgc1a* promoter were designed using the PyroMark Assay Design 2.0 software and are shown in Table S3. DNA samples (500 ng) were bisulfite converted using the EpiTect DNA kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. A thermal cycler was used to convert the DNA with two cycles of denaturation at 95 °C for 5 min and incubation at 60 °C for 10 min, followed by column-based DNA clean-up. PCR of 25 ng of bisulfite converted DNA was carried out using the PyroMark PCR Kit (Qiagen, Hilden, Germany) under the following PCR conditions: 95 °C for 15 min; 45 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s, and final elongation of 72 °C for 5 min. PCR reactions were carried out in a Veriti 96-well Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) and the quality and size of amplicons were assessed by electrophoresis using a 1.5% (w/v) agarose gel. Pyrosequencing was carried out using the PyroMark Gold Q96 Reagents (Qiagen, Hilden, Germany) on a PyroMark ID pyrosequencer (Qiagen, Hilden, Germany), and the methylation level was calculated using the PyroMark Q96 software (Version 1.0.10; Qiagen). All pyrosequencing assays were validated using different ratios of methylated: unmethylated bisulfite converted DNA (0, 10, 25, 50, 75, 90 and 100%) (Merck, Kenilworth, NJ, USA), from which standard curves were constructed to determine primer sensitivity. Each pyrosequencing run contained negative controls and bisulfite conversion controls were incorporated within each assay sequence to assess conversion efficiency.

2.9. In silico prediction of transcription factor binding sites

In silico analysis was performed to identify potential transcription factors that bind the region spanning the −219 CpG site within the promoter of *Pgc1a*. Since the −219 CpG site is conserved between rats and humans, the human CpG (located at −260) was also investigated to explore conserved transcription factor binding. PROMO [25] and Alibaba2.1 [26] transcription factor prediction software were used to identify putative transcription factors within the region spanning −290 to −230 bp from the transcription start site of the human *PGC1A* gene and −249 to −189 bp from the transcription start site of the rat *Pgc1a* gene.

2.10. Statistical analysis

Data presented in this study were captured in Microsoft Excel (Microsoft Office 2016) and statistical analysis conducted in GraphPad Prism software (GraphPad Prism® version 6.01, GraphPad Software, San Diego, CA, USA). Data are represented as the mean ± standard deviation (SD). Normally distributed data, as determined by the Shapiro-Wilk test, were analysed using the unpaired Student's t-test, while skewed data were analysed using the Mann-Whitney test. A p-value ≤0.05 was considered statistically significant.

3. Results

3.1. Animal characteristics

The characteristics of the animals used in this study have been reported previously (Table 1) [20]. After three months, HFHS diet-fed rats were heavier ($p < 0.001$), had higher HOMA-IR values ($p = 0.040$), greater retroperitoneal fat pad mass ($p < 0.010$) and liver mass ($p < 0.010$) compared to STD diet-fed rats. While no difference in blood glucose concentrations were observed between the groups, the HFHS diet-fed rats exhibited increased insulin ($p = 0.042$), triglyceride ($p < 0.010$) and leptin ($p < 0.001$) concentrations compared to STD diet-fed rats. No difference in total calorie consumption was observed between the groups.

3.2. HFHS diet induces hepatic steatosis

Morphological differences in the livers of HFHS- and STD-diet fed rats were visualised by H&E staining. Results showed that three months of HFHS feeding induced intracellular lipid accumulation of a mixed micro- and mediovesicular pattern, characterised by predominantly small and medium sized lipid droplets, accentuated in the areas around the centrilobular and intermediary zones in HFHS-fed rats (Fig. 1A–D). Measurement of hepatic triglycerides showed a 1.8-fold ($p < 0.001$) increase in HFHS- compared to STD-diet fed rats (Fig. 1E). Oil Red O staining and quantification of liver lipid droplets confirmed the presence of more lipid droplets in HFHS- compared to STD-diet fed rats (Fig. S1).

