ROLE OF EPIGENETIC MODIFICATIONS ON DIET-INDUCED METABOLIC DISEASES

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ROLE OF EPIGENETIC MODIFICATIONS ON DIET-INDUCED METABOLIC DISEASES

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EDITORIAL
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Editorial: Role of Epigenetic Modifications on Diet-Induced Metabolic Diseases

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Editorial on the Research Topic

Role of Epigenetic Modifications on Diet-Induced Metabolic Diseases

The prevalence of diet-induced metabolic diseases, including insulin resistance, type 2 diabetes (T2D), hypertension, cardiovascular disease, non-alcoholic fatty liver disease (NAFLD) and metabolic syndrome, is rising rapidly worldwide, with obesity representing a major cause of these diseases (Han and Lean, 2016). Moreover, obesity enhances the likelihood of developing numerous blood-borne and solid cancers, thus undoubtedly increasing morbidity and mortality in an unprecedented manner (Islami et al., 2019).

The pathogenesis of metabolic diseases that are triggered by excessive caloric intake remains to be fully elucidated, although researchers have recently highlighted that organ and systemic damage associated with these pathologies might be the result of a complex network of interactions between genetics and environment, potentially orchestrated by epigenetic mechanisms (Sorrenti et al., 2017).

The eukaryotic epigenome is believed to interact with environmental stimuli through alterations of several chromatin features, and by differential silencing or activation of gene expression. Cells exhibit three major interconnected systems that are able to turn on/off genes: (1) DNA methylation, (2) histone modifications, and (3) incorporation of histone variants into the chromatin and non-coding RNAs (including microRNAs) (Jiménez-Chillarón et al., 2012). Whether dietary habits or specific nutrients during prenatal and postnatal life may trigger specific epigenetic mechanisms, which in turn are involved in the development of disease phenotypes, has not yet been fully established.

A deeper understanding of the mutual influence between dietary patterns and epigenetic modifications could have important diagnostic and therapeutic implications.

In this Research Topic, we present a series of both review articles and original research manuscripts that illuminate the role and effects of epigenetic modifications occurring in diet-induced metabolic diseases.

Epidemiological evidence has revealed that health and diseases in later life are also linked to fetal origins and early life factors, such as maternal health status and diet (Vinciguerra and Cordero Sanchez, 2020). Indeed, adverse events during prenatal life may result in permanent changes in the physiology and metabolism of the offspring, which in turn lead to programming and an increased risk of a variety of metabolic diseases.

In our Research Topic, Deodati et al. provide evidence of the epigenetic regulation (mainly DNA methylation and microRNAs) of the developmental programming of metabolism that may be related to the insurgence of cardiometabolic disease. Some external environmental factors (i.e.,

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air pollution) coupled with a wrong diet might increase the risk of cardiovascular diseases and metabolic disorders. Barchitta et al., in their original research, evaluate the effect of particulate matter (PM) exposure and adherence to a Mediterranean Diet (MD) on the methylation of interspersed nucleotide elements 1 (LINE-1). Interestingly, this surrogate marker of global genomic DNA methylation is associated with cardiovascular disease and cancer. The authors found an inverse association between the adherence to MD and exposure to PM10 and LINE-1 methylation levels. Beside pollution, specific genotoxic insults may also affect diet and induce epigenetic modifications that may have consequences on the onset of multifactorial diseases. ADP-ribosylation is an important post-translational protein modification that regulates diverse biological processes, controlled by dedicated transferases and hydrolases. Gene copy number variants of mono-ADP-Ribosylhydrolase *MACROD2* are associated with early onset obesity and insulin resistance (Pettersson et al., 2017). However, the physiopathological relevance of these findings remain elusive. In their original research, Lo Re et al., demonstrate that newborn *MACROD2*-knock-out mice actually display no differences in irradiation-induced lethality and DNA damage, high fat-diet induced obesity, or insulin and glucose/insulin intolerance, when compared to wild type littermates. The latter study underlies the importance of *in vivo* testing, using suitable animal models, to confirm epidemiologic and *in vitro* studies.

In addition to environmental factors and diet, the complex network of interactions established between the host, its microbes, and their products within the human intestine (i.e., the gut microbiota) may influence epigenetic mechanisms in eukaryotes and induce different obesity-related diseases. In a review article, Sharma et al. discuss the potential pathophysiological effects of the gut microbiome and epigenetic crosstalk on obesity and type 1/type 2 diabetes.

DNA methylation patterns in genomic and intergenic regions might also be a contributing factor in T2D, thus suggesting that epigenetic marks could represent potential biomarkers of disease. Importantly, Willmer et al., reviewed the most recent lines of evidence about the potential use of DNA methylation patterns obtained from human blood samples as semi-invasive candidate biomarkers for T2D prevention and treatment. This tool could be particularly relevant for the evaluation of the beneficial effects of some natural and/pharmacological anti-diabetic agents (Wen et al.) by monitoring epigenetic modifications from readily available body fluids, such as blood or urine.

However, an outstanding open question remains: are epigenetic modifications causative or simply associated with the cardiometabolic risk of T2D? Greco et al. attempts to reply to this question by providing an overview of the epigenetic role and the effects of some specific nutrients and other chemicals in the regulation of metabolic chronic disorders. In any case, it appears clear that dietary habits during pregnancy may influence offspring health at birth and later in life. Accordingly, in a piece of original research, Bianchi et al. demonstrate that maternal intake of N-3 polyunsaturated fatty acids, profiled at pregnancy, impacts on the offspring's DNA methylation status analyzed by cord blood mononuclear cells. In particular, the authors show that differentially methylated genes belonged prevalently to cell signaling pathways and metabolic processes, and identified four genes (i.e., *MSTN, IFNA13, ATP8B3*, and *GABBR2*) as potential determinants of the infant's metabolic programming.

Lipid imbalance, elicited also by a threonine-deficient diet in animal models, may also increase the risk of NAFLD. Threonine and S-adenosyl-methionine metabolism can influence histone methylation. In a piece of original research, Jiang et al. showed that a threonine-deficient diet in ducks caused NAFLD by triggering a differential expression of genes that regulate triglyceride metabolism in the liver. Among the epigenetic mechanisms involved in NAFLD, there is not enough attention on histone modifications and their dynamic changes. Barbaro et al. commented on a recent original study that demonstrates that the histone demethylase plant homeodomain finger 2 protects the liver from NAFLD progression.

The understanding of how epigenomic regulation might affect individual risk of developing obesity-related comorbidities represents a major challenge, especially in the study of genes that are involved in disease phenotypes (i.e., genes that are involved in feeding and related to endocannabinoid and the opioid systems). In this respect, Pucci et al. showed that a high-fat-diet-induced epigenetic modulation of type 1 cannabinoid receptor gene and mu opioid receptor gene expression provides a new robust hypothalamic biomarker for the early development of obesity.

Finally, as we recalled before, obesity increases death rates of different types of cancers, including NAFLD-related hepatocellular carcinoma (HCC). It is widely accepted that in NAFLD-related HCC pathogenesis multiple interactions between genetics, epigenetics, and diet play a key role. In this respect, Dreval et al. showed that NAFLD-related HCC in a genetic animal model is characterized by the progressive accumulation of alterations in DNA methylation and altered gene expression patterns, and highlighted that the progressive changes in *TUBB2B* expression/methylation might be a contributor to hepatocarcinogenesis. Other authors reviewed genes and epigenetic mechanisms that are linked to HCC development (Farcas et al.; Krupenko and Horita).

In conclusion, this Research Topic was aimed at providing up-to-date evidence of the link between diet, environmental factors, epigenetic modifications, and the onset or progression of several obesity-related diseases. However, we would like to highlight here that epigenetic marks, especially those detectable in the circulation by non-invasive high-throughput approaches, might become efficient and effective predictive biomarkers of the stratification and progression of several chronic diseases that represent a costly healthcare endeavor.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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GENERAL COMMENTARY
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Commentary: The histone demethylase Phf2 acts as a molecular checkpoint to prevent NAFLD progression during obesity

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Keywords: NAFLD (non-alcoholic fatty liver disease), NASH (non-alcoholic steatohepatitis), Phf2, histone modification, epigenetics (MeSH)

A Commentary on

The histone demethylase Phf2 acts as a molecular checkpoint to prevent NAFLD progression during obesity

by Bricambert, J., Alves-Guerra, M. C., Esteves, P., Prip-Buus, C., Bertrand-Michel, J., Guillou, H., et al. (2018) Nat. Commun. 9:2092. doi: 10.1038/s41467-018-04361-y

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Non-alcoholic fatty liver disease (NAFLD) is a multi-faceted and multi-factorial hepatic disease, characterized by different patterns of histological damage, which range from hepatosteatosis to non-alcoholic steatohepatitis (NASH). NASH is histologically characterized by hepatosteatosis, hepatocellular ballooning, lobular inflammation, and fibrosis, which may progress to cirrhosis and, potentially, end-stage liver disease and hepatocellular carcinoma (Yeh and Brunt, 2014).

It is widely accepted that NAFLD could be a heritable disease in which genetic variants, including those of *PNPLA3, TM6SF2,* and *MBOAT7* genes, and epigenetic drivers linked to over-nutrition closely interact to determine the disease phenotype and its progression (Eslam et al., 2018). Thus, epigenetic modifications, including chromatin remodeling, histone modifications, DNA methylation and non-coding RNAs, make a major contribution in determining the NAFLD onset and progression (Eslam et al., 2018). A genome-wide integrated methylome/transcriptome analysis has demonstrated that genes involved in the methylation process, inflammation, and fibrogenesis showed a stage-dependent regulation, suggesting that epigenetic changes are involved in the progression of NAFLD (Murphy et al., 2013).

The recently-emerged fact that nutritional epigenetics may explain the gene-diet interactions, further elucidating the modulatory role of nutrition in diseases, is noteworthy. In line, NAFLD may exhibit a different pattern and level of severity of tissue damage in function of the excess of specific nutrients in the diet. Several studies have showed that histone modifications, such as acetylation and methylation, may contribute to diverse diet-induced metabolic dysfunctions (Murphy et al., 2013). However, a comprehensive analysis of the histone modifications and their dynamic changes in NAFLD is infrequent, hampering our understanding of the role of diet-related epigenetic mechanisms in the development and progression of this disease.

In this regard, a recent article published in Nature Communication (Bricambert et al., 2018) demonstrated that the histone demethylase Plant homeodomain finger 2 (Phf2) protects liver from NAFLD progression. The authors found that an over-expression/induction of Phf2 protected the liver from lipotoxicity and oxidative stress in models of a high-fat/high-sucrose diet (HF/HSD) dependent NAFLD. The proposed mechanism was that a Phf2 over-expression regulated the promoters of several genes including PKLR, ACLY, ACCA, FASN, SCD1, ELOVL6, TXNIP, RGS16,

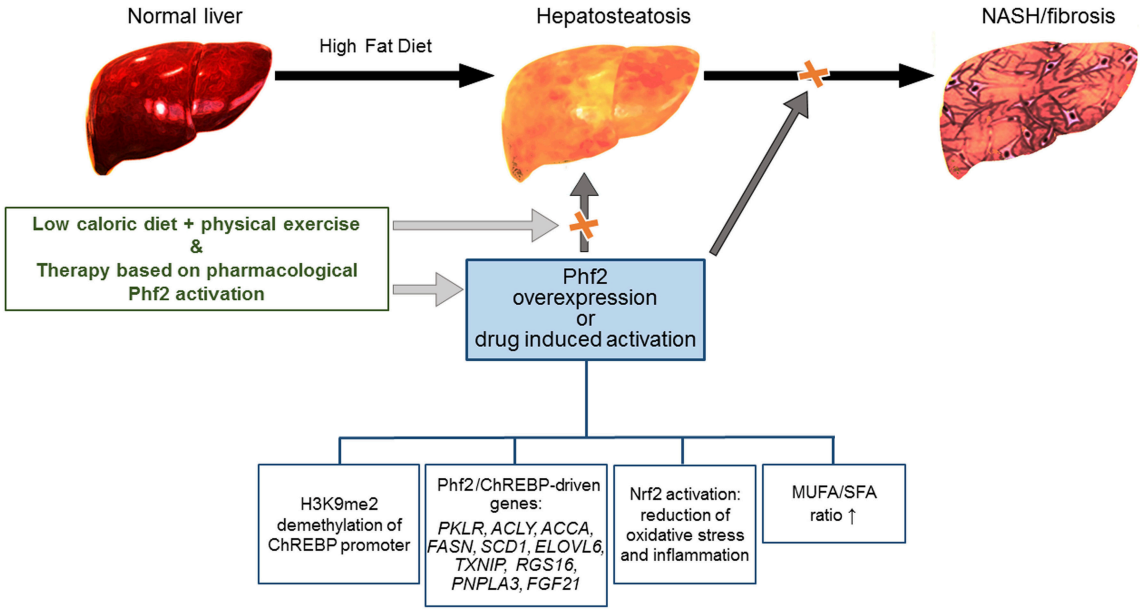

FIGURE 1 | Schematic representation of the Phf2 action in NAFLD. Phf2 activation, through H3K9me2 demethylation at specific ChREBP-regulated gene promoters, increases MUFA synthesis and provides protection from the progression of hepatosteatosis toward NASH. Therapy based on Phf2, in combination with a low-caloric diet and physical exercise could be an active therapeutic approach against NAFLD.

PNPLA3, and FGF21 by facilitating H3K9me2 histone demethylation at a carbohydrate-responsive element binding protein (ChREBP). Previous studies investigated the Phf2 role in metabolism (Baba et al., 2011; Okuno et al., 2013). Baba et al. (2011) suggested that Phf2 is inactive by itself, but becomes an active H3K9Me2 demethylase through PKA-mediated phosphorylation, with a major role in the induction of gluconeogenic genes (Baba et al., 2011). More recently, Okuno et al. (2013) demonstrated a role for Phf2, in the regulation of adipogenesis. Specifically, the authors demonstrated in Phf2-knockout mice, that Phf2 potentiates adipogenesis through an interaction with the transcription factor, the CCAAT/enhancer-binding protein alpha, indicating Phf2 as a potential new therapeutic target in the treatment of obesity and the metabolic syndrome (Okuno et al., 2013).

Importantly, the article by Bricambert et al. (2018) fits well into the deepening of the role of Phf2 in metabolic diseases. Specifically, in a mouse model of HF/HSD-induced NAFLD, the authors demonstrated that even by the triggering of diet-induced hepatic steatosis, Phf2 concomitantly reduced lipotoxicity by increasing the production of mono-unsaturated fatty acids (MUFA), thus enhancing the MUFA/saturated fatty acids (SFA) ratio. Phf2 over-expression also reduced oxidative stress by improving the activity of the NF-E2-related factor 2 (Nrf2). Findings by Bricambert et al. (2018) highlighted that both Phf2 and ChREBP are functionally co-recruited to the Nrf2 promoter in response to glucose, thus increasing the Nrf2 expression and its activity on the genes of specific targets. Indeed, the authors reported that the Phf2-mediated activation of Nrf2, redirects glucose toward the pentose phosphate pathway and glutathione biosynthesis, defending the liver from the accumulation of reactive oxygen species and consequently, oxidative stress.

The Phf2-dependent reduction of oxidative stress rebounded like a cascade on several other genes that control hepatic inflammation and fibrosis, implying a consequent great reduction of their expression. Previous studies highlighted the crucial role of Nrf2 and its signaling pathways in protecting hepatic cells from oxidative damage during the development of common chronic liver diseases, indicating Nrf2 as a therapeutic target (Meakin et al., 2014; Sharma et al., 2017). Meakin et al. (2014) showed that Nrf2 deficiency made the mice more sensitive to develop NASH when placed on an HFD, by the induction of lipogenesis genes and the suppression of β-oxidation genes. More recently, the same group of authors (Sharma et al., 2017) demonstrated that a potent pharmacologic activator of Nrf2 (TBE-31) ameliorated experimental NASH and liver fibrosis reducing insulin resistance, suppressing hepatic steatosis, and inhibiting inflammatory response and oxidative stress. However, the existence of some adverse effects of Nrf2 activation cannot be ignored. In fact, among others, a phase 3 clinical trial, evaluating the Nrf2-pathway activator Bardoxolone methyl, in type 2 diabetes and stage 4 chronic kidney disease, has been interrupted as it did not reduce the risk of end-stage renal disease/death from cardiovascular causes (de Zeeuw et al., 2013). These findings are important, considering that Phf2 acts on Nrf2. Moreover, the fact that a Phf2 over-expression promotes hepatic fat accumulation should not be underestimated.

Several prevailing drugs, evaluated in clinical trials on NASH patients have been proven to slightly improve only one feature of

the hepatic disease without exacerbating the others (Townsend and Newsome, 2017).

On the other hand, the Phf2-dependent increase of hepatosteatosis is a no-progressive feature in the pre-clinical study by Bricambert et al. (2018), suggesting the possibility to employ Phf2 pharmaceutical targeting as a therapeutic approach.

Hopefully it might be hypothesized that the anti-fibrogenic/inflammatory therapies based on Phf2 activation could be an option, in combination with diet, physical exercise and nutritional supplements that are recognized as anti-steatotic (**Figure 1**) (Romero-Gómez et al., 2017).

In conclusion, the article by Bricambert et al. (2018) provides a great contribution to the large landscape of epigenetics, and could serve as another brick in the field dealing with the use of epigenetic drugs as one of the multiple treatments to be included in multi-target therapeutic strategies in our fight against the development and progression of NAFLD.

AUTHOR CONTRIBUTIONS

BB, IR, and AA wrote the text. BB and IR designed the figure. AA critically revised the final manuscript. All authors have approved the manuscript.

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Mediterranean Diet and Particulate Matter Exposure Are Associated With LINE-1 Methylation: Results From a Cross-Sectional Study in Women

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Emerging evidence suggests that air pollution increases the risk of cardiovascular disease (CVD) and metabolic disorders, adding to the global burden of disease attributable to lifestyle and behavioral factors. Although long interspersed nucleotide elements 1 (LINE-1) methylation has been associated with these disorders, no studies have simultaneously examined the effects of diet and air pollution exposure on DNA methylation. Herein, we evaluated the association of particulate matter (PM with aerodynamic diameters of less than 10 mm) exposure and adherence to Mediterranean Diet (MD) with LINE-1 methylation. Healthy women (n = 299), aged 15 to 80 years, were enrolled in a cross-sectional study. Dietary data and adherence to MD were assessed by a Food Frequency Questionnaire (FFQ) and Mediterranean Diet Score (MDS). PM10 levels during 1-month before recruitment were recorded by monitoring stations and assigned to each woman based on their residential address and day of recruitment. LINE-1 methylation in blood samples was assessed by pyrosequencing and reported as percentage of 5-methylcytosine (5mC). The Mann–Whitney U test, Spearman's rank correlation test and linear regression models were applied. Our results demonstrated, for the first time, an inverse association between adherence to MD and exposure to PM10 with LINE-1 methylation: while higher monthly PM10 exposure decreases LINE-1 methylation level ($\beta = -0.121$; $p = 0.037$), the adherence to MD increases it ($\beta = 0.691$; $p < 0.001$). MDS seemed to interact with PM10 levels ($p = 0.002$) on LINE-1 methylation, as such we confirmed that the effect of MD decreased with increasing PM10 levels ($\beta = 0.657$; $p < 0.001$ in the first tertile; $\beta = 0.573$; $p < 0.001$ in the second tertile; $\beta = 0.551$; $p < 0.001$ in the third tertile). Thus, we suggest that LINE-1 methylation is a possible mechanism underpinning environment-related health effects, and encourage further research to evaluate whether the adherence to the MD could counteract the negative effect of PM10 exposure.

Keywords: epigenetics, dietary habits, air pollution, gene–diet interaction, cardiovascular disease, metabolic disorders, cancer, hypomethylation

INTRODUCTION

Air pollution constitutes a global health hazard, which may activate several pathways involving oxidative stress, vascular dysfunction, and metabolic impairment. In fact, air pollution increases the risk of cardiovascular disease (CVD) and metabolic disorders, adding to the global burden of disease attributable to lifestyle and behavioral factors (Stansfeld, 2015). The effect of chronic environmental exposure is complex since it occurs within the framework of the cumulative impact of exposures over time (Münzel et al., 2017). This approach recognizes that environmental-related diseases are the result of the totality of a person's environmental exposures, from all sources and routes, across the lifespan – the so-called exposome (Wild, 2012).

In the last decades, several lines of evidence suggested that molecular effects of environment might extend beyond the interaction with the DNA sequence (Reamon-Buettner et al., 2008; Baccarelli and Bollati, 2009). In this context, epigenetics - the study of heritable changes in gene expression that occur without changes in DNA sequence (Wolffe and Guschin, 2000) – provides opportunities to understand the mechanistic underpinnings of environment-related health effects (Angrish et al., 2018). Epigenetic signatures, including DNA methylation, histone modifications, histone variants, and chromatin remodeling, are dynamic in response to environmental signals, modifiable during cell differentiation and heritable in daughter cells (Hammoud et al., 2013). Among these, DNA methylation can be potentially modified by environmental and lifestyle factors, reprogramming the genome of exposed individuals and future generations (Burris and Baccarelli, 2014). DNA methylation can regulate gene transcription and chromosome stability via the addition of methyl groups to cytosine residues. In mammalian, 5-methylcytosines (5mC) represent 2–5% of all cytosines and are mainly found on CpG dinucleotides, also called CpG sites. Although hypermethylation of CpG sites, located in promoter regions, leads to decreased expression of the genes (Orphanides and Reinberg, 2002), more than 90% of CpG sites are located in transposable repetitive elements (Yang et al., 2004). Hypomethylation of transposable repetitive elements has been associated with chromosomal instability and aberrant genome function (Schulz, 2006; Slotkin and Martienssen, 2007). Methylation level of transposable sequences, including both long interspersed nucleotide elements 1 (LINE-1) and Alu sequences, has been used as a surrogate marker of global genomic DNA methylation (Yang et al., 2004). Although LINE-1 methylation is not an universally accepted marker of global methylation, aberrant methylation of these sequences was shown to be associated with cancer (Woo and Kim, 2012; Barchitta et al., 2014b), CVD (Baccarelli et al., 2010), and degenerative diseases (Maugeri et al., 2018a,b).

Exposure to air pollution, especially to particulate matter, has been linked to epigenetic changes by several epidemiological studies. For instance, a study by Tarantini et al. (2009) showed that long-term exposure to particulate matter with aerodynamic diameters of less than 10 mm (PM10) was negatively associated with Alu and LINE-1 methylation. Consistently, results from the Normative Aging Study demonstrated that exposure to black carbon, a marker of traffic particles, was also negatively associated with LINE-1 methylation (Baccarelli et al., 2009). These findings point out the need of further research to determine whether air pollution leads to LINE-1 methylation changes. However, since nutritional factors may modulate the response to environmental exposure, current environmental health research should incorporate nutrition and dietary practices. For instance, several classes of nutrients (i.e., folate, polyphenols, selenium, retinoid, fatty acids, isothiocyanates, and allyl compounds) can affect DNA methylation via different mechanisms (Ong et al., 2011). Among these, folate is a fundamental methyl donor for cellular replication and maintenance via modulating DNA methylation, synthesis, and repair. However, since current evidence is controversial (Moore et al., 2008; Choi et al., 2009; Zhang et al., 2011b, 2012; Ono et al., 2012; Piyathilake et al., 2012; Agodi et al., 2015a), its effect remains to be completely elucidated. Focusing on dietary patterns, the adherence to diet characterized by a high intake of vegetables and fruits has been associated with LINE-1 methylation (Zhang et al., 2011b; Agodi et al., 2015a; Delgado-Cruzata et al., 2015). Despite these findings, lack of evidence still exists about the effect of Mediterranean Diet (MD) – widely recognized as the optimal diet for disease prevention and global health – on LINE-1 methylation. To our knowledge, no studies have simultaneously examined the effects of diet and air pollution exposure in experimental models or humans. The questions that need to be addressed include the interactive effects of both factors on surrogate molecular markers of diseases. Given this scenario, we recently designed the present project, aiming to evaluate whether adherence to MD, as well as the intake of specific nutrients, might modulate the association between air pollution and methylation in healthy women living in Catania (Barchitta et al., 2017b). In previous studies, we demonstrated the effect of both diet and epigenetic mechanisms in several female physiological and pathological conditions (Agodi et al., 2014, 2015a,b; Barchitta et al., 2014a, 2017a,c, 2018). Herein, we evaluated the association of PM10 exposure and adherence to MD, 1 month before recruitment, with LINE-1 methylation, taking into account their potential interaction and the effect of socio-economic and lifestyle factors.

MATERIALS AND METHODS

Study Design

Women referred to two clinical laboratories of Catania (Italy) were fully informed of the purpose and procedures and invited to participate in this cross-sectional study. This study was carried out in accordance with the recommendations of the involved institution. The protocol was approved by the ethics committee of the involved institution. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The inclusion criteria were: (i) non-pregnant women (ii) with no current or previous self-reported history of severe diseases, as cancer, CVD, type 2 diabetes, neurodegenerative and autoimmune diseases (iii) who signed a written consent to participate in the study. Information on sociodemographic and lifestyle data were collected by trained epidemiologists

using a structured questionnaire. Educational level was classified as low (primary school, i.e., ≤8 years of school) and high (high school education or greater, i.e., >8 years of school). Women were also classified as employed or unemployed (including students and housewives). Body mass index (BMI) was calculated as weight (kg) divided by height (m^2) and classified based on criteria from the World Health Organization (1995). No exclusion criteria for BMI categories were applied.

PM10 Exposure Assessment

In Catania, air pollutants are continuously measured by a network of five automatic monitoring sites located in the urban area. PM10 levels, recorded by the Ecology and Environment Office of the Municipality of Catania through these monitoring stations and available online as daily means, were assigned to each woman based on their residential address and day of recruitment. Briefly, monitoring stations and residential addresses were geocoded and PM10 measurements from the nearest monitoring station to residential address were assigned. For each woman, we recorded the daily mean of PM10 level the day of recruitment and back to 30 days before. We also calculated the monthly mean PM10 level referred to the month before recruitment. Missing values were imputed by an algorithm integrating the annual average of the incomplete series and the PM10 concentrations of the nearest and more correlated monitors (Cattani et al., 2010).

Dietary Assessment

Dietary data were obtained by a 95-item semi-quantitative Food Frequency Questionnaire (FFQ), using the previous month as the reference period (Agodi et al., 2011). For each food item, women were asked to report the frequency of consumption and portion size. To estimate the amount of each food item and to minimize inaccuracies, an indicative photograph atlas was used. Frequencies of food consumption were classified into 12 categories, ranging from "almost never" to "two or more times a day." The medium serving sizes were described by natural portions or standard weight and volume measures of the servings commonly consumed in the Italian population. Accordingly, portion size was classified into three categories: small (half a medium serving size), medium, and large (1.5 times or more than a medium serving size). The food intakes derived from the FFQ were calculated by multiplying the frequency of consumption with the daily portion size of each food group. Folate and total caloric intakes were calculated using the USDA Nutrient Database[1] adapted to the Italian food consumption. Intake of folic acid from supplements was specifically addressed as previously described (Agodi et al., 2014). Prevalence of folate deficiency was estimated by comparing folate intake with the Estimated Average Requirements (EAR) (Institute of Medicine (US) Food and Nutrition Board, 1998), taking into account the use of folic acid supplements.

[1]http://ndb.nal.usda.gov/

TABLE 1 | Characteristics of study population.

Population characteristics (*N* = 299)	Mean *(SD)* or proportion
Age, years	38.99 (16.53)
Low educational level	30.1%
Unemployed	54.2%
Current smokers	21.2%
BMI, kg/m^2	24.55 (5.04)
Underweight	4.7%
Normal Weight	57.4%
Overweight	24.0%
Obese	13.9%
Dietary folate intake, μg/day	295.36 (132.62)
Folic acid supplement users	18.1%
Folate deficiency	47.5%
MDS	4.64 (1.78)
Low adherence	27.1%
Medium adherence	58.2%
High adherence	14.7%
LINE-1 methylation level, % 5mC	67.66 (7.48)
CpG site 1, % 5mC	80.47 (3.14)
CpG site 2, % 5mC	57.11 (9.20)
CpG site 3, % 5mC	65.39 (7.48)

SD, standard deviation; BMI, body mass index; 5mC, 5-methylcytosine.

Mediterranean Diet Score

Adherence to MD was assessed using the Mediterranean Diet Score (MDS) (Trichopoulou et al., 1995; Couto et al., 2011) which includes 9 components: fruits and nuts, vegetables, legumes, cereals, lipids, fish, dairy products, meat products, alcohol and the ratio of unsaturated to saturated lipids. For components that are more consumed in Mediterranean countries (vegetables, legumes, fruits and nuts, cereals, fish, and a high ratio of unsaturated to saturated lipids), women whose consumption was below or equal to the median value of the population were assigned a value of 0, and a value of 1 was assigned otherwise. For components consumed less frequently in Mediterranean countries (dairy and meat products), women whose consumption was below the median were assigned a value of 1, and a value of 0 was assigned otherwise. A value of 1 was given to women consuming a moderate amount of alcohol (5 to <25 g per day). Accordingly, MDS ranges from 0 (no-adherence) to 9 (perfect adherence). MD adherence was categorized, according to the MDS, as follows: low adherence (MDS range: 0–3), medium adherence (MDS range: 4–6), or high adherence (MDS range: 7–9) (Barchitta et al., 2014a).

DNA Extraction and Methylation Analysis

Whole blood samples, collected into EDTA tubes from each participant, were centrifuged at 2500 rpm for 15 min. The buffy coat fraction was transferred to a cryovial and immediately frozen at −20°C until use. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Italy) according to the manufacturer's protocol. LINE-1 methylation levels were measured by pyrosequencing-based methylation analysis, using the PyroMark Q24 instrument

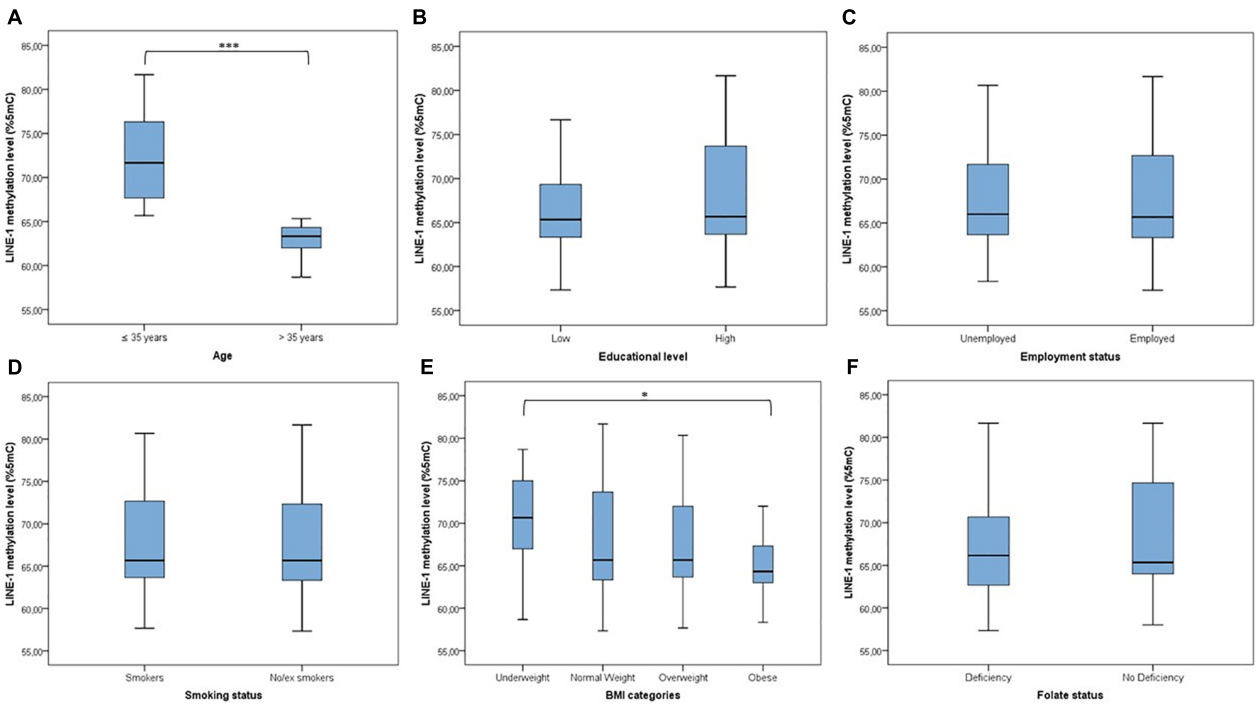

FIGURE 1 | Distribution of LINE-1 methylation by population characteristics. **(A)** ≤Median age (35 years) vs. >median age; **(B)** low educational level (≤8 years of school) vs. high educational level (>8 years of school); **(C)** unemployed vs. employed (including part-time and full-time); **(D)** smokers vs. no/ex-smokers; **(E)** comparison across BMI categories; **(F)** folate deficiency vs. no folate deficiency (taking into account the use of supplements). ***$p < 0.001$ and *$p < 0.05$ based on the Mann–Whitney *U* test or the Kruskal–Wallis test.