3.3. Altered hepatic gene expression in HFHS-fed rats

Next, we explored gene expression differences in the livers of HFHS-

Table 1
Metabolic characteristics of HFHS and STD diet-fed rats.

Variable	STD (n = 10)	HFHS (n = 10)	P-value
Initial bodyweight (g)	370.20 ± 27.76	364.40 ± 13.54	p = 0.560
Final bodyweight (g)	474.00 ± 33.30	567.50 ± 27.86	**p<0.001**
HOMA-IR	1.00 ± 0.53	1.65 ± 0.76	**p = 0.040**
Retroperitoneal fat mass (g)	3.85 ± 0.90	12.50 ± 4.66	**p<0.010**
Liver mass (g)	14.23 ± 0.79	16.51 ± 2.13	**p<0.010**
Fasting insulin (ng/ml)	3.78 ± 1.97	6.14 ± 2.79	**p = 0.042**
Fasting glucose (mmol/L)	5.94 ± 0.43	5.98 ± 0.51	p = 0.851
Triglycerides (mM)	0.50 ± 0.10	1.20 ± 0.10	**p<0.010**
Leptin (ng/ml)	4.91 ± 2.36	24.81 ± 6.47	**p<0.001**
Total calorie intake (kcal/rat)	7224.5 ± 713.1	6692.2 ± 377.7	p = 0.052

Abbreviations: HFHS – High fat, high sugar diet, HOMA-IR – Homeostatic model assessment for insulin resistance, STD – Standard rodent diet.