(Qiagen, Italy), as previously reported (Agodi et al., 2015a; Barchitta et al., 2017c). Briefly, bisulfite conversion and clean-up of DNA for methylation analysis of 30–40 ng of DNA were completed using the EpiTect Bisulfite Kit (Qiagen, Italy) and the converted DNA was eluted in 20 μl of Elution Buffer. PCR was conducted in a reaction volume of 25 μl, using the PyroMark PCR Kit (Qiagen, Italy). According to the manufacturer's instructions, each reaction mixture contained 1.5 μl of bisulfite-converted DNA, 12.5 μl of PyroMark PCR Master Mix 2×, 2.5 μl of Coral Load Concentrate 10×, and 2 μl of the forward primer (5′-TTTTGAGTTAGGTGTGGGATATA-3′) and the reverse-biotinylated primer (5′-biotin-AAAATCAAAAAATTCCCTTTC-3′) (0.2 μM for each) (Agodi et al., 2015b). Hot start PCR cycling conditions were 1 cycle at 95°C for 15 min, 40 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. Then, the PCR product underwent pyrosequencing using 0.3 mM of the sequencing primer (5′-AGTTAGGTGTGGGATATAGT-3′). All runs included 0 and 100% methylated human DNA as positive controls as well as a negative control. To confirm reproducibility every sample was tested two times and failed assays were repeated. Overall, intra-observer coefficient of variability between the two replicates of LINE-1 methylation measurements was 3.2% (SD = 3.0%). LINE-1 methylation levels were calculated as percentage of methylated cytosines over the sum of methylated and unmethylated cytosines, and reported for each CpG site as well as the average of the three CpG sites (GenBank Accession No. X58075).

Statistical Analyses

Statistical analyses were performed using the SPSS software (version 22.0, SPSS, Chicago, IL, United States). Descriptive statistics were presented as frequencies, means ± standard deviations (SDs), median values and interquartile range (IQR). Prior to analysis, the normal distribution of all variables was checked using the Kolmogorov–Smirnov test. Since LINE-1 methylation exhibited non-normal distribution, differences by population characteristics were tested using the Mann–Whitney *U* test or the Kruskal–Wallis test. Correlation of daily and monthly PM10 levels with LINE-1 methylation was evaluated using the Spearman's rank correlation coefficient and presented as a correlation matrix. To evaluate the association of monthly PM10 levels or MDS with LINE-1 methylation, we used the following linear regression models: the age-adjusted model; the multivariable model adjusted for covariates that were selected *a priori* (i.e., age, educational level, employment status, smoking, BMI, season of recruitment, dietary folate intake and use of supplements). In linear regression models, continuous variables, i.e., LINE-1 methylation level, PM10 level, MDS, age, BMI and dietary folate intake were used. Interaction of PM10 levels and MDS with LINE-1 methylation was investigated by using the general linear model, adjusting for age, educational level, employment status, smoking, BMI, and folate deficiency.

TABLE 2 | Daily mean PM10 levels during 30 days before recruitment.

Days before recruitment	Mean	*SD*	Median	IQR (percentiles)	
				25th	75th
30 days	24.26	9.06	23.00	18.44	29.20
29 days	24.38	10.15	23.00	18.44	29.60
28 days	25.16	10.00	23.70	19.11	29.20
27 days	24.54	9.16	23.50	18.44	29.10
26 days	25.17	11.61	23.00	18.60	28.60
25 days	25.04	11.02	23.00	18.44	28.60
24 days	24.07	9.63	21.29	18.44	28.60
23 days	24.32	8.84	23.60	18.44	29.10
22 days	25.32	11.53	23.80	18.44	29.20
21 days	26.52	12.60	24.20	18.44	31.90
20 days	27.50	15.84	23.90	18.44	31.30
19 days	27.17	15.80	23.00	18.44	30.70
18 days	25.44	11.33	22.70	18.44	28.60
17 days	24.90	9.66	23.00	18.44	29.10
16 days	24.39	9.07	22.00	18.44	30.20
15 days	23.73	8.42	22.00	18.44	27.70
14 days	23.83	8.40	21.60	18.44	29.10
13 days	24.03	8.45	21.60	18.44	27.80
12 days	23.97	8.13	21.80	18.44	27.80
11 days	23.70	8.80	20.70	18.44	27.60
10 days	23.67	8.72	21.00	18.44	29.20
9 days	23.16	8.32	21.80	18.44	27.70
8 days	22.28	7.52	21.30	18.44	25.20
7 days	22.64	7.29	20.70	18.44	26.50
6 days	22.75	7.48	20.47	18.44	27.70
5 days	23.00	10.11	20.47	18.44	26.60
4 days	22.39	7.66	20.47	18.44	25.70
3 days	23.51	7.43	21.30	18.44	27.60
2 days	22.77	7.91	20.70	18.44	26.20
1 day	22.66	8.45	20.50	18.44	26.10
Day of recruitment	23.39	8.59	21.10	18.44	27.30

SD, standard deviation; IQR, interquartile range.

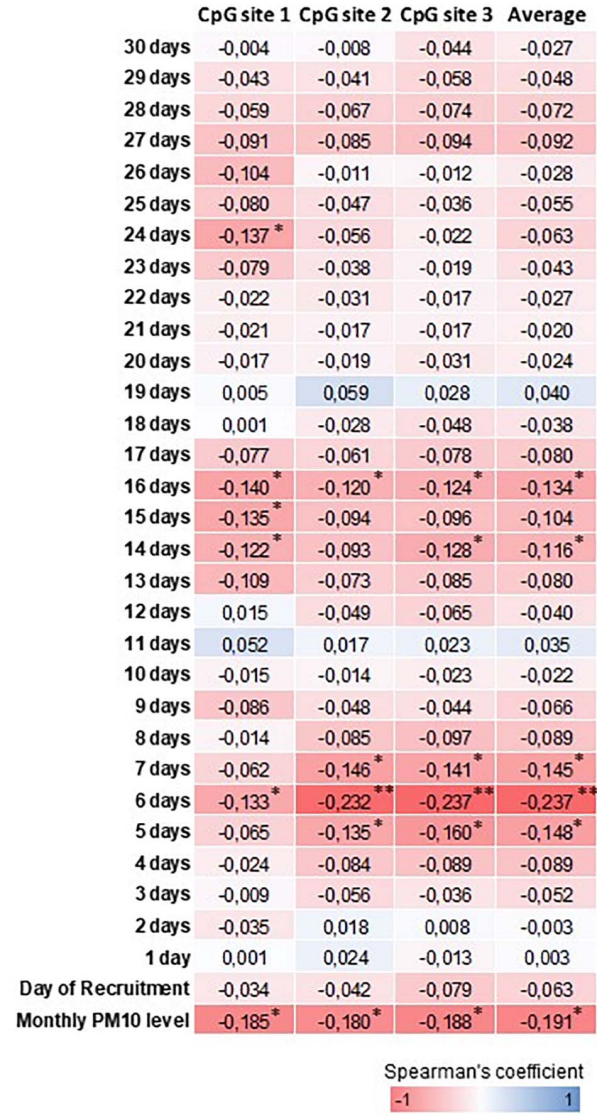

FIGURE 2 | Heatmap representation of Spearman's correlation coefficients between PM10 and LINE-1 methylation levels. ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$.

Since interaction was statistically significant, we tested the association of MDS with LINE-1 methylation by using a multivariable-adjusted model stratified by tertiles of PM10 exposure. Correction for multiple comparisons was performed by the Bonferroni method and adjusted *p*-values were calculated by multiplying the unadjusted *p*-value by the number of comparisons. Overall, results were reported as beta regression coefficient (β) expressing the change in LINE-1 methylation level associated with a standard deviation increase in PM10 levels or MDS. A *p*-value of 0.05 was considered as statistically significant.

RESULTS

The main characteristics of the 299 women, aged from 15 to 80 years, included in the present study are given in **Table 1**. In summary, mean age was 38.99 years (*SD* = 16.53; median = 35), 69.9% reported high educational level and 54.2% were unemployed. With regard to lifestyle factors and nutritional status, 21.2% were current smokers and 37.9% were overweight or obese. Mean folate intake was 295.36 μg/day, and only 18.1% reported the use of folic acid supplements. Taking into account the use of supplements, prevalence of folate deficiency was 47.5%.

LINE-1 methylation levels at the three CpG sites correlated with each other (r_{1-2} = 0.398; r_{1-3} = 0.940; r_{2-3} = 0.394; $p < 0.001$), showing mean values of 80.47 (*SD* = 3.14), 57.11 (*SD* = 9.20) and 65.39 (*SD* = 7.48), respectively. The mean LINE-1 methylation level was 67.66 (*SD* = 7.48). **Figure 1** shows that distribution of LINE-1 methylation levels differed by age ($p < 0.001$) and BMI categories ($p = 0.047$), while no significant differences by educational

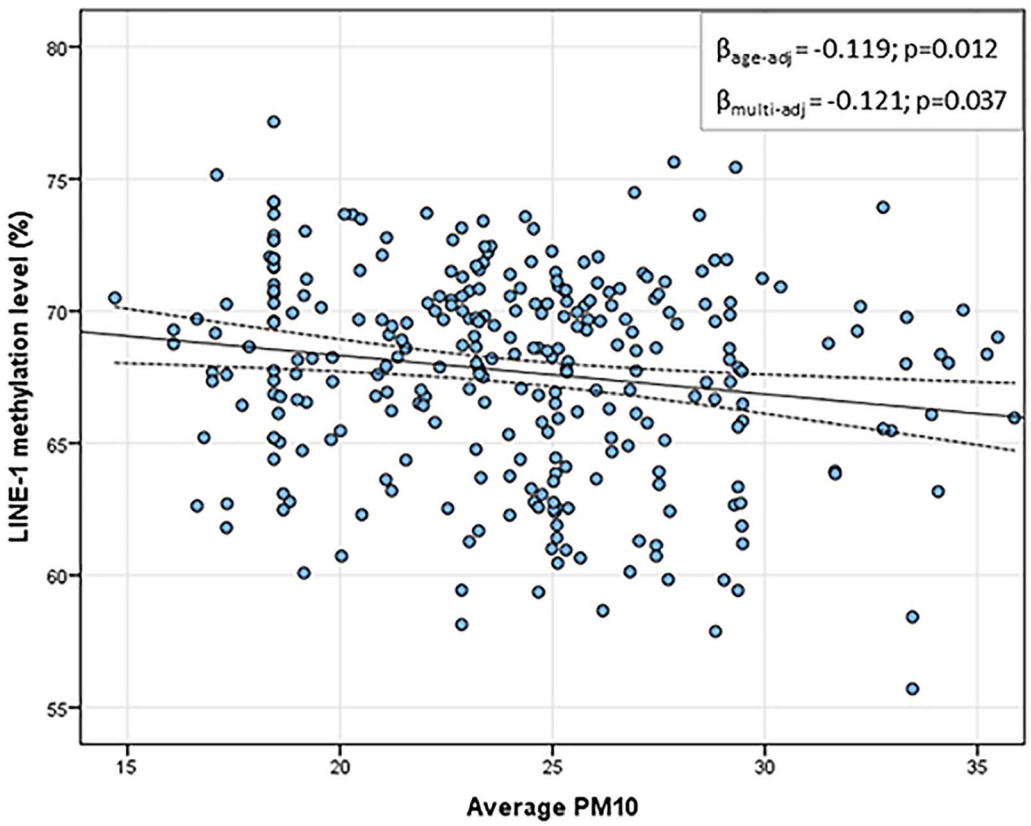

FIGURE 3 | Scatter plot of the linear association between monthly mean PM10 and LINE-1 methylation levels. In the *x*-axis the monthly average PM10 levels; in the *y*-axis the predicted LINE-1 methylation level adjusted for age, educational level, employment status, smoking, BMI, season of recruitment, dietary folate deficiency and use of supplements.

level, employment status, smoking and folate deficiency were evident.

Table 2 shows the daily PM10 levels attributed to each woman and evaluated during the 30 days before recruitment. PM10 levels ranged from 22.28 (8 days before recruitment) to 27.50 ng/m^3 (20 days before recruitment). Accordingly, the monthly mean PM10 level was 24.18 ng/m^3 (*SD* = 4.29). In order to assess whether PM10 levels were associated with LINE-1 methylation levels, we first analyzed the correlation between daily PM10 levels, as well as the monthly mean, and LINE-1 methylation levels. **Figure 2** shows that PM10 levels during 16, 14, 7, 6, and 5 days before recruitment were weakly but significantly correlated with LINE-1 methylation levels. Consistently, a negative significant correlation with monthly mean PM10 level was shown. Scatter plot representing the linear association of monthly mean PM10 level with LINE-1 methylation is shown in **Figure 3**. In the age-adjusted model, monthly mean PM10 level was significantly and negatively associated with LINE-1 methylation ($\beta = -0.119$; $p = 0.012$). A negative association was also observed in the multivariable regression analysis ($\beta = -0.121$; $p = 0.037$), adjusting for age, educational level, employment status, smoking, BMI, season of recruitment, dietary folate deficiency and use of supplements. Interestingly, this model also showed that LINE-1 methylation was negatively associated with age ($\beta = -0.519$; $p < 0.001$) and dietary folate intake ($\beta = -0.111$; $p = 0.001$).

According to MDS (mean = 4.64; *SD* = 1.78), adherence to MD has been classified as low (27.1% of women), medium (58.2% of women), and high (14.7% of women). The distribution of LINE-1 methylation by categories of adherence to MD shows that LINE-1 methylation levels increased with increasing MD adherence ($p = 0.004$) (**Figure 4**). Results from linear regression analysis demonstrated that MDS was significantly and positively associated with LINE-1 methylation levels both in the age-adjusted ($\beta = 0.606$; $p < 0.001$) and multivariable-adjusted model ($\beta = 0.691$; $p < 0.001$). By contrast, age was significantly and negatively associated with LINE-1 methylation in both models ($\beta = -0.787$; $p < 0.001$; $\beta = -0.581$; $p < 0.001$).

In order to assess whether the adherence to MD could influence the relationship between PM10 exposure and LINE-1 methylation, we tested the interaction between MDS and monthly mean PM10 level. Since MDS seemed to interact with PM10 levels ($p = 0.002$), we conducted a multivariable linear regression analysis stratified by tertiles of PM10 exposure. Our results confirmed that MDS was significantly and positively associated with LINE-1 methylation, and that its effect decreased with increasing PM10 levels ($\beta = 0.657$; $p < 0.001$ in the first

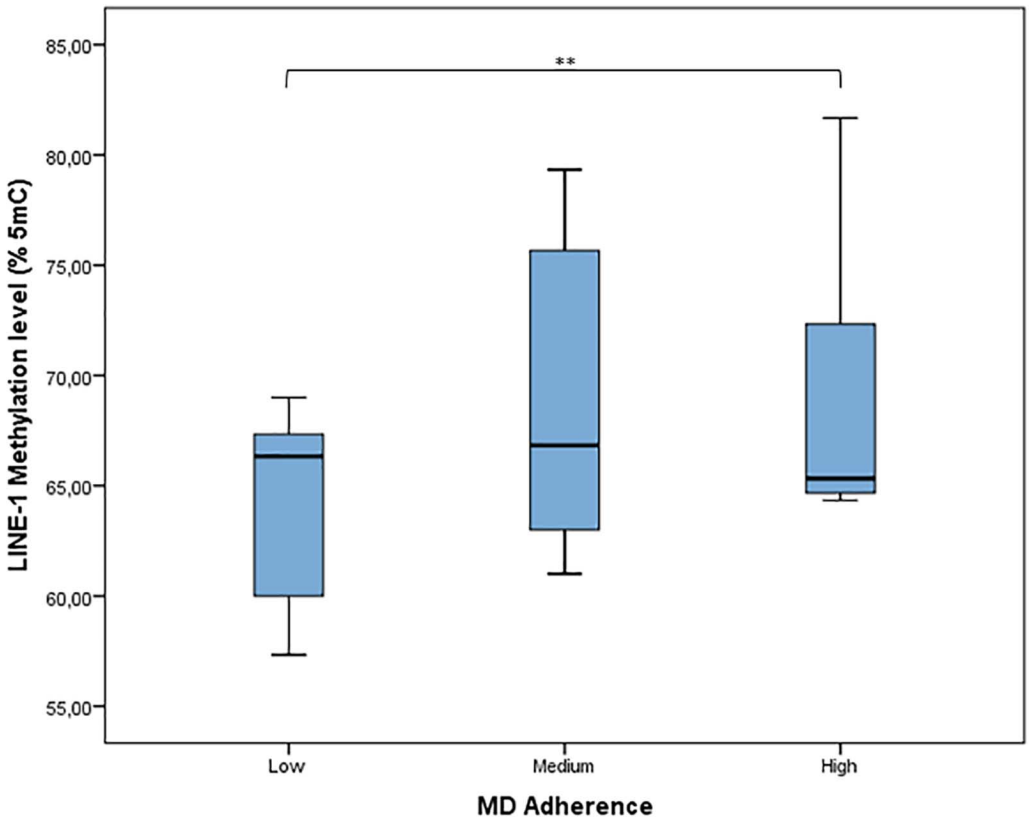

FIGURE 4 | Distribution of LINE-1 methylation by categories of adherence to MD. MD adherence was categorized, according to the MDS, as low adherence (MDS range: 0–3), medium adherence (MDS range: 4–6), or high adherence (MDS range: 7–9). $^{**}p < 0.01$ based on the Kruskal–Wallis test.

tertile; $\beta = 0.573$; $p < 0.001$ in the second tertile; $\beta = 0.551$; $p < 0.001$ in the third tertile).

DISCUSSION

Several lines of evidence indicate that dynamic changes in LINE-1 methylation – a well-established event in cancer and CVD (Baccarelli et al., 2010; Woo and Kim, 2012; Barchitta et al., 2014b) – appear to be influenced by environmental and lifestyle risk factors. In this framework, our findings support the relationship between air pollution exposure and epigenetic changes (Baccarelli and Bollati, 2009; Burris and Baccarelli, 2014). In line with previous work (Baccarelli et al., 2009; Tarantini et al., 2009; Madrigano et al., 2011), we observed that exposure to PM10 is negatively associated with LINE-1 methylation in healthy women. The study by Tarantini et al. (2009) conducted in an electric furnace steel plant, demonstrated that PM10 levels impacted on genomic DNA methylation of workers over an extended time frame. In particular, they reported that long-term, but not short-term, exposure to PM10 was inversely associated with methylation of Alu and LINE-1 sequences (Tarantini et al., 2009). This evidence, though from a different setting with peculiar exposure, led us to evaluate the correlation of 1-month daily PM10 exposure with LINE-1 methylation. Particularly, we observed that the effect was maximum for the exposure of 6 days before recruitment and spanned from days 5 to 7. Since a negative correlation with monthly mean PM10 level was also evident, we tested the association of monthly PM10 exposure and LINE-1 methylation. Notably, we demonstrated, for the first time, that monthly mean PM10 level was significantly and inversely associated with LINE-1 methylation both in the age- and multivariable-adjusted model.

In our analysis, we also observed that age was negatively associated with LINE-1 methylation. Although some studies reported no effect of aging on LINE-1 methylation (Chalitchagorn et al., 2004; El-Maarri et al., 2011; Zhang et al., 2011a), our result was in line with previous works, which demonstrated that methylation levels in the repetitive elements, including LINE-1, significantly decreased with increasing age (Bollati et al., 2009; Zhu et al., 2012; Cho et al., 2015). However, the observed effect of age on LINE-1 methylation appeared stronger if compared to evidence from previous similar investigations.

Several scientific evidences also suggested that dietary folate status, as well as amounts of other methyl donors, could affect global DNA methylation. However, these findings were conflicting: while the majority of studies demonstrated that global DNA methylation levels increased with increasing folate intake (Moore et al., 2008; Choi et al., 2009; Zhang et al., 2011b, 2012;

Piyathilake et al., 2012; Agodi et al., 2015a), others observed an inverse relationship (Ono et al., 2012). This controversy might be explained by differences in unmeasured factors such as ethnicity, genetic variants in one-carbon metabolism, lifestyles, physiological and pathological conditions, which in turn can affect DNA methylation process.

The study of dietary patterns plays an important role in evaluating causes and consequences of PM exposure for human health and disease. This concept raised from the need of a more complete environmental exposure assessment in epidemiological studies, providing a comprehensive description of lifelong exposure history (Wild, 2012). In this context, there is growing interest in determining how dietary patterns may affect global and gene-specific DNA methylation. To our knowledge, the present study was the first to demonstrate that adherence to MD was positively associated with LINE-1 methylation levels, after adjusting for age, educational level, employment status, smoking, BMI, season of recruitment, dietary folate deficiency and use of supplements. This was consistent with previous studies showing that healthy women with high intake of vegetables and/or fruits had a lower risk of LINE-1 hypomethylation (Zhang et al., 2011b; Agodi et al., 2015a). The biological basis of this relationship could be attributed to the wide variety of nutrients and bioactive compounds provided by MD, such as phytochemicals (phenolics, flavonoids, and carotenoids), vitamins (vitamin C, folate, and pro-vitamin A), minerals (potassium, calcium, and magnesium), and fibers, which in turn act on multiple signal transduction pathways and epigenetic mechanisms (Saura-Calixto and Goñi, 2009; Liu, 2013).

Since we demonstrated the inverse association of exposure to PM10 and adherence to MD with LINE-1 methylation levels, we finally aimed to assess whether adherence to MD could influence the relationship between monthly PM10 levels and LINE-1 methylation. Our findings confirmed not only the interaction between MDS and PM10 exposure, but also that the positive effect of MD on LINE-1 methylation decreased with increasing PM10 levels.

This study had some limitations. Although hypomethylation of transposable repetitive elements, including LINE-1 sequences, has been associated with chromosomal instability and aberrant genome function (Schulz, 2006; Slotkin and Martienssen, 2007) and with different chronic degenerative diseases (Baccarelli et al., 2010; Woo and Kim, 2012; Barchitta et al., 2014b; Maugeri et al., 2018b), mechanisms of DNA hypomethylation are not fully understood. Therefore, the cross-sectional design of our analysis arises the need of further prospective studies and of functional test to explore the causes and the consequences of these modifications and to better understood the biological significance of LINE-1 methylation alterations. Moreover, the peculiar geological and environmental conditions, due to natural sources of particles from the near Mount Etna volcano, sea salt and Sahara dust could be a potential confounder in our study. Although PM10 exposure assessment method has been used in other studies (Cattani et al., 2010; De Prins et al., 2013), this method has not been previously validated in our population characterized by specific PM exposure. Thus, to validate the PM10 exposure assessment method, including both volcanic and usual urban PM exposure, further research should develop a standardized protocol to characterize the mineralogical-chemical composition of PM and to better define exposure. In addition, limiting our investigation PM10 exposure avoided potential concerns related to the effect of other environmental and work-related chemicals that modify epigenetic marks, including metals (cadmium, arsenic, nickel, chromium, and methylmercury), air pollutants (PM2.5, black carbon, and benzene), and toxicants (diethylstilbestrol, bisphenol A, persistent organic pollutants, and dioxin). Other potential weaknesses regard the dietary assessment and the observed relationship between MD and LINE-1 methylation. Although dietary assessment through FFQ does not preclude measurement errors and inaccuracies, the FFQ used in the present study has been previously developed and validated for use among our population (Agodi et al., 2011). However, the relationship between MD and LINE-1 methylation may be also affected by genetic factors (i.e., polymorphisms in the *MTHFR* gene), which in turn may interact with folate status and methylation process (Agodi et al., 2015c). With regard to molecular analysis, precision and reproducibility of the DNA methylation assay should be considered when interpreting results of the present study. Reliability and flexibility have made pyrosequencing of bisulfite-treated DNA the "gold standard," and a high-throughput and replicable methodology to evaluate LINE-1 methylation (Beck and Rakyan, 2008; Rakyan et al., 2011). However, in the present study, the methylation analysis was performed on white blood cell DNA, including several cell type subsets. Previous studies reported small differences in LINE-1 methylation levels according to target CpG site and blood cell composition (Nelson et al., 2011; Zhu et al., 2012; Koestler et al., 2013; Tarantini et al., 2013; Nüsgen et al., 2015). Thus, the distinctiveness of LINE-1 methylation patterns discourages the comparison between results from studies, which evaluated LINE-1 methylation status at different CpG sites and reinforces the importance of accounting for cellular heterogeneity in future research.

CONCLUSION

In conclusion, results of the present study, conducted in a sample of healthy women, demonstrate the inverse association of adherence to MD and exposure to PM10 with LINE-1 methylation levels. Moreover, our findings suggest that LINE-1 methylation is a possible mechanism underpinning environment-related health effects and thus, further research are encouraged to evaluate whether the adherence to the MD could counteract the negative effect of PM10 exposure.

AUTHOR CONTRIBUTIONS

AA is the principal investigator of the project. AA, GB, PM, GDG, and NC conceived and designed the research. MB, AM, and AQ performed the experiments and statistical analyses. MB, AM,

and AA wrote the original draft. All the authors reviewed, edited, and approved the final draft.

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REVIEW
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Blood-Based DNA Methylation Biomarkers for Type 2 Diabetes: Potential for Clinical Applications

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Type 2 diabetes (T2D) is a leading cause of death and disability worldwide. It is a chronic metabolic disorder that develops due to an interplay of genetic, lifestyle, and environmental factors. The biological onset of the disease occurs long before clinical symptoms develop, thus the search for early diagnostic and prognostic biomarkers, which could facilitate intervention strategies to prevent or delay disease progression, has increased considerably in recent years. Epigenetic modifications represent important links between genetic, environmental and lifestyle cues and increasing evidence implicate altered epigenetic marks such as DNA methylation, the most characterized and widely studied epigenetic mechanism, in the pathogenesis of T2D. This review provides an update of the current status of DNA methylation as a biomarker for T2D. Four databases, Scopus, Pubmed, Cochrane Central, and Google Scholar were searched for studies investigating DNA methylation in blood. Thirty-seven studies were identified, and are summarized with respect to population characteristics, biological source, and method of DNA methylation quantification (global, candidate gene or genome-wide). We highlight that differential methylation of the *TCF7L2*, *KCNQ1*, *ABCG1*, *TXNIP*, *PHOSPHO1*, *SREBF1*, *SLC30A8,* and *FTO* genes in blood are reproducibly associated with T2D in different population groups. These genes should be prioritized and replicated in longitudinal studies across more populations in future studies. Finally, we discuss the limitations faced by DNA methylation studies, which include including interpatient variability, cellular heterogeneity, and lack of accounting for study confounders. These limitations and challenges must be overcome before the implementation of blood-based DNA methylation biomarkers into a clinical setting. We emphasize the need for longitudinal prospective studies to support the robustness of the current findings of this review.

Keywords: global DNA methylation, gene-specific DNA methylation, genome-wide DNA methylation, blood, type 2 diabetes, biomarkers

INTRODUCTION

Diabetes mellitus is a leading cause of death and disability worldwide, affecting 415 million people in 2017, and this figure is expected to increase to 592 million by 2035 (1, 2). Type 2 diabetes (T2D) accounts for over 90% of diabetes mellitus cases and its incidence is increasing globally in response to escalating rates of obesity and insulin resistance. Indeed, according to the World Health Organization, over 90% of patients with T2D are overweight or obese (3). T2D is a progressive, chronic disorder with a long asymptomatic phase. The early stages of disease can remain undetected for many years, during which time micro- and macro-vascular complications may occur (4, 5). Identification of individuals during the asymptomatic phase would not only permit opportunities for early interventions to prevent the development of overt diabetes, but may also lead to better management of the disease. A major area of current research has thus been to search for robust, sensitive, and readily accessible biomarkers of T2D. In this regard, a striking amount of evidence has accumulated to suggest that changes to the epigenetic landscape in insulin-responsive tissues play an important role in the pathogenesis of obesity, insulin resistance, and T2D, and if reflected in blood, may represent potential biomarker candidates (6–8).

Epigenetics is defined as heritable changes that affect gene expression without altering the underlying genomic sequence (9). These processes include DNA methylation, chromatin modifications such as histone acetylation and methylation, and non-coding RNAs that act as regulatory molecules (9). DNA methylation is the most widely studied and best characterized epigenetic mechanism, and involves the covalent addition of a methyl group to carbon C5 of cytosine nucleotides to create 5-methylcytosine (5 mC) (10). Cytosine methylation occurs in cytosine-guanine dinucleotides (CpG) sites, which tend to cluster together as repetitive sequences known as CpG islands, which are primarily found within promoter regions of genes, or regions with increased centromeric tandem repeat units (9, 10). CpG methylation within promoter regions is generally associated with gene silencing, although recent studies have provided evidence of the importance of non-CpG and non-promoter methylation in development and disease (11, 12). DNA methylation alterations can occur in response to biological (13, 14), lifestyle (15, 16), and environmental (17) factors and associate with gene expression changes and pathological dysfunctions. Moreover, DNA methylation is reversible, and therefore aberrant DNA methylation modifications have attracted increased interest as drug targets. As such, the interest in dissecting the impact of epigenetic variation on human diseases, including T2D, has increased over the last decade (18–23).

For numerous epidemiological studies, it is not always possible to access human tissues central to the pathogenesis of disease. Importantly, T2D-associated DNA methylation changes in pancreatic ß-cells and insulin-responsive tissues (liver, muscle, and adipose tissue) have been reported to be reflected in the blood, thus offering an opportunity to use alternative, non-invasive clinical samples for methylation analysis (7, 8, 24). Peripheral blood is relatively quick and easy to collect, with minimal side effects and much higher patient acceptability. As blood collection is already a part of routine medical checkups in both developed and developing countries, identification of blood-based DNA methylation alterations would greatly facilitate biomarker discovery for T2D screening. This, together with the chemical and biological stability of DNA methylation signatures, mark them as attractive and feasible prognostic/diagnostic tools.

Despite the exponential rise in epigenetic research over the last decade, the current status of DNA methylation alterations in blood from human T2D subjects still remains limited. Indeed, most recent reviews have focused on DNA methylation signatures in pancreatic islets and peripheral tissues (liver, skeletal muscle, and adipose tissue), which are not feasible tissue sources for biomarker generation. The aim of this review is to summarize, discuss, and integrate the most recent available evidence of the potential of blood-based DNA methylation alterations as candidate biomarkers for T2D prevention and treatment.

Four databases, Scopus, PubMed, Cochrane Central, and Google Scholar, were searched to identify published studies reporting DNA methylation changes in blood between January 2008 to July 2018. The following keywords: "DNA methylation," AND "blood," OR "peripheral blood," OR "peripheral blood mononuclear cells," OR "peripheral blood leukocytes" OR "peripheral blood lymphocytes" OR "white blood cells" AND "type 2 diabetes" AND "human" were used. Studies were considered eligible if they were original articles, investigated

Abbreviations: T2D, Type 2 diabetes; FFAs, free fatty acids; 5mC, 5-methylcytosine; CpG, Cytosine-guanine dinucleotides; GWAS, genome-wide association studies; ELISA, enzyme-linked immunosorbent assay; HDL, high-density lipoprotein; NOS3, nitric oxide synthase 3; LINE-1, long interspersed nuclear element-1; LDL, low-density lipoprotein; Alu, Arthrobacter luteus; MeDIP, methylated DNA immunoprecipitation; FTO, obesity-associated protein; PPARγ, peroxisome proliferator–activated receptor gamma; PDK4, pyruvate dehydrogenase lipoamide kinase isozyme 4; GCK, glucokinase; PRCKZ, protein kinase C zeta; GIPR, gastric inhibitory polypeptide receptor; IGFNP-7, insulin-like growth factor-binding protein 7; PTPN1, protein tyrosine phosphatase, non-receptor type 1; MCP-1, monocyte chemoattractant protein-1; SLC30A8, solute carrier family 30 member 8; BCL11A, B-cell lymphoma/leukemia 11A; TCF7L2, transcription factor 7-like 2; CALM2, calmodulin 2; CRY2, CRY2 cryptochrome circadian regulator 2; CMAK1D, Ca2+/calmodulin-dependent protein kinase 1 subfamily of serine/threonine kinases; TLR2, toll-like receptor 2; TLR3, toll-like receptor 3; FFAR3, free fatty acid receptor 3; PI3K, phosphatidylinositol 3-kinase; GLUT4, glucose transporter type 4; SNP, single nucleotide polymorphisms; HOMA-IR, homeostatic model assessment for insulin resistance; ROS, reactive oxygen species; GI, gastrointestinal; BMI, body mass index; SCFA, short chain fatty acids; FFARs, free fatty acid receptors; OGTTs, oral glucose tolerance tests; KCNQ1, potassium voltage-gated channel subfamily KQT member 1; THADA, thyroid adenoma associated protein; JAZF1, juxtaposed with another zinc finger protein 1; TXNIP, thioredoxin-interacting protein; ABCG1, ATP-binding cassette sub-family G member 1; PHOSPHO1, phosphoethanolamine/phosphocholine phosphatase 1; SOCS3, suppressor of cytokine signaling 3; SREBF1, sterol regulatory element-binding transcription factor 1; HbA1c, hemoglobin A1c; VEGF, vascular endothelial growth factor; DQX1, DEAQ-box RNA dependent ATPase 1; SAMD12, Sterile Alpha Motif Domain Containing 12; MALT1, mucosa-associated lymphoid tissue lymphoma translocation protein 1; GPR6, G-protein receptor 6; TG, triglyceride; BCL3, B-Cell CLL/Lymphoma 3; IL23A, Interleukin 23 Subunit Alpha; F2RL1, F2R Like Trypsin Receptor 1; S100A12, S100 Calcium Binding Protein A12; TNFRSF10B, TNF Receptor Superfamily Member 10b; NEK6, NIMA Related Kinase 6; RNF31, Ring Finger Protein 31; SLC35B2, Solute Carrier Family 35 Member B2; IRAK1BP1, Interleukin 1 Receptor Associated Kinase 1 Binding Protein 1.

DNA methylation patterns in relationship with T2D and if the study was published in English. Reference lists of included studies were also hand-searched to identify other potentially eligible studies. Both cross-sectional, case control, and longitudinal studies that provided sufficient information were included. The study methods included global DNA methylation studies, candidate-gene methylation studies, and genome-wide association studies (GWAS).

GLOBAL DNA METHYLATION STUDIES

Global DNA methylation, referred to as the total methylation status that occurs across the genome, has been reported to be one of the earliest molecular changes in the transition of a cell from a normal to a diseased state (25). Technological advances have resulted in an increase in global DNA methylation studies. Current methods to quantify global DNA methylation include enzyme-linked immunosorbent assays (ELISA), methylation-sensitive restriction enzymes, liquid chromatography coupled with mass spectrometry, flow cytometry, and quantification of DNA methylation within repetitive elements using bisulfite pyrosequencing (26). Global DNA methylation studies are advanced in cancer research, with a blood-based candidate diagnostic biomarker for colorectal cancer already commercially available (27). In recent years, global DNA has attracted considerable interest as a biomarker for T2D. Studies that have quantified global DNA methylation in peripheral blood of T2D subjects are summarized in **Table 1**.