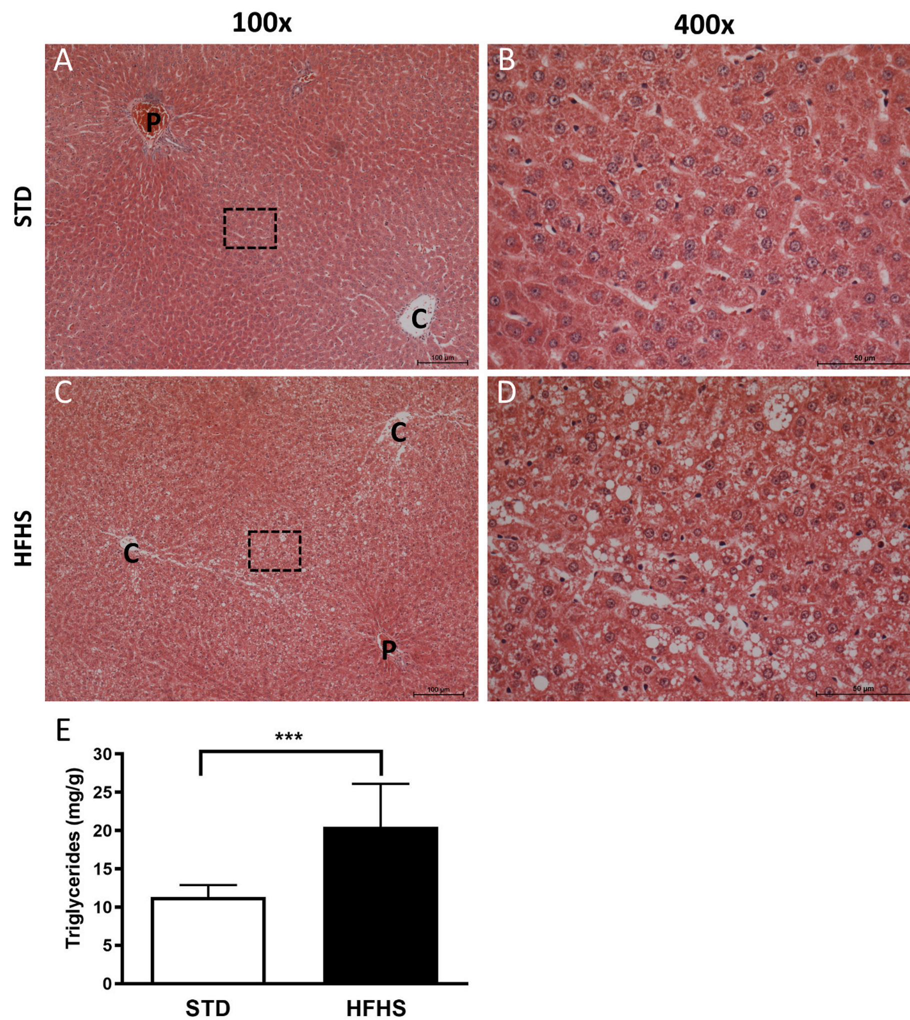

Fig. 1. Hepatic histology showing altered hepatocyte morphology in HFHS diet-fed rat livers. Liver sections from rats fed a STD **(A, B)** or HFHS diet **(C, D)** for three months stained with H&E (x100 magnification with box insert at x400 magnification). C - central vein, P - portal tract. E) Bar graph showing hepatic triglyceride concentrations within liver tissue. Data are presented as the mean ± SD (n = 10), ***p < 0.001.

and STD-diet fed rats using commercial PCR arrays. Results showed that the expression of eight genes were increased, and 11 genes were decreased in response to the HFHS diet (Fig. 2A, Table 2). The expression of *Pgc1a* showed the largest fold-difference between HFHS and STD-fed rats (5.2-fold, p = 0.021). Based on this difference and the key role of PGC-1α in the development of hepatic steatosis, subsequent analysis was focussed on this gene. Gene expression analysis by qPCR using fluorescent probes confirmed the downregulation of *Pgc1a* expression in the livers of HFHS- compared to STD diet-fed rats (2.6-fold, p < 0.010) (Fig. 2B). The expression of uncoupling protein 2 (*Ucp2*), a downstream target of downstream of *Pgc1a* [27], was similarly decreased in response to HFHS diet (1.7-fold, p < 0.001), while the expression of *Srebf1* (2.0-fold, p < 0.001) and *Pparg* (1.7-fold, p = 0.017), whose expression positively correlates with hepatic steatosis [11,28], were increased in HFHS diet-fed rats compared to STD diet-fed rats.

3.4. HFHS diet induced hypermethylation of the Pgc1a promoter

To gain insight into the mechanism underlying the altered expression of *Pgc1a,* we quantified DNA methylation using pyrosequencing. Our analysis focused on a CpG site, situated 219 base pairs upstream of the transcription start site of *Pgc1a,* that is conserved between humans and rats. Results showed hypermethylation of this CpG in the livers of HFHS-compared to STD diet-fed rats (52.0 ± 3.6% vs 41.8 ± 12.3%, p = 0.018) (Fig. 3A and B).

3.5. Transcription factor binding sites

To explore the functional significance of differential *Pgc1a* methylation, *in silico* analysis was conducted to identify transcription factors that bind to the region spanning CpG −219 in rats and humans. Putative binding sites for CCAAT/enhancer-binding protein beta (C/EBPß) and Hepatocyte nuclear factor 1 (HNF1) in proximity to CpG-219 were identified in both rats and humans (Fig. 4A and B). In addition, species-specific transcription factor binding sites were also observed. Activator protein 1 (AP-1), CCAAT/enhancer-binding protein alpha (C/EBPα) and Upstream stimulatory factor 2 (USF2) were specific to rats (Fig. 4A), while sites Forkheadbox P3 (FOXP3), Retinoic acid receptor beta/Retinoid X receptor alpha (RARß/RXRα), Yin Yang 1 (YY1), Glucocorticoid receptor (GR), Nuclear factor kappa-B (NF-κB) were specific to humans (Fig. 4B).

4. Discussion

The current study investigated hepatic gene expression and DNA methylation patterns during the development of diet-induced obesity in Wistar rats, to elucidate potential mechanisms through which a HFHS diet mediates lipid accumulation in the liver. We show that consumption of a HFHS diet increased hepatic lipid accumulation, which was accompanied by decreased expression of *Pgc1a* and the hypermethylation of an evolutionarily conserved CpG site within its promoter.