Luttmer et al. quantified global DNA methylation levels in peripheral blood leukocytes of 738 individuals from the Netherlands Hoorn Study cohort and reported a progressive decrease in global DNA methylation in individuals with T2D compared to those with impaired glucose tolerance and normoglycaemia. Moreover, DNA hypomethylation in these subjects was independently associated with hyperglycaemia and high-density lipoprotein (HDL) cholesterol (28). In contrast, a Colombian study using a smaller patient group, observed a global increase in DNA methylation in 44 subjects with T2D compared to 35 healthy controls, which correlated with the percentage of glycated hemoglobin A1c (HbA1c) (29). Similar findings were reported by Matsha et al. using a South African population consisting of 158 individuals with T2D, 119 with dysglycaemia, and 287 healthy controls. They showed that levels of global DNA methylation were higher in individuals with impaired glucose tolerance or treatment-naïve T2D compared to those with normoglycaemia (29, 30). Interestingly, no difference in global DNA methylation was observed between diabetic individuals on treatment and normoglycaemic subjects, prompting the authors to speculate that glucose management caused the reversal of aberrant DNA methylation patterns during T2D (30).

An innovative study by Simar et al. measured global DNA methylation levels of different subtypes of peripheral blood mononuclear cells in obese and T2D individuals, using a flow cytometry bead-based method. They reported that global DNA methylation levels were increased in B cells from obese individuals and subjects with T2D, and in natural killer lymphocytes from patients with T2D, while no overall difference was observed in the mixed population of blood mononuclear cells from these individuals. DNA methylation in B cells and natural killer lymphocytes correlated positively with insulin resistance, suggesting an association between DNA methylation alterations, immune function, and metabolic disorders (31). These findings highlight the importance for not only tissue specific but also cell type specific epigenetic studies, which may improve sensitivity and specificity.

Repetitive elements, such as *long interspersed nuclear element-1* (*LINE-1*) are highly represented throughout the genome and as such, methylation of these elements is generally considered to correlate with global genomic DNA methylation (38). Martin-Núñez (33) demonstrated that *LINE-1* methylation was decreased in peripheral blood from a small Spanish group of 12 individuals with T2D compared to 12 normoglycaemic individuals (33). Conversely, a study using 228 non-diabetic individuals reported that *LINE-1* DNA hypermethylation is associated with increasing fasting glucose, total cholesterol, triglycerides, low-density lipoprotein (LDL) cholesterol, and risk of developing T2D (34). These findings were later supported by Wu and colleagues who reported a significant increase in *LINE-1* methylation in a group of 205 Chinese patients with T2D compared to 213 healthy controls (35). Using a similar approach, Zhao and colleagues assessed methylation of *Arthrobacter luteus* (*Alu*) elements as a proxy for global DNA methylation by performing quantitative bisulfite pyrosequencing. They found that hypermethylation of these elements in peripheral blood leukocytes from 84 monozygotic twin pairs discordant for T2D was significantly associated with insulin resistance (36). More recently, *Alu* methylation levels were also investigated in white blood cells from 85 individuals with T2D, 113 with impaired glucose tolerance, and 42 healthy control subjects, using *Alu*-Combined Bisulfite Restriction Analysis (COBRA). Interestingly, in contrast to the findings of Zhao et al. (36), they reported that individuals with T2D exhibited the lowest *Alu* methylation compared to controls, which directly correlated with higher fasting blood glucose and HbA1c concentrations and high blood pressure (37). Taken together, the results from global DNA methylation analysis are mostly inconsistent and more studies are needed to consolidate the findings on the association between blood-based global DNA methylation and T2D.

CANDIDATE GENE STUDIES

Despite being a robust measure of overall genomic methylation, global DNA does not have the resolution to measure methylation within specific genes (39). Thus, a candidate gene approach to quantify the methylation status of specific CpG sites within genes associated with T2D are increasingly being investigated (40). Methods used in these studies include methylated DNA immunoprecipitation (MeDIP), methylation specific PCR, mass spectrometry combined with RNA base-specific cleavage, as well as bisulfite pyrosequencing (41). Genes investigated include *fat mass and obesity-associated protein* (*FTO*), *peroxisome proliferator–activated receptor gamma* (*PPARγ*), *pyruvate*

TABLE 1 | Main findings from T2D studies investigating global DNA methylation in blood.

Author (year)	Country	Sample size	Gender	Biological source	Method	Study outcome
Luttmer et al. (28)	Netherlands	IGT = 172 T2D = 286 Controls = 280	M and F	PBL	5 mC/C ratio measurement by LCMS	Global DNA hypomethylation in IGT and individuals with T2D compared to control subjects. Methylation negatively associated with fasting blood glucose concentrations and positively associated with HDL.
Pinzon-Cortes et al. (29)	Colombia	T2D = 44 Controls = 35	Unknown	PB	5 mC measurement using colorimetric methylated DNA quantification	Global hypermethylation in patients with T2D compared to controls.
Matsha et al. (30)	South Africa	IGT = 119 T2D = 158 Controls = 287	M and F	PBMCs	5 mC measurement using Imprint DNA methylation ELISA	Global hypermethylation in pre-diabetic and treatment naïve T2D individuals compared to controls while no significant difference observed in global DNA methylation between individuals with T2D on treatment and those with normoglycaemia. NOS3 G894T polymorphism an independent determinant of global DNA methylation.
Simar et al. (31)	Denmark	T2D = 12 Obese = 14 Controls = 7/11	M	PBMCs/monocytes, lymphocytes/T cells	5 mC measurement using bead-based flow cytometry	Increased global DNA methylation levels in B cells from obese and T2D subjects and in natural killer lymphocytes from T2D patients. No overall association between PBMC methylation levels and T2D/obesity.
Zhang et al. (32)	China	T2D = 75 Controls = 29	M and F	PB	5 mC measurement using HPLC	No association between DNA methylation and T2D between groups.
Martin-Nunez et al. (33)	Spain	T2D = 12 Controls = 12	M	PB	LINE-1 measurement using pyrosequencing	LINE-1 DNA methylation inversely correlated with T2D risk.
Pearce et al. (34)	England	228 non-diabetic	M and F	PB	LINE-1 measurement using pyrosequencing	Increased methylation associated with increasing fasting glucose concentrations, total cholesterol, total triglycerides, and LDL cholesterol. No differences in LINE-1 methylation between M and F.
Wu et al. (35)	China	T2D = 205 Controls = 213	M and F	PBL	Quantitative methylation-specific PCR	LINE-1 DNA methylation positively correlated with T2D risk.
Zhao et al. (36)	Vietnam	84 monozygotic twin pairs, 11.4% diabetic	M	PBL	*Alu* repetitive elements using pyrosequencing	Global *Alu* hypermethylation positively associated with insulin resistance.
Thongsroy et al. (37)	Thailand	IGT = 113 T2D = 85 Controls = 42	M and F	WBC	*Alu* repetitive elements using COBRA	Global *Alu* hypomethylation positively associated with high fasting blood glucose, HbA1c and high blood pressure.

Alu, Arthrobacter luteus; COBRA, ALU-Combined Bisulfite Restriction Analysis; ELISA, enzyme-linked immunosorbent assay; HbA1c, glycated hemoglobin A1c; HDL, High-density lipoprotein; HPLC, high-performance liquid chromatography; IGT, impaired glucose tolerance; LCMS, Liquid chromatography mass spectrometry; LDL, Low-density lipoprotein; LINE-1, Long interspersed nuclear element-1; NOS3, nitric oxide synthase 3; PB, Peripheral blood; PBL, Peripheral blood leukocytes; PBMCs, Peripheral blood mononuclear cells; T2D, Type 2 Diabetes; WBC, White blood cells; 5 mC, 5 methyl cytosine.

dehydrogenase lipoamide kinase isozyme 4 (*PDK4*), *transcription factor 7-like 2* (*TCF7L2*), *monocyte chemoattractant protein-1* (*MCP-1*), *glucokinase* (*GCK*), *protein kinase C zeta* (*PRCKZ*), *B-cell lymphoma/leukemia 11A* (*BCL11A*), *gastric inhibitory polypeptide receptor* (*GIPR*), *solute carrier family 30 member 8* (*SLC30A8*), *insulin-like growth factor-binding protein 7* (*IGFNP-7*), *protein tyrosine phosphatase, non-receptor type 1* (*PTPN1*), *calmodulin 2* (*CALM2*), *CRY2 cryptochrome circadian regulator 2* (*CRY2*), *Ca2+/calmodulin-dependent protein kinase 1 subfamily of serine/threonine kinases* (*CAMK1D*), *toll-like receptor* (*TLR*) *2,* and *4*, and *free fatty acid receptor 3* (*FFAR3*), which are discussed in further detail below and summarized in **Table 2**

Genes Involved in Glucose and Lipid Metabolism

The *FTO* gene encodes a 2-oxoglutarate-dependent nucleic acid demethylase and various studies have reported that variants in the *FTO* locus are strongly linked with obesity and can predict risk of T2D and cardiovascular disease (53–57). The methylation status of *FTO* was analyzed by van Otterdijk et al. who identified hypermethylation of one CpG locus in the promoter region in peripheral blood leukocytes from 25 individuals with T2D compared to 11 control healthy subjects (40). The same study also identified hyper- and hypomethylation of CpG sites in the promoters of the *PPARγ* and *PDK4* genes, respectively, in patients with T2D vs. healthy controls. PPARγ is a transcription factor that plays major roles in adipogenesis and insulin sensitivity, and agonists are currently being used as anti-diabetic agents (58). PDK4 is reported to play a role in the regulation of glucose metabolism and mitochondrial function, and hypomethylation of this gene has also been reported in skeletal muscle of diabetic patients compared to controls (59). TCF7L2 is involved in glucose homeostasis and was reported to be differentially methylated in 13 of its promoter CpGs (eight hypermethylated and five hypomethylated) between treatment-naïve patients with T2D and matched controls (42). Furthermore, methylation at specific CpG sites of the *TCF7L2* promoter correlated significantly with fasting glucose concentrations, total cholesterol, LDL-cholesterol, as well as the homeostatic model assessment for insulin resistance (HOMA-IR) (47).

MCP-1 is a chemokine that regulates macrophage migration and infiltration into adipose tissue and in this way, contributes to insulin resistance and decreased glucose uptake during obesity and T2D (60). Interestingly, hypomethylation of the *MCP-1* promoter associated with increased serum MCP-1 levels, HbA1c, and fasting blood glucose levels in patients with T2D compared to healthy controls (43). The *GCK* gene encodes glucokinase, a key glycolytic enzyme that catalyzes the first step in hepatic and pancreatic islet glucose utilization pathways (61). Tang et al. (44) evaluated *GCK* methylation in T2D subjects and matched controls, and identified significant hypermethylation of one intragenic CpG site exclusively in male patients with T2D compared to healthy controls, which also correlated with total cholesterol levels. The results for this association indicate an interaction between gender and T2D-associated methylation alterations.

Genes Involved in Insulin Secretion and Function

PRKCZ, a member of the PKC family of serine/threonine kinases, functions downstream of phosphatidylinositol 3-kinase (PI3K) to positively regulate the insulin signaling pathway and contributes to the translocation of glucose transporter type 4 (GLUT4) from the cytoplasm to the membrane, where it facilitates glucose uptake (62). A comparison between the *PRKCZ* promoter sequence in peripheral blood leukocytes from Chinese individuals with either T2D or normoglycaemia showed that seven CpG sites were methylated in the T2D group whereas only one CpG site was methylated in the control group (45). Furthermore, the protein expression levels of PRKCZ in the serum of the group with T2D was significantly reduced compared to the control group, suggesting that *PRKCZ* promoter activity and gene expression are regulated by methylation (45). The *BCL11A* gene, encoding a CH2H2 type zinc-finger transcription factor, has also been associated with T2D risk. BCL11A plays a normal physiological role in lymphocyte production but variants of this gene have been shown to affect insulin response to glucose, as well glucagon secretion (63, 64). Tang et al. investigated the correlation between *BCL11A* methylation at one intragenic and four promoter CpG sites and T2D risk (46). They found a significant decrease in the mean DNA methylation levels across these CpG sites in males with T2D compared to normoglycaemic controls (46). Interestingly, these differences were not observed in females.

The *GIPR* gene encodes a receptor of the incretin, GIP, a gastrointestinal hormone that stimulates insulin response after ingesting food. Canivell et al. performed DNA methylation profiling of the *GIPR* promoter in peripheral blood DNA and identified differential methylation at nine CpG sites located upstream of the first exon between patients with T2D and controls. On average, these nine CpG sites were hypomethylated in patients with T2D and significantly correlated with waist circumference and fasting glucose concentrations (47). *SLC30A8* encodes a pancreas-specific, zinc efflux transporter, and reduced levels or activity of SLC30A8 hinder glucose-induced insulin secretion, as zinc is required for the crystallization of insulin within secretory granules (65). Seman et al. analyzed DNA methylation alterations in the *SLC30A8* promoter in peripheral blood from a large Malay population and identified hypermethylation of five CpG sites in patients with T2D compared to controls (48).

Another insulin associated gene that has been linked to T2D is *IGFBP-7*, a member of the insulin growth factor binding family. The expression of IGFBP-7 was previously found to be increased in the serum of subjects with T2D compared to controls, and significantly associated with insulin resistance (49, 66, 67). Gu et al. studied the correlation between *IGFBP-7* promoter methylation and T2D in peripheral blood from a large Swedish cohort of subjects. They identified increased *IGFBP-7* methylation in three CpG sites in newly diagnosed men with T2D, but not in women, compared to non-diabetic individuals (49). Interestingly, no significant differences in serum IGFBP-7 expression were observed between groups (49). These results are conflicting with previous studies that reported increased serum

TABLE 2 | Main findings from T2D studies investigating candidate gene methylation in blood.

Author (year)	Genes investigated	Country	Sample size	Gender	Tissue type	Method	Study outcome
van Otterdijk et al. (40)	*KCNJ11*, *PPARγ*, *PDK4*, *KCNQ1*, *SCD1*, *PDX1*, *FTO* and *PEG3*	Germany	T2D = 25 Controls = 11	M and F	PBL	Bisulphite pyrosequencing	Hypermethylation of *FTO*, hypermethylation of *PPARγ*, and hypomethylation *PDK4* associated with metabolic syndrome, T2D and both metabolic syndrome and T2D, respectively.
Canivell et al. (42)	*TCF7L2* (22 CpGs)	Spain	T2D = 93 Controls = 93	M and F	WB	LCMS and RNA base-specific cleavage	Hypermethylaion of 8 CpGs and hypomethylation of five CpGs were observed in T2D patients compared to controls. Differential methylation of CpGs at −382, +5, +96, and +186 (relative to ATG) associated with fasting glucose and CpG at +137 associated with total cholesterol and LDL-cholesterol.
Liu et al. (43)	*MCP-1*	China	T2D = 32 Controls = 15	M and F	PBMCs	Methylation specific PCR	Hypomethylation of *MCP-1* in T2D patients compared to controls.
Tang et al. (44)	*GCK* (4 CpGs)	China	T2D = 48 Controls = 48	M and F	PB	Bisulfite pyrosequencing	Hypermethylation of one CpG site in *GCK* in T2D subjects compared to controls. Association specific to males.
Zou et al. (45)	*PRKCZ* (9 CpGs)	China	T2D = 152 Controls = 120	M and F	PBL	Bisulfite pyrosequencing	Hypermethylation of seven CpG sites in T2D patients compared to controls.
Tang et al. (46)	*BCL11A* (5 CpGs)	China	T2D = 48 Controls = 48	M and F	PB	Bisulfite pyrosequencing	Significant association between mean DNA hypomethylation of *BCL11A* CpGs and T2D in males but not females.
Canivell et al. (47)	*GIPR*	Spain	T2D = 93 Controls = 93	M and F	WB	LCMS and RNA base-specific cleavage	Hypomethylation of *GIPR* promoter associated with increased fasting blood glucose levels and HOMA-IR.
Seman et al. (48)	*SLC30A8* (6 CpGs)	Malaysia	T2D = 509 Controls = 441	M and F	PB	Bisulfite pyrosequencing	Hypermethylation at five CpGs in T2D subjects compared to controls. Combined methylation scores of all 6 CpGs significantly increased in T2D subjects compared to controls.
Gu et al. (49)	*IGFBP-7* (3 CpGs)	Sweden	T2D TN = 100 T2D T = 140 Controls = 100	M and F	PB	Bisulfite-pyrosequencing	Hypermethylation of three CpG sites observed in newly diagnosed, treatment naïve T2D patients compared to controls. Combined methylation scores from all three CpGs showed increased genomic methylation levels in T2D compared to normoglycaemic controls.
Huang et al. (50)	*PTPN1* (8 CpGs)	China	T2D = 97 Controls = 97	M and F	PBMCs	Bisulfite-pyrosequencing	Hypermethylation of all eight CpGs correlated with T2D risk and inversely associated with low-density lipoprotein and total cholesterol in females.
Cheng et al. (51)	*CAMK1D* (9 CpGs), *CRY2* (5 CpGs), *CALM2* (4 CpGs)	China	T2D = 48 Controls = 48	M and F	PB	Bisulfite pyrosequencing	Hypomethylation in promoters of all three genes observed in T2D subjects compared to controls.
Remely et al. (52)	*TLR2* (7 CpGs), *TLR4* (4 CpGs)	Austria	T2D = 24 Obese = 14 Controls = 18	M and F	WB	Bisulfite pyrosequencing	Mean methylation of all four CpGs in the first exon of *TLR4* were significantly reduced in obese subjects compared to T2D subjects, while no differences in mean methylation were observed between T2D subjects and lean controls. Reduced methylation of seven CpGs in the *TLR2* promoter observed in T2D vs. lean group, while no differences observed between obese group and lean controls.
Remely et al. (52)	*FFAR3*	Austria	T2D = 24 Obese = 14 Controls = 18	M and F	WB	Bisulfite pyrosequencing	Significantly reduced methylation in T2D subjects compared to controls.