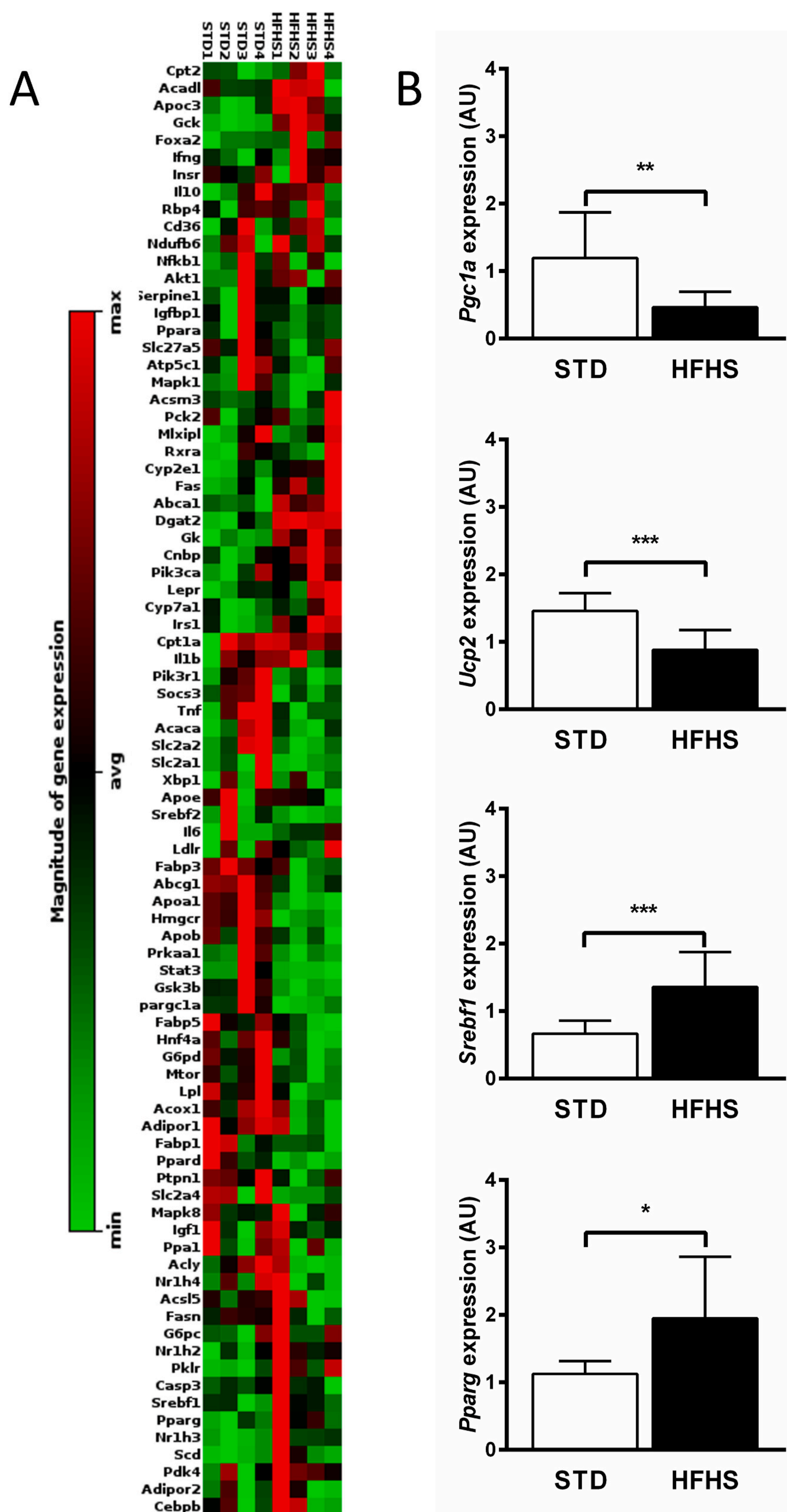

Fig. 2. Altered hepatic gene expression of HFHS diet-fed rats. A) Heatmap of up- and down-regulated genes, and those with unchanged expression as determined by the Rat Fatty Liver RT^2 Profiler PCR Array (n = 4). **B)** TaqMan® gene expression validation of *Pgc1a*, *Ucp2*, *Srebf1* and *Pparg*. Data are presented as mean ± SD (n = 10). B) Assays. *p < 0.050, **p < 0.010, ***p < 0.001.

Table 2
Differentially expressed hepatic genes as assessed by Rat Fatty Liver PCR array.

Gene symbol	Gene Name	Fold Regulation	P-value
Pgc1a	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha	−5.24	0.021
Ppard	Peroxisome proliferator-activated receptor delta	−3.06	0.018
Apoa1	Apolipoprotein A1	−2.93	<0.001
Gck	Glucokinase	2.76	0.002
Socs3	Suppressor of cytokine signalling 3	−2.29	0.031
Fabp5	Fatty acid binding protein 5	−2.11	0.032
Fas	Fas cell surface death receptor	2.08	0.022
Hmgcr	3-Hydroxy-3-methylglutaryl-CoA reductase	−2.05	<0.001
Abcg1	ATP binding cassette subfamily G member 1	−1.95	0.003
Pklr	Pyruvate kinase L/R	1.89	0.014
Fabp3	Fatty acid binding protein 3	−1.83	0.035
Lpl	Lipoprotein lipase	−1.83	0.02
Gsk3b	Glycogen synthase kinase 3 beta	−1.78	0.011
Cyp2e1	Cytochrome P450 family 2 subfamily E member 1	1.77	0.02
G6pd	Glucose-6-phosphate dehydrogenase	−1.67	0.008
Dgat2	Diacylglycerol O-acyltransferase 2	1.59	<0.001
Nr1h3 (Lxra)	Nuclear receptor subfamily 1 group H Member 3	1.58	0.047
Abca1	ATP binding cassette subfamily A member 1	1.57	0.001
Gk	Glycerol kinase	1.5	<0.001

The expression of *Srebf1* and *Pparg*, regulators of hepatic lipogenesis, were increased in HFHS- compared to STD diet-fed rats while the expression of *Ucp2*, a downstream target of *Pgc1a* whose expression is positively correlated with lipid oxidation, was decreased.

It has been widely reported that the chronic overconsumption of HFHS diets alters lipid homeostasis leading to hepatic steatosis and metabolic dysregulation [29–31]. In a liver-specific hemizygotic murine model, Estall et al. (2009) showed that hemizygotic disruption of *Pgc1a* expression led to hepatic steatosis, disrupted insulin signalling and hypertriglyceridaemia [31]. However, in contrast to our results, both wildtype and hemizygotic mice showed elevated levels of *Pgc1a* expression in response to high fat diet feeding. PGC-1α is a transcriptional coactivator that plays a critical role in the regulation of glucose, lipid and energy metabolism [32,33]. We also observed increased expression of the *de novo* lipogenic transcription factors *Srebf1* and *Pparg*. SREBF1 is upregulated in the liver in response to high-fat feeding and hepatic steatosis [34], and is a key regulator for induction of lipogenic enzymes genes [10]. PPARG is a master regulator of adipocyte differentiation and promotes the uptake of triglycerides from serum to be stored in adipose tissue to reduce lipotoxicity [35]. Its upregulation in liver tissue is proposed to be a possible adaptive mechanism to increase the uptake of serum free fatty acids, so as to protect other tissues from the effects of lipid accumulation and insulin resistance [13]. The expression of *Ucp2* was also decreased in our study, which may be attributed to decreased expression of *Pgc1a*, as *Ucp2* has been proposed to be a direct target of PGC-1α transcriptional regulation [27]. Murine knockout models of *Ucp2* found that knockout mice exhibited a reduction in hepatic beta oxidation and fatty acid substrate usage [36]. Our finding that decreased expression of *Pgc1a* is associated with hepatic steatosis and the increased expression of *Srebf1* and *Pparg*, suggests that hepatic steatosis may develop due to the inability of PGC-1α to inhibit lipogenesis and activate fatty acid oxidation, thus leading to excessive lipid accumulation. However, further investigation is required to explore this hypothesis.