BCL11A, B-cell lymphoma/leukemia 11A; CALM2, calmodulin 2; CAMK1D, Ca2+/calmodulin-dependent protein kinase 1 subfamily of serine/threonine kinases; CRY2, CRY2 cryptochrome circadian regulator 2, FFAR3, free fatty acid receptor 3; FTO, fat mass and obesity-associated protein; GCK, glucokinase; GIPR, gastric inhibitory polypeptide receptor; HOMA-IR, homeostatic model assessment-insulin resistance; IGFNP-7, insulin-like growth factor-binding protein 7; IGT, impaired glucose tolerance; LCMS, Liquid chromatography mass spectrometry; MCP-1, monocyte chemoattractant protein-1; PB, Peripheral blood; PBL, Peripheral blood leukocytes; PBMCs, Peripheral blood mononuclear cells; PDK4, pyruvate dehydrogenase lipoamide kinase isozyme 4; PPARγ, peroxisome proliferator–activated receptor gamma, PRCKZ, protein kinase C zeta; PTPN1, protein tyrosine phosphatase, non-receptor type 1; SLC30A8, solute carrier family 30 member 8; TCF7L2, transcription factor 7-like 2; TLR2, toll-like receptor 2, TLR4, toll-like receptor 4. T2D, Type 2 Diabetes; WB, Whole blood.

IGFBP-7 concentrations in T2D subjects compared to healthy individuals. The discrepancies in these results may be due to the use of a much larger sample size in the study by Gu et al. (140 T2D subjects and 100 controls), as well as differences in the selection criteria of the patients with T2D between studies (49, 66, 67). While Gu et al. (49) studied newly diagnosed, treatment naïve T2D subjects, the previous studies included participants on chronic pharmacological therapies, including insulin, oral hypoglycaemic agents, statins, fibrates, blood pressure–lowering agents, and aspirin (49, 66, 67).

More recently, Huang et al. investigated the methylation status of another key regulator of the insulin signaling pathway, *PTPN1*, in relation to T2D susceptibility (50). *PTPN1* encodes the protein-tyrosine phosphatase 1B protein, which attenuates the insulin signaling pathway by decreasing the phosphorylation of the insulin receptor and/or insulin receptor substrate 1 (68). In the study, DNA methylation of the *PTPN1* promoter region was quantified in peripheral blood mononuclear cells from 97 Chinese patients with T2D and 97 age- and gender-matched healthy controls, using bisulfite pyrosequencing. The results revealed a significant correlation between *PTPN1* promoter methylation and increased T2D risk in females, but not in males. Furthermore, *PTPN1* methylation was also inversely associated with low-density lipoprotein and total cholesterol levels in females. These results indicate that *PTPN1* promoter hypermethylation is a risk factor for T2D in the female Chinese population.

Genes Associated With Pancreatic and Cardiovascular Function

Cheng et al. investigated DNA methylation in the promoters of *CALM2*, *CRY2,* and *CAMK1D*, based on previous reports linking variants of these genes with T2D susceptibility (51). They demonstrated that four, five, and nine CpGs within the *CALM2*, *CRY2,* and *CAMK1D* gene promoters, respectively, were significantly hypomethylated in the peripheral blood of subjects with T2D compared to healthy controls. CRY2 plays a role in circadian rhythm which, when desynchronized, results in metabolic disturbances including increased insulin and postprandial glucose levels, increased arterial blood pressure, and decreased leptin levels, which may predispose individuals to T2D (69). Variants in the *CALM2* gene, a member of the calmodulin family, have been associated with dialysis survival in T2D-associated renal disease, as well as arrhythmia susceptibility in infants (70). CAMK1D plays a key role in granulocyte function and reactive oxygen species (ROS) inhibition through the chemokine signal transduction pathway, and consequently, non-functional variants or hypomethylation of this gene may result in apoptosis and consequently, reduced β-cell mass (71).

Genes Associated With Gut Microbiota

Remely et al. investigated DNA methylation of two genes involved in innate immunity and inflammation, *TLR 2,* and *4*, in response to changes in gut microbiota in individuals with T2D (72–74). This investigation was prompted by a spate of recent studies to show that changes in gut microbiota composition can lead to chronic low-grade inflammation, metabolic dysregulation, and T2D (75, 76). Remely et al. investigated three groups of subjects: patients with T2D using glucagon-like peptide-1 (GLP-1) agonist therapy, obese individuals without established insulin resistance, and a normal-weight control group. The authors identified four significantly hypomethylated CpGs in the first exon of *TLR4* in obese individuals compared to healthy controls, while methylation of seven CpGs in the promoter region of *TLR2* was significantly lower in subjects with T2D compared to obese subjects and normal-weight controls, which correlated with body mass index (BMI) (52). Furthermore, distinct changes in gut microbiota composition were observed between the three groups, the most significant being a high abundance of lactic acid bacteria in individuals with T2D.

Gut microbiota contribute to energy metabolism through the production of short chain fatty acids (SCFA) during fermentation in the colon. SCFAs are believed to alter DNA methylation patterns of genes involved in inflammatory reactions, including genes encoding free fatty acid receptors (FFARs) (77, 78). Based on this as well as their previous findings, a follow up study conducted by Remely et al. investigated the effect of gut microbiota and SCFA production on DNA methylation of *FFAR3* in blood from the same cohort described previously (52). Their results showed differential composition of gut microbiota in the T2D and obese subjects, and significantly higher methylation in five CpGs in the *FFAR3* promoter region in normal-weight controls compared to obese subjects with the lowest methylation in subjects with T2D (77). Taken together, these two studies provide evidence that differential composition of gut microbiota in obesity and T2D is associated with epigenetic gene regulation. The authors thus proposed that improvements in diet targeted to restore gut microbial balance may ameliorate aberrant epigenetics and be effective as a preventative treatment for metabolic syndrome (52).

GENOME-WIDE ASSOCIATION STUDIES

With technological advances, the focus of epigenetics studies has shifted from candidate regions to high throughput, genome wide association studies (GWAS). In the past few years, as a result of a widespread use of techniques, including the Infinium Beadchip Arrays and methylation pull-down sequencing assays, major insights into DNA methylation changes associated with T2D have been obtained, which are summarized in **Table 3**.

Microarray-based Methylation Assays

Microarray-based methylation assays use the ratio between hybridization intensities of DNA samples before and after digestion with a cocktail of methyl-sensitive restriction enzymes to generate quantitative methylation scores (91). This technique was used in one of the first GWAS to compare T2D-associated genome-wide methylation alterations in human blood. Toperoff et al. assessed pooled, peripheral blood DNA methylation in a Jewish cohort of 710 T2D and 459 control subjects (79). Their analysis covered 1 461 753 DNA genomic fragments containing 3,359,645 CpG methylation sites and results showed that differentially methylated sites were enriched in genomic regions that had previously been associated with T2D. The most

TABLE 3 | Main findings from T2D studies investigating genome-wide DNA methylation in human population-based studies.

Author (year)	Population	Sample size	Gender	Tissue type	Method	Study outcome
Toperoff et al. (79)	Jewish	T2D = 710 Controls = 459	M and F	WB	Microarray-based methylation assays	Differential methylation identified in 13 CpGs, mapping to *SLC30A8*, *TCF7L2*, *KCNQ1*, *FTO*, *THADA,* and *JAZF1* genes in T2D subjects compared to controls.
Chambers et al. (80)	Indian Asian and European	Indian Asian: T2D = 1,608 Controls = 11 927 European: T2D = 306 Controls = 6,760	M and F	PB	450 K	Differential methylation identified in five regions mapping to *TCF7L2*, *FTO*, *KCNQ1*, *TXNIP ABCG1*, *PHOSPHO1*, *SOCS3,* and *SREBF1* genes, replicated in two cohorts.
Dayeh et al. (81)	European	T2D = 19 Controls = 19	M and F	WB	450 K	*ABCG1*, *PHOSPHO1* associated with future T2D risk but not *SOCS3*, *SREBF1* or *TXNIP. ABCG1* hypermethylation positively associated with HbA1c and fasting insulin levels.
Kriebel et al. (82)	German	1,448 non-diabetic (FBG and HbA1c) 1,440 non-diabetic (FI and HOMA-IR) 617 non-diabetic (2-h insulin)	M and F	WB	450 K	DNA methylation at cg06500161 (*ABCG1*) associated with fasting glucose, fasting insulin, and HOMA-IR.
Hidalgo et al. (83)	American	[a]Healthy individuals = 544 [b]Healthy individuals = 293	M and F	WB	450 K	*ABCG1* hypermethylation associated with fasting insulin and HOMA-IR.
Walaszczyk et al. (84)	Dutch	T2D = 100 Controls = 100	M and F	WB	450 K	Differential methylation of *ABCG1*, *LOXL2*, *TXNIP*, *SLC1A5,* and *SREBF1* associated with T2D.
Muftah et al. (19)	[a]Arab, [b]Caucasian	[a]T2D = 30 Controls = 93 [b]180 twins from TwinsUK cohort	M and F	WB	450 K	Differential methylation identified in *TXNIP* and *DQX1* genes in T2D subjects compared to controls.
Kulkarni et al. (85)	Mexican-American	T2D = 174 Controls = 676	M and F	PB	450 K	*TXNIP*, *ABCG1*, *SAMD12* associated with T2D, FBG, and HOMA-IR.
Soriano-Tarraga et al. (86)	Caucasian, (Spain)	[a]T2D = 151 Controls = 204 [b]T2D = 59 (BISMAR cohort) Controls = 108 [b]T2D = 63 (REGICOR cohort) Controls = 582	M and F	WB	450 K	One differentially methylated region in the *TXNIP* gene, replicated in 2 independent cohorts.
Florath et al. (87)	German	[a]T2D = 154 Controls = 835 [b]T2D = 87 Controls = 527	M and F	WB	450 K	Differential methylation of *TXNIP* associated with T2D in discovery and replication cohorts.
Jeon et al. (88)	Korean	[a]High-glucose group- 8 T2D = 5 Controls = 13 [b]T2D = 220 Controls = 220	M and F	PB	[a]450 K [b]Bisulfite Pyrosequencing	[a]MSI2 hypomethylated by 11% in T2D cases and 7% in high glucose cases (p-value = 0.038). CXXC4 hypomethylated by 15% in T2D cases (p-value = 0.044), and 12.8% in high glucose cases (p-value = 0.033). [b]MSI2 hypomethylation significantly correlated with T2D.
Yuan et al. (89)	European	[a]T2D = 23 Controls = 31 [b]T2D = 42 Controls = 221	M and F	WB	[a]MeDIP-seq and [b]450 K	Two DMS within a 2 kb region upstream of the transcriptional start site of the *MALT1* gene on T2D subjects compared to controls.

(Continued)

TABLE 3 | Continued

Author (year)	Population	Sample size	Gender	Tissue type	Method	Study outcome
Matsha et al. (90)	South African, mixed ancestry.	T2D = 3 Prediabetes = 3 Controls = 3	F	PB	MeDIP-seq	1,415 DMS in the promoter regions of T2D subjects compared to normoglycaemic controls. Genes associated with cell surface signaling, glucose transport, insulin signaling, pancreas development, and the immune system.
Pheiffer et al. (11)	South African, mixed ancestry.	T2D = 3 Prediabetes = 3 Controls = 3	F	PB	MeDIP-seq	3,081 DMS in T2D and prediabetic subjects occurred within non-promoter regions, including sites encoding miRNAs.

[a]Discovery cohort; [b]Validation cohort; DMS, differentially methylated sites; F, female; FBG, fasting blood glucose; FI, fasting insulin; HbA1c, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment-insulin resistance; M, male; MeDIP-seq, Methylated DNA immunoprecipitation sequencing; PB, Peripheral blood; T2D, Type 2 Diabetes; WB, Whole blood; 450 K, Infinium Human-Methylation450 BeadChip.

significant methylation differences between T2D and control subjects mapped to the *SLC30A8*, *TCF7L2*, *FTO*, *potassium voltage-gated channel subfamily KQT member 1* (*KCNQ1*), *thyroid adenoma associated protein* (*THADA*), and jux*taposed with another zinc finger protein 1* (*JAZF1*) genes (79). The authors validated these methylation changes using bisulfite sequencing, which also revealed that hypomethylation of a CpG site in the first intron of the *FTO* gene, was significantly associated with T2D risk. Furthermore, these findings were reproduced by the same group in an independent population cohort (Jerusalem LRC longitudinal Study) of young individuals who later developed T2D, indicating that hypomethylation of specific genomic sites may be an early risk factor that predisposes individuals to T2D later in life.

Beadchip Arrays

Bead array-based DNA methylation analysis is designed to provide single-base resolution and quantitative evaluation of specific cytosines in multiple samples (92). The Infinium HumanMethylation BeadChip was developed by Illumina and interrogates over 485,000 methylation sites and covers 96% of CpG islands, as well as additional island shores (i e., regions flanking 2 kb of CpG islands) (93). Using the HumanMethylation450 BeadChip, Chambers et al. investigated T2D-associated DNA methylation alterations in peripheral blood from 2,664 Indian Asians and replicated the study findings in 1,141 Europeans (80). Differentially methylated CpG sites were identified within 853 genes in individuals with T2D, including known T2D-associated loci, *TCF7L2*, *FTO,* and *KCNQ1*. The authors also found that CpG sites in *thioredoxin-interacting protein* (*TXNIP*), *ATP-binding cassette sub-family G member 1* (*ABCG1*), *phosphoethanolamine/phosphocholine phosphatase 1* (*PHOSPHO1*), *suppressor of cytokine signaling 3* (*SOCS3*), and *sterol regulatory element-binding transcription factor 1* (*SREBF1*) were significantly associated with the future development of T2D (80). In addition, the combined methylation scores for these five loci were associated with future T2D incidence independently of the established T2D risk factors—family history of T2D, physical activity, BMI, waist:hip ratio, HbA1c, and glucose and insulin concentrations.

The loci identified by the Chambers study were later evaluated in an independent cohort (the Botnia prospective study) by Dayeh et al. (81), who confirmed an association between *ABCG1* and *PHOSPHO1* methylation in whole blood and future T2D risk but not *SOCS3*, *SREBF1*, or *TXNIP*. They found that *ABCG1* hypermethylation positively associated with HbA1c and fasting insulin levels. Furthermore, the methylation status of *ABCG1* could be replicated in the blood of diabetic twins compared to their non-diabetic counterparts (81). The association between *ABCG1* hypermethylation and fasting blood glucose and insulin levels has also been reported in three other GWAS studies (82–85). The *ABCG1* gene encodes a protein involved in cholesterol transport (94). Since cholesterol abnormalities is a hallmark of T2D, it is tempting to speculate that hypermethylation of this gene may modulate circulating cholesterol levels, and thus have an impact on initiation and progression of T2D, as well as T2D-associated cardiovascular complications (94).

Dayeh et al. also found that DNA hypermethylation at the *PHOSPHO1* locus positively correlated with HDL and associated with decreased T2D risk (81). The *PHOSPHO1* gene encodes a hydrolase enzyme and is involved in skeletal and vascular mineralization (95, 96). Cardiovascular calcification is a common consequence of aging, diabetes and hypercholesterolemia, and PHOSPHO1 has thus been marked as an attractive target for cardiovascular therapy (96). The observation that *PHOSPHO1* hypermethylation correlated with HDL and reduced T2D risk provides additional evidence for its candidacy as a diagnostic marker for T2D–associated CVD complications.