Epigenetic modifications are a mechanism through which environmental exposures may affect gene expression, thus impacting susceptibility to metabolic diseases [37]. There is increasing evidence in both humans and rodents that consuming HFHS diets causes DNA methylation changes associated with obesity and metabolic dysfunction [38, 39]. Using pyrosequencing, we show hepatic hypermethylation at an evolutionarily conserved CpG site located 219 bp upstream of the transcription start site of *Pgc1a* in response to HFHS feeding. Barrès et al. (2009) similarly reported hypermethylation of this conserved CpG site

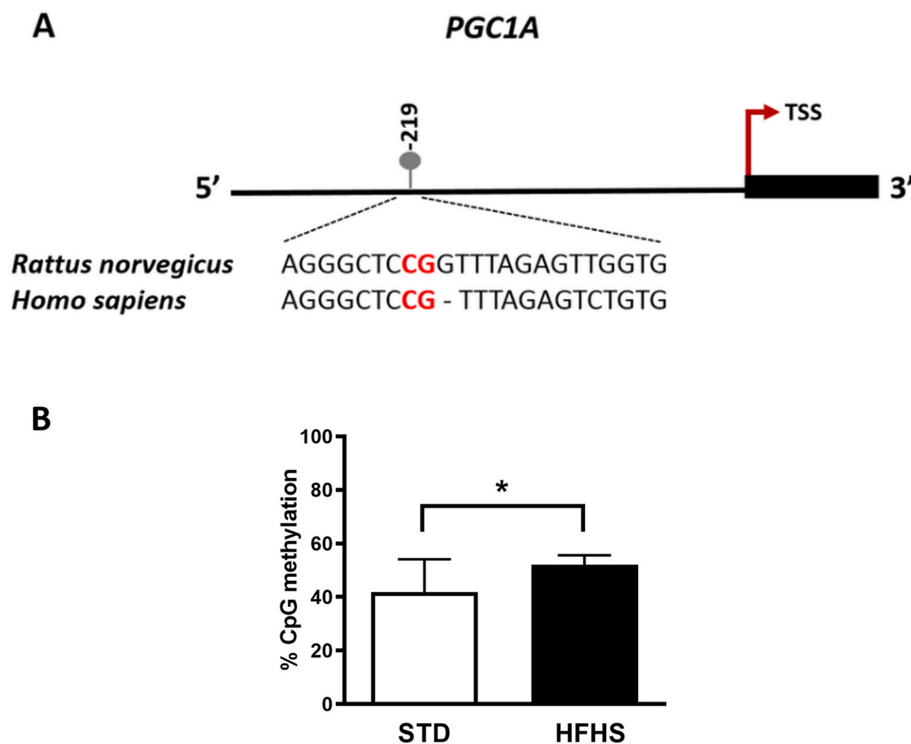

Fig. 3. Schematic diagram and methylation status of conserved *Pgc1a* CpG site. A) Location of the conserved CpG site in the *Pgc1a* promoter. **B)** Hepatic methylation of CpG −219 methylation of *Pgc1a*. Data are presented as the mean ± SD (n = 10), *p < 0.050.

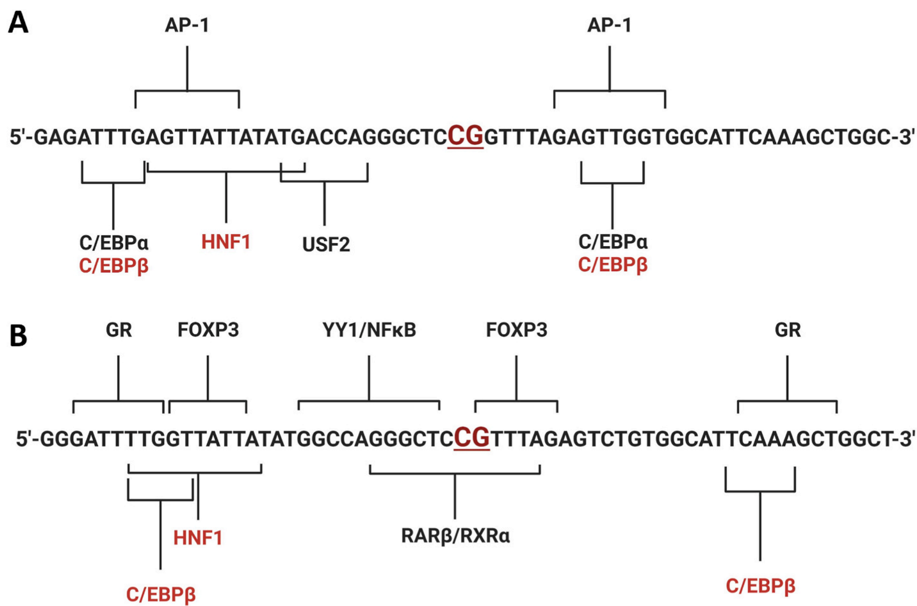

Fig. 4. Schematic illustration showing predicted transcription factors to the *PGC1A* region investigated by pyrosequencing. Diagrams showing predicted transcription factor binding sites in **A)** rats and **B)** humans. Abbreviations: C/EBPα – CCAAT/enhancer-binding protein alpha, C/EBPß – CCAAT/enhancer-binding protein beta, HNF1 – Hepatocyte nuclear factor 1, AP-1 – Activator protein 1, USF2 – Upstream stimulatory factor 2, FOXP3 – Forkheadbox P3, RARß/RXRα – Retinoic acid receptor beta/Retinoid X receptor alpha, YY1 – Yin Yang 1, GR – Glucocorticoid receptor, NF-κB – Nuclear factor kappa-B.

(−260 bp upstream of the transcription start site in humans) in skeletal muscle biopsies from male patients with type 2 diabetes (T2D) [40]. In addition, the authors showed that the fatty acids palmitate and oleate, which are major lipid components of high fat diets, induced hypermethylation of the *PGC1A* promoter in primary skeletal muscle cells obtained from biopsies of normal, glucose tolerant individuals. Others have similarly reported hypermethylation of the *PGC1A* promoter in liver biopsies from patients with NAFLD and pancreatic islets from patients with T2D, which was inversely associated with *PGC1A* expression [17,41]. However, these studies explored a different promoter region to the conserved CpG site investigated in the current study. The prior studies [17,41] were performed in patients with T2D and NAFLD, while our study for the first time shows hypermethylation of *Pgc1a* during hepatic steatosis that was associated with hyperinsulinaemia but not hyperglycaemia. Although the findings of this study are purely correlative, it is possible that this change may represent an early event, preceding the development of T2D and NAFLD. Longitudinal studies to investigate *Pgc1a* promoter methylation during the development of steatosis and T2D are required to confirm this.

In silico analysis of the *Pgc1a* promoter sequence revealed putative transcription factor binding sites for HNF1 and C/EBPß in both rat and human sequences. These transcription factors have been proposed to play a role in endoplasmic reticulum (ER) stress, inflammation and hepatic triglyceride concentrations [41,42]. While knockout mouse models showed that both HNF1 and C/EBPß are associated with hepatic steatosis [43,44], we cannot rule out the effects of other *trans*-acting proteins and therefore chromatin immunoprecipitation assays investigating the region containing the conserved CpG site should be conducted. As *Pgc1a* is a potent coactivator of transcription, investigating the effects of its dysregulation on its targets in metabolically significant pathways will form part of future work.