Muftah et al. investigated DNA methylation patterns in the whole blood of 123 subjects from an Arab cohort and replicated eight known CpG associations with T2D/BMI identified in Caucasians, including an association of *TXNIP* hypomethylation with T2D (reported by the Chambers study) (19, 80). Hypomethylation of *TXNIP* in T2D subjects has since been reported in four additional studies using HumanMethylation450 BeadChip arrays (84–87). Interestingly, *TXNIP* expression is induced by glucose, as a result of a carbohydrate response element in its promoter and *TXNIP* overexpression has been reported in both diabetic animals and humans (97). Furthermore, *TXNIP* has been linked to vascular complications through its ability to modulate angiogenesis by repressing vascular endothelial growth factor (VEGF) (97). Muftah et al. also identified a significant association between methylation at a novel CpG site within the *DEAQ-box RNA dependent ATPase* 1 (*DQX1*) gene and T2D in both the Arab and Caucasian cohorts (19). *DQX1* encodes an RNA-dependent ATPase, which is highly expressed in the liver and muscle, however its role in T2D remains to be elucidated (98).

A family-based study by Kulkarni et al. analyzed the association of DNA methylation at 446,356 sites in peripheral blood from 850 pedigreed Mexican-American individuals (85). They found differential methylation of 51 CpG sites that significantly associated with T2D, 19 with increased fasting blood glucose concentrations and 24 with HOMA-IR (85). Interestingly, the five CpG sites that were most significantly associated with T2D-related traits mapped to three genes, including the previously identified *TXNIP* and *ABCG1* genes, and *Sterile Alpha Motif Domain Containing 12* (*SAMD12*). *SAMD12* has been identified as a target of gene fusion in breast cancer (99), however its role in T2D still needs to be explored.

More recently, Jeon et al. (88) investigated genome-wide DNA methylation changes in peripheral blood related to hypoglycaemia in a longitudinal Korean population-based cohort (88). They identified hypomethylation of two genes, *Musashi RNA-Binding Protein 2* (*MSI2*) and *CXXC-Type Zinc Finger Protein 4* (*CXXC4*), in individuals with T2D and impaired glucose tolerance, compared to healthy controls. They further assessed these findings in an additional cross-sectional replication cohort of subjects with T2D and healthy controls, using targeted pyrosequencing. Here, only *MSI2* hypomethylation could be validated, which significantly associated with T2D. Interestingly, the same association was observed in pancreatic islet DNA from subjects with T2D, indicating that *MSI2* methylation may be biologically relevant. This is in line with expression studies performed by Szabat et al. (100), who demonstrated that *MSI2* could be upregulated in response to lipotoxicity and endoplasmic reticulum (ER) stress, and that knockdown/overexpression of MSI2 in mouse pancreatic beta cells resulted in significantly altered insulin expression, suggesting a potential modulatory role for MSI2 in T2D.

Methylated DNA Immunoprecipitation Sequencing

Methylated DNA immunoprecipitation sequencing (MeDIP-seq) is a versatile, unbiased approach for detecting methylated DNA and involves the use of a monoclonal antibody that specifically recognizes 5 mC to enrich for methylated DNA, after which the immunoprecipitated fraction can be analyzed by large-scale sequencing (101). This approach is particularly useful because it bypasses the need for bisulfite conversion and is able to distinguish between 5 mC and 5-hydroxymethylcytosine, an oxidation product of 5 mC (101).

Yuan et al. (89) investigated epigenome-wide methylation patterns in whole blood from monozygotic twins discordant for T2D using MeDIP-sequencing, after which the top scoring results were replicated in a separate cohort of twins using the Illumina Human Methylation 450 K array. In the first cohort of twins, they identified T2D-associated differentially methylated regions located within 3,597 genes, which were hypermethylated in two-thirds of cases (89). Furthermore, 30% of the differentially methylated regions could be replicated in the additional twin cohort. Importantly, the top two differentially hypermethylated regions identified in the study were found to reside within a 2 kb region upstream of the transcriptional start site of the *mucosa-associated lymphoid tissue lymphoma translocation protein 1* (*MALT1*) gene. Studies in *MALT1* knockout mice have demonstrated its critical roles in antigen- receptor-induced activation of NF-κB (89). NF-κB has well established roles in T2D–associated chronic inflammation, however, the effects of *MALT1* hypermethylation or transcript depletion on NF-κB signaling and associated inflammation in humans has not yet been explored (102). In the same study, Yuan et al. also identified hypermethylation in the promoter region of the *G-protein receptor 6* (*GPR6*) gene, encoding a member of the G protein-coupled receptor family of transmembrane receptors. Interestingly, *GPR6* knockout mice exhibit hyperphagia-induced obesity and higher liver triglyceride content, plasma insulin, and leptin levels compared to wild-type mice (103). These findings suggest that GPR6 plays a role in the regulation of food intake and body weight, and may thus be an important molecular target for obesity or hyperphagia.

Matsha et al. (90) performed GWAS analysis on DNA methylation patterns in peripheral blood from a small cohort of South African women of mixed ethnic ancestry, consisting of 3 subjects with T2D, 3 with pre-diabetes, and 3 with normoglycaemia. They identified 1,415 differentially methylated sites in the promoter regions of T2D subjects compared to normoglycaemic controls, of which over 80% were hypermethylated, including the following genes: *B-Cell CLL/Lymphoma 3* (*BCL3*), *Interleukin 23 Subunit Alpha* (*IL23A*),

F2R Like Trypsin Receptor 1 (*F2RL1*), *S100 Calcium Binding Protein A12* (*S100A12*), *TNF Receptor Superfamily Member 10b* (*TNFRSF10B*), *NIMA Related Kinase 6* (*NEK6*), *Ring Finger Protein 31* (*RNF31*), *Solute Carrier Family 35 Member B2* (*SLC35B2*), and *Interleukin 1 Receptor Associated Kinase 1 Binding Protein 1* (*IRAK1BP1*). Interestingly, when grouped according to chromosomal location it was found that, compared to controls and pre-diabetic subjects, individuals with T2D had hypermethylated regions that were more common in chromosomes 3, 6, 11, 13, and 17, while more hypomethylated methylated regions were found in chromosome 1. Furthermore, these identified hypermethylated regions mapped to pathways related to T2D, including cell surface signaling, glucose transport, insulin signaling, pancreas development, and the immune system, whereas hypomethylated regions related to the pro-inflammatory NF-κB cascade, as well as metabolism pathways for polyunsaturated omega-6 fatty acids, linoleic acid, and arachidonic acid (90). Interestingly, excess consumption of polyunsaturated fatty acids, particularly found at high concentrations in the western diet, can result in increased inflammation and contribute to the onset of chronic diseases including obesity and T2D (104). Importantly, Matsha et al. (90) demonstrated that linoleic acid and arachidonic acid metabolism pathways were also associated with hypomethylated differentially methylated regions in subjects with prediabetes compared to controls, suggesting that alterations in the methylation state of these genes may occur before the onset of overt T2D.

An additional investigation by the same group focused on identifying T2D-associated DNA methylation changes in intergenic regions compared to promoter and gene body regions, as it now appreciated that methylation within intergenic regions regulate RNA processing, as well as high-copy interspersed or tandem DNA repeats (10). Using peripheral blood DNA from the same South African patient cohort as described above (90), they showed increased DNA methylation in intergenic regions compared to gene body and promoter regions (11). Furthermore, 3,081 of the differentially methylated regions were associated with microRNAs. Importantly, a subset of miRNAs identified in the study, including miR-9, miR-34, miR-124, and miR-1297, have already been linked to T2D and associated traits in human and animal diabetic models. Since dysregulated miRNAs have an established role in T2D (105), those identified by Pheiffer et al. merit further evaluation as novel disease risk biomarkers.

INTERACTIONS BETWEEN GENETICS AND EPIGENETICS IN T2D

There is evidence to suggest that single nucleotide polymorphisms (SNPs) may be associated with altered epigenetic signatures (106). Indeed, it has been suggested that up to 25% of all SNPs in the genome either introduce or remove CpG sites (106, 107). In this regard, CpG-SNPs have been suggested to be a potential mechanism through which SNPs affect gene function via epigenetics, highlighting the complex interaction between genetics and epigenetics (107). While CpG-SNPs have been reported in numerous obesity-associated genes, few studies have examined the association between SNPs and T2D risk through effects on DNA methylation (108).

An investigation of T2D-associated DNA methylation candidates reported in this review revealed that 20 genes were indeed associated with SNPs. *FTO,* and *TCF7L2* have been deemed two of the most important T2D susceptibility genes to date (54–57, 109, 110), while additional SNPs in *MCP-1*, *SLC30A8, GCK*, *PRKCZ*, *GIPR*, *IGFBP-7*, *PTPN1*, *PPARγ*, *KCNQ1*, *BCL11A*, *CALM2*, *CRY2, CAMK1D, THADA, ABCG1, SOCS3, SREBF1, TXNIP* have either been associated with glycaemic traits, T2D or risk of T2D-linked complications (63, 64, 110–119). It is still unknown whether the above associated SNPs may directly cause differential DNA methylation of genes that contribute to the pathogenesis of T2D, or if SNPs within regulatory regions change the affinity and/or binding of transcription factors, which in turn influence the recruitment of epigenetic machinery. Future work should be directed at combining genetic information and methylation marks when comparing individuals with T2D to those without disease.

Interestingly, SNPs may also be associated with altered global DNA methylation. Matsha et al. performed genetic screening of polymorphisms in the *nitric oxide synthase 3* (*NOS3*) gene using peripheral blood from South African subjects with T2D, pre-diabetes or normoglycaemia, and reported that the *NOS3* G894T polymorphism was independently associated with global DNA methylation. NOS3 has previously been reported to be affected by supplementation with folate, a dietary methyl donor (120). Although the potential role played by NOS3 in global DNA methylation is unclear, it encodes an enzyme involved in endothelial function and may thus potentially contribute to T2D-associated vascular complications (121).

CHALLENGES AND LIMITATIONS IN DNA METHYLATION STUDIES

The current review highlighted variation in the outcome of DNA methylation studies, however, it is important to note that the range of methods employed to measure DNA methylation is vast, as are the sources of DNA (cell type), DNA isolation method and methods of data analysis (122). This large heterogeneity complicates the direct comparison of findings between studies, particularly for those published more than a decade ago, as the epigenetics field is expanding at such a rapid rate. The standardization of experimental and analysis approaches, as well as internal and external validation of study findings will be an important step in improving the reproducibility and biological relevance of these findings.

The inability of some of the reported studies to replicate methylation associations could be explained by differences in the groups of participants analyzed, as the majority of findings emanated from small cross-sectional or case-control studies in varying populations. To advance reproducibility in different populations, more robust longitudinal studies are required, which would involve prospective recruitment of a large cohort of healthy individuals at baseline, and the follow-up of these individuals over several decades to track T2D incidence.

However, due to higher costs and study duration, longitudinal studies for complex diseases such as T2D still remain scarce. The only longitudinal studies reported in this review were that of Chambers et al. (80) and Jeon et al. (88). Interestingly, differentially methylated genes identified in the Chambers study were replicated in cross-sectional studies (*TXNIP*, *ABCG1*, *PHOSPHO1,* and *SREBF1*) (19, 81–87). The ability of these changes to be captured across studies of different time lengths may be attributed to the stable nature of DNA methylation marks after disease onset.

An additional limitation in some of the reported studies was failure to include in-depth demographic, lifestyle, and health data and consequently, lack of consideration or adjustment for potential confounding factors. This is crucial for data interpretation as it is now widely appreciated that DNA methylation signatures vary with gender, age, and ethnicity and are sensitive to many environmental influences (32). Indeed, in cases where gender was considered, differences in DNA methylation patterns were reported for *BCL11A*, *GCK*, *IGFBP-7,* and *PTPN1* (44, 46, 49). These observations are consistent with findings on gender-specific DNA methylation marks in other diseases, such as *PLA2G7* in cardiovascular disease and *MTHFR* in schizophrenia (123, 124). Furthermore, gender-specific differences in glucose homeostasis and T2D risk have been reported, which may be related to the levels of sex-hormones such as estrogen and testosterone (125). The confounding effect of chronic medication was also highlighted by Matsha et al. (30), who reported an association between global DNA hypomethylation in T2D individuals and the use of glucose-controlling agents. Indeed, the widely used anti-diabetic drug, metformin, was recently shown to promote global DNA methylation in cancer cell lines by modulating the intracellular ratio of S-adenosylhomocysteine (SAH) and S-adenosylmethionine (SAM) (126). Thus, the observed methylation differences and associations between methylation changes and T2D risk might be confounded by medications, such as metformin, which should be taken into consideration in future studies. Furthermore, these findings may offer opportunities for the use of DNA methylation for monitoring of management and response to T2D medications.

DNA methylation varies with cell type and thus cellular homogeneity within a tissue is an important characteristic for a DNA methylation biomarker. While the finding in this review strengthen the candidacy of blood-based markers, it is important to note that blood exhibits cellular heterogeneity, as it consists of a wide variety of cell types including erythrocytes, basophils, neutrophils, eosinophils, monocytes, lymphocytes, natural killer cells, and platelets (127). Each of these cells possess a unique epigenetic signature and this can lead to variation between studies. Only one study reported in this review controlled for the estimated proportion of different blood cell types, and indeed proved that epigenetic heterogeneity in whole blood cell constituents impacts on data interpretation (31). Thus, it is plausible that differences in cell composition between groups may drive false associations or mask potential differences between groups. In this regard, there are several additional methods that can be used to avoid potential confounding effects of the blood cell composition, such as the measurement of DNA methylation in individual cell types following sorting of the cells, adjustment for direct measured cell count or the use of *post-hoc* regression models, as described by Houseman et al. (128).

It is important to note that while DNA methylation in gene promoters has consistently been linked with gene silencing, some studies could not correlate promoter methylation with gene or protein expression (49). This could either be a result of DNA methylation at the reported CpGs being ineffective to reduce transcript levels, or due to alternative transcriptional or post-transcriptional influences. Indeed, a growing body of evidence suggests that miRNAs and histone modifications are also highly involved in T2D pathogenesis, for which comprehensive reviews have been published (105, 129, 130).

CONCLUSIONS AND FUTURE PERSPECTIVES

The current review identified 37 articles investigating DNA methylation markers for T2D detection or risk evaluation, using DNA isolated from blood. Based on reproducible findings from the reviewed studies in different population groups, differentially methylated sites in *TCF7L2*, *KCNQ1*, *ABCG1*, *TXNIP*, *PHOSPHO1*, *SREBF1*, *SLC30A8,* and *FTO* are potentially associated with T2D and their predictive powers may hold irrespective of different genetic backgrounds and different lifestyle or environmental pressures. A model for the role of these DNA methylation alterations in the pathogenesis of T2D is depicted in **Figure 1**. Although these alterations were

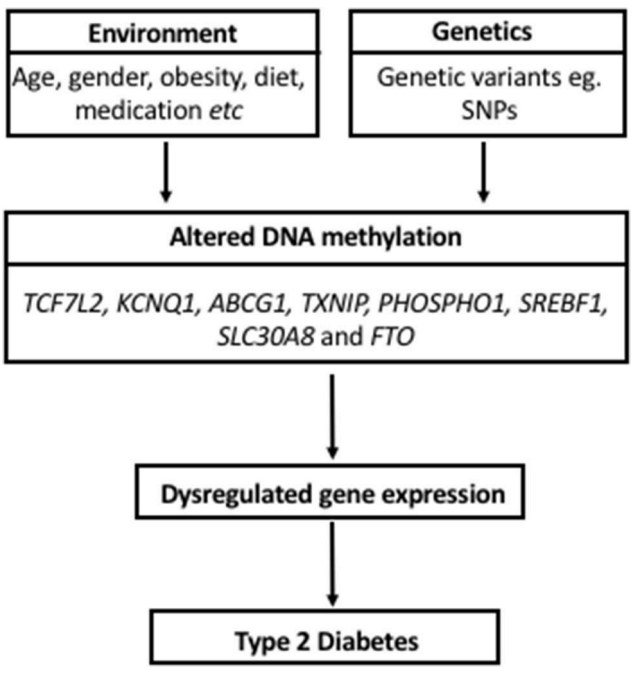

FIGURE 1 | Model proposing a role for DNA methylation in the pathogenesis of Type 2 Diabetes and its interaction with environmental factors and genetics.

detected in blood, which is not an insulin-responsive tissue, the implicated genes have been shown to play a role in critical biological processes that are deregulated during T2D development, including energy intake and expenditure (*FTO*), lipogenesis and glycolysis (*SREBF1*), glucose homeostasis and carbohydrate metabolism (*TXNIP, TCF7L2*), lipid transport (*ABCG1*) pancreatic insulin secretion (*SLC30A8, KCNQ1*), and cardiovascular function (*PHOSPHO1*). It is thus plausible that these blood-based epigenetic markers mirror tissues with deteriorated metabolic function, and are prime non-invasive candidates for T2D biomarkers.

Some limitations of the current review are the exclusion of studies that were not published in English and the use of only four databases of published literature. Positive publication bias also should be considered, as studies with negative findings may not have been published. Finally, the current review only focused on DNA methylation, given the large scope of studies already published in this field. Although it was beyond the scope of this review, we cannot rule out other possible epigenetic biomarker candidates of T2D, such as non-coding RNAs and histone modifications, for which evidence is rapidly accumulating (105, 129, 130).