The study has a few limitations to consider. Due to its cross-sectional nature, we are not able to determine whether the altered DNA methylation and/or gene expression patterns observed in this study play a causal role in metabolic dysregulation. Future longitudinal studies are necessary to elucidate the functional consequences of these changes. We did not assess changes in protein levels of the genes that were altered in response to HFHS diet-feeding in this study. Another limitation was the lack of assessing high-density (HDL) and low-density (LDL) lipoprotein concentrations, which is another mechanism by which hepatic lipid homeostasis is maintained. Sexual dimorphism in DNA methylation and cardiometabolic health risk have been reported and as a result, the data obtained in this study may not reflect the pathophysiology in both sexes [45,46]. *Pgc1a,* in particular, has been shown to have sex-specific effects on hepatic lipid homeostasis in response to diets high in fat in rodent models [47,48]. Therefore, future studies should investigate the effect of HFHS diet-feeding on *Pgc1a* methylation in both sexes. In conclusion, our study showed that a HFHS diet led to hepatic steatosis and changes in the transcriptional co-activator *Pgc1a* gene expression and methylation. We propose that the HFHS diet increases visceral adiposity, induces hyperinsulinaemia and hypertriglyceridaemia. These conditions may lead to hypermethylation of the conserved CpG site within the *Pgc1a* promoter, and we hypothesise that this hypermethylation decreases its gene expression by altering the binding of *trans*-acting proteins to this region. This occurs in conjunction with increased activation of pro-lipogenic pathways through SREBF1 and PPARG which disrupts lipid homeostasis and stimulates lipid and fatty acid uptake into the liver, leading to steatosis (Fig. 5). Although further mechanistic experiments are required to confirm our hypothesis, our novel findings link HFHS feeding, hepatic steatosis and altered DNA methylation and gene expression of *Pgc1a*.

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Author contributions

CP conceptualized the study and methodology; YA, TW, OP, CM and CP conducted the laboratory work and data analysis. YA and CP contributed to manuscript writing. EB, SW, RJ, TW, CM and OP reviewed and edited the manuscript. All authors read and approved the final manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

Data availability statement

The datasets generated for this study are available from www.kaggle.com/dataset/e76461efceddbae54b4f6b5b32441cd5eecd212558a3d16

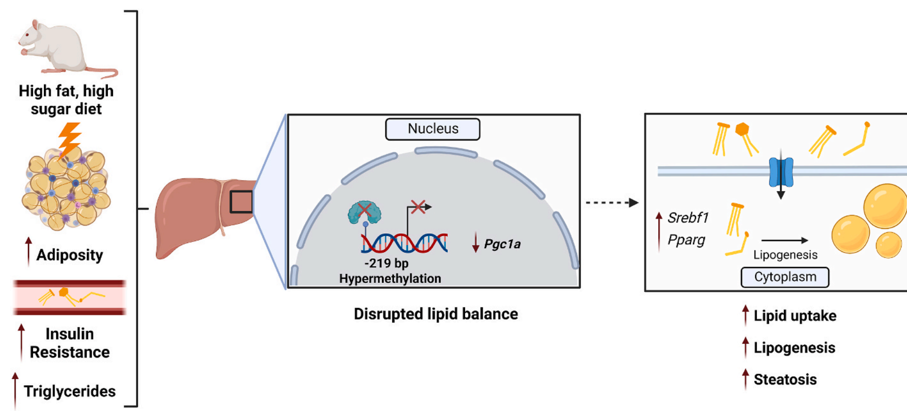

Fig. 5. Schematic proposed model showing how HFHS induced hypermethylation of the *Pgc1a* promoter leads to increased lipid accumulation in the liver. Abbreviations: *Srebf1* – Sterol regulatory element-binding transcription factor 1, *Pparg* – Peroxisome proliferator-activated receptor gamma, *Pgc1a* – Peroxisome proliferator-activated receptor gamma coactivator 1 alpha. This image was created using BioRender (https://biorender.com).

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2023.09.004.

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