The major strength of this review is the central focus on blood-based DNA methylation signatures in T2D, which will have important implications for the development of non-invasive T2D screening tests, given the difficulty in accessing T2D-associated tissues, particularly for longitudinal studies. To the best of our knowledge, this is the only review solely examining associations of T2D with DNA methylation profiles in peripheral blood. We also highlighted specific methylation patterns that associated with T2D risk factors such as BMI, HbA1c levels, and HDL/LDL, which further supports the hypothesis that profiling DNA methylation in blood could be used to monitor high risk individuals and delay or prevent T2D by facilitating early intervention strategies. In this regard, the candidate markers in this review need to be further validated in additional prospective study cohorts and tested in large screening populations by high quality studies. At present, no epigenetic biomarkers for T2D have yet entered clinical trials, however, there is hope that initiatives such as next generation sequencing and the use of longitudinal study designs, will uncover important predictive T2D biomarkers.

AUTHOR CONTRIBUTIONS

TW designed the study and extracted the data. TW wrote the manuscript. CP and RJ corrected the manuscript. All authors read and approved the final manuscript.

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Corrigendum: Blood-Based DNA Methylation Biomarkers for Type 2 Diabetes: Potential for Clinical Applications

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In the original article, there was an error. "Colombian" was misspelled as "Columbian."

A correction has been made to the **Introduction**, **Global DNA Methylation Studies**, paragraph two.

"Luttmer et al. quantified global DNA methylation levels in peripheral blood leukocytes of 738 individuals from the Netherlands Hoorn Study cohort and reported a progressive decrease in global DNA methylation in individuals with T2D compared to those with impaired glucose tolerance and normoglycaemia. Moreover, DNA hypomethylation in these subjects was independently associated with hyperglycaemia and high-density lipoprotein (HDL) cholesterol (28). In contrast, a Colombian study using a smaller patient group, observed a global increase in DNA methylation in 44 subjects with T2D compared to 35 healthy controls, which correlated with the percentage of glycated hemoglobin A1c (HbA1c) (29). Similar findings were reported by Matsha et al. using a South African population consisting of 158 individuals with T2D, 119 with dysglycaemia, and 287 healthy controls. They showed that levels of global DNA methylation were higher in individuals with impaired glucose tolerance or treatment-naïve T2D compared to those with normoglycaemia (29, 30). Interestingly, no difference in global DNA methylation was observed between diabetic individuals on treatment and normoglycaemic subjects, prompting the authors to speculate that glucose management caused the reversal of aberrant DNA methylation patterns during T2D (30)."

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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Mono-ADP-Ribosylhydrolase MACROD2 Is Dispensable for Murine Responses to Metabolic and Genotoxic Insults

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ADP-ribosylation is an important post-translational protein modification that regulates diverse biological processes, controlled by dedicated transferases, and hydrolases. Disruption in the gene encoding for MACROD2, a mono-ADP-ribosylhydrolase, has been associated to the Kabuki syndrome, a pediatric congenital disorder characterized by facial anomalies, and mental retardation. Non-coding and structural mutations/variations in MACROD2 have been associated to psychiatric disorders, to obesity, and to cancer. Mechanistically, it has been recently shown that frequent deletions of the MACROD2 alter DNA repair and sensitivity to DNA damage, resulting in chromosome instability, and colorectal tumorigenesis. Whether MACROD2 deletion sensitizes the organism to metabolic and tumorigenic stressors, in absence of other genetic drivers, is unclear. As MACROD2 is ubiquitously expressed in mice, here we generated constitutively whole-body knock-out mice for MACROD2, starting from mouse embryonic stem (ES) cells deleted for the gene using the VelociGene® technology, belonging to the Knockout Mouse Project (KOMP) repository, a NIH initiative. MACROD2 knock-out mice were viable and healthy, indistinguishable from wild type littermates. High-fat diet administration induced obesity, and glucose/insulin intolerance in mice independent of MACROD2 gene deletion. Moreover, sub-lethal irradiation did not indicate a survival or lethality bias in MACROD2 knock-out mice compared to wild type littermates. Altogether, our data point against a sufficient role of MACROD2 deletion in aggravating high-fat induced obesity and DNA damage-associated lethality, in absence of other genetic drivers.

Keywords: metabolic stress, obesity, MACROD2, irradiation, genotoxic stress response, knock out mouse model

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INTRODUCTION

ADP-ribosylation, the addition of one or more ADP-ribose moieties from nicotinamide adenine dinucleotide (NAD+) to a protein or a target molecule, was first described in the early 1960's. ADP ribosylation is a reversible post-translational modification regulating critical cellular pathways in eukaryotes, such as DNA repair and apoptosis, and underlies as well the pathogenicity of certain

bacteria (Belenky et al., 2007; Cohen and Chang, 2018). Improper ADP-ribosylation has been implicated in metabolic, inflammatory diseases and several cancers (Bai, 2015). ADP-ribosylation levels are defined by the activities of specific transferases and hydrolases. ADP-ribosylation can be catalyzed by poly(ADP-ribose) polymerases (PARPs), which transfer ADP-ribose chains, or by mono(ADP-ribose) polymerase that add single ADP-ribose blocks. MACROD2 is of one of the tree monoADP-ribosylases (MARs) in humans, together with macroD1 and C6orf130, which possesses reversible ADP-ribosyl hydrolase activity and is required for its recruitment to DNA lesions induced by laser microirradiation (Jankevicius et al., 2013; Rosenthal et al., 2013; Barkauskaite et al., 2015). Abnormalities in the sequence of the gene locus of MACROD2, most often deletions or single nucleotide polymorphisms (SNPs), have been consistently associated with cancers, neurological and psychiatric disorders (Anney et al., 2010; Lionel et al., 2011; Perlis et al., 2012; Cheng et al., 2013; Jahanshad et al., 2013; Linnebacher et al., 2013; Tsang et al., 2013; Jones et al., 2014; Mohseni et al., 2014; Briffa et al., 2015; van den Broek et al., 2015; Hu et al., 2016; Sakthianandeswaren et al., 2018). Beyond the *in vitro* and clinical association studies discussed above, the *in vivo* function and the exact molecular functions of MACROD2 are poorly understood.

A study by Chang et al. (2018) aiming at identifying the genetic loci for circulating VAP-1 levels (Vascular adhesion protein-1, a membrane-bound amine oxidase highly expressed in mature adipocytes and released into the circulation) in 1,100 Han Chinese individuals from 398 families, demonstrated a strong association with MACROD2; siRNA-mediated knockdown of MACROD2 significantly suppressed the expression of key adipogenic genes FABP4, ADIPOQ, CEBPA, PPARG2, and SREBP1 in primary human pre-adipocytes isolated from the visceral adipose tissue, thus maintaining them in an undifferentiated state. Therefore MACROD2 could act as a transcriptional regulator of adipogenesis and obesity, in turn a major metabolic risk factor for developing cancer. In this respect, a recent study demonstrated how MACROD2 haploinsufficiency alters DNA repair and sensitivity to DNA damage, and results in chromosome instability in almost 1/3 of colorectal patients (Sakthianandeswaren et al., 2018). Whereas previous studies investigating the role of MACROD2 on cancer development used nude mice xenograft with human cells harboring altered expression of MACROD2 (Mohseni et al., 2014), the latter was the first study reporting the phenotype of MACROD2 knock-out mice [obtained from the Knockout Mouse Project Repository (KOMP), Jackson Laboratory], which developed normally into adulthood, and then crossed with the ApcMin/+ mouse model. ApcMin/+ mice harbor a truncating germline mutation in Apc, and intestinal tumors arise spontaneously from loss of heterozygosity of the wild-type Apc allele, a mechanism found in human colorectal cancer. Heterozygous and homozygous depletion of MACROD2 enhanced significantly intestinal tumorigenesis in this susceptible genetic background (Sakthianandeswaren et al., 2018). Accordingly, at the clinical level, low nuclear expression of MACROD2 is associated with poor prognosis of patients with stage III primary colon cancer (van den Broek et al., 2018).

Despite these evidences, whether MACROD2 deletion sensitizes the organism to metabolic and tumorigenic stressors, in absence of other genetic drivers, is unclear. Here we generated constitutively whole-body knock-out mice for MACROD2. MACROD2 knock-out mice were viable and healthy, indistinguishable from wild type littermates. High-fat diet administration induced obesity, and glucose/insulin intolerance in mice independent of MACROD2 gene deletion. Moreover, sub-lethal irradiation did not indicate a survival or lethality bias in MACROD2 knock-out mice compared to wild type littermates. Altogether, our data point to a dispensable role for MACROD2 deletion in aggravating the effects of metabolic and tumorigenic stressors, in absence of other genetic drivers.

MATERIALS AND METHODS

MACROD2 Knock-Out Mice Generation

MACROD2 knock-out (KO) in VGB6 embryonic stem (ES) cells were purchased from the University of California (UC) Davis KOMP: http://velocigene.com/komp/detail/12650, a trans National Institute of Health (NIH) initiative aiming at generating a comprehensive and public resource comprised of mouse ES cells containing a null mutation in every gene in the mouse genome (www.komp.org). Constitutive KO of MACROD2, located on chromosome 2, was achieved using the VelociGene® technology (Valenzuela et al., 2003), according to the design illustrated in **Figure 1B**. The genotype strategy is illustrated in Supplemental **Figure 1**. Accordingly, the genotyping primer sequences (5'-3') were as it follows:

SU, TTCCTGAGCTCCGTGAATG, SD, TCTTTCAAGC TGACTGTGGG;
LacInR, TTGACTGTAGCGGCTGATGTTG, LacInf, GG TAAACTGGCTCGGATTAGGG;
NeoInR, TACTTTCTCGGCAGGAGCAAGGTG, Neo InF, TTCGGCTATGACTGGGCACAACAG;
LacInZRev, GTCTGTCCTAGCTTCCTCACTG, Neo Fwd, TCATTCTCAGTATTGTTTTGCC.

Targeted VGB6 ES cells were then injected into C57BL/6 eight cell-stage embryos. Mice heterozygous for the macroD2 allele were further crossed between each other to generate KO mice. All mice used were on a C57Bl/6 genetic background, and were bred and maintained at the EMBL Mouse Biology Unit, Monterotondo, or at Plaisant Srl (Rome, Italy), in accordance with current Italian legislation (article 9, 27 January 1992, number 116) under license from the Italian Health Ministry. EchoMRI quantitative magnetic resonance and CT scan were performed as previously described (Pazienza et al., 2016).

Irradiation

For survival experiments, total body irradiation was administered to wild type macroH2A1 heterozygous and macroH2A1 KO mice (n = 25–30 per group), restrained in holders, using a MK-1-68A Cesium-137 Gamma animal irradiator (J.L. Shepherd and

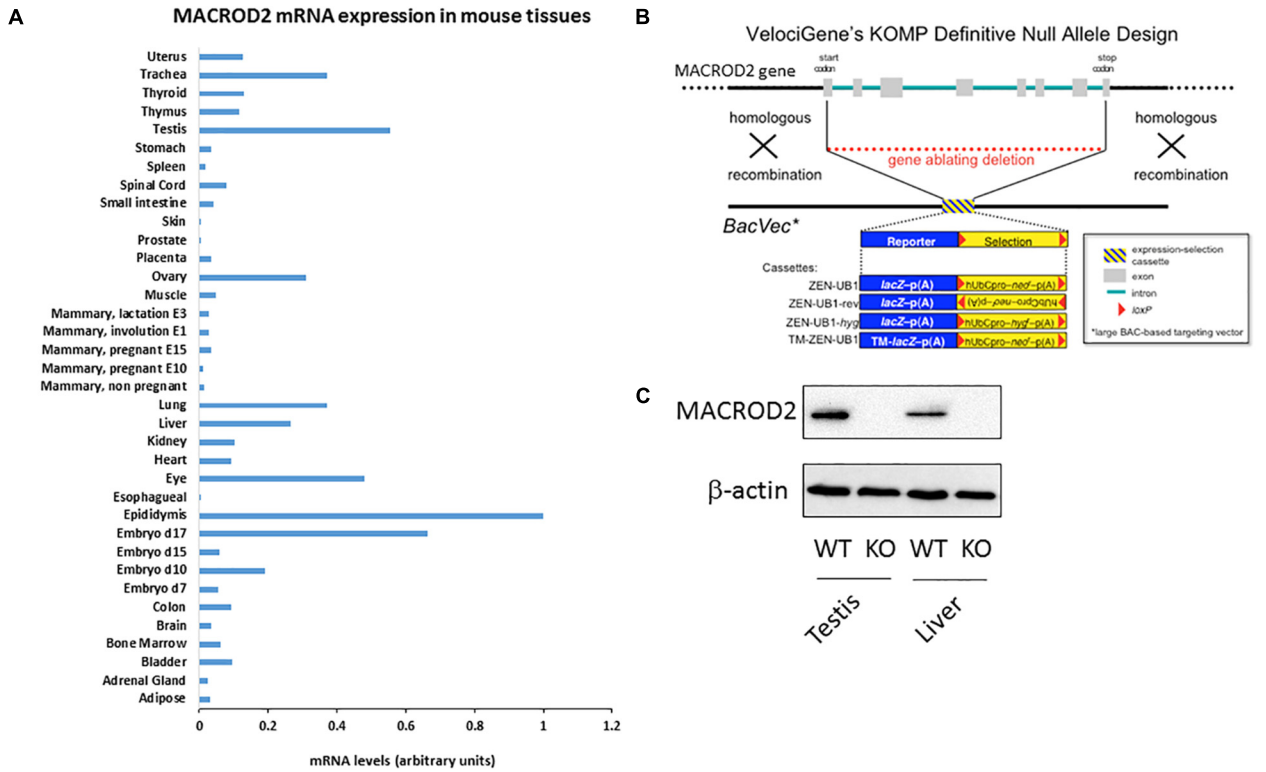

FIGURE 1 | Please provide promoter the meaning of "*" specified in Figure 1. **(A)** qPCR screening of MACROD2 mRNA expression levels normalized to GAPDH across 36 murine somatic and reproductive organs and tissues. **(B)** Generation of MACROD2 mice. Design diagram of VelociGene® KOMP constitutive MACROD2 deletion. LacZ, β-galactosidase; TM-lacZ, transmembrane sequence fused to β-galactosidase; neo or hyg, coding sequences for neomycin or hygromycin phosphotransferases; hUbCpro, promoter from the human ubiquitin C gene; p(A), polyadenylation signal. **(C)** Immunoblot analysis. Protein extracts (10 μg/lane) of liver (L) and kidney (K) were prepared from wild-type (WT) and macroD2 KO (KO) mice and analyzed using anti-macroD2 and anti-β-actin. *BacVec = BAC based targeting vector.

Associates), with 1000 rad in a single dose. After irradiation all animals were returned to the animal facility.

qPCR

MACROD2 mRNA expression screen was performed on a cDNA library (96 wells coated plates, normalized against GAPDH) from ORIGENE (MNRT101) – TissueScan Mouse Normal Tissue qPCR Array, according to manufacturer's instructions. The array included cDNA from Adipose Tissue, Adrenal Gland, Bladder, Bone Marrow, Brain, Colon, Embryo d7, Embryo d10, Embryo d15, Embryo d17, Epididymis, Esophagus, Eye, Heart, Kidney, Liver, Lung, Mammary gland (not pregnant), Mammary gland (pregnant E10), Mammary gland (pregnant E15), Mammary gland (involution E1), Mammary gland (lactation E3), Muscle, Ovary, Placenta, Prostate, Skin, Small intestine, Spinal Cord, Spleen, Stomach, Testis, Thymus, Thyroid, Trachea, and Uterus. Real Time-PCR was performed in triplicate utilizing StepOnePlus™ Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) and SYBRTM Select Master Mix (ThermoScientific). Primer sequences were as it follows (5'-3'): MACROD2: sense, GCCTGAGACGGTTATGGAAA; antisense, TGTCTCCCACCCTTCTTGTC. 18S: sense: AGTCCCTGC CCTTTGTACACA; antisense: CGATCCGAGGGCCTCACTA. GAPDH: sense: CGTCCCGTAGACAAAATGGT; antisense: TCAATGAAGGGGTCGTTGAT. Fold changes between groups were calculated using the $2^{-\Delta\Delta ct}$ method.

Immunoblotting

Protein extraction and immunoblotting analyses were performed as previously described (Benegiamo et al., 2012; Borghesan et al., 2016). Primary antibodies were as follows: anti-macroD2 (ThermoScientific, Cat. PA5-45950), anti-β-actin (Cell Signaling, Cat. 4967).

Comet Assay

Mouse embryonic fibroblasts (MEFs) were isolated as previously described (Di Biase et al., 2017) from day 10.5 gestation embryos, washed in phosphate-buffered saline, and disaggregated with an 18-gauge needle. Following three washes in Dulbecco's modified Eagle medium (DMEM), the suspension was plated in a 60-mm dish in DMEM containing 15% fetal calf serum. MEF were isolated at 37°C and 5% CO2 in either 3 or 21% oxygen. After 24 h cells were trypsinized and replated to enrich for fibroblasts in DMEM containing 10% fetal calf serum. Until the genotypes (WT or MACROD2 KO) were confirmed, each embryo was cultured separately. After 2 days, cells with an identical genotype

were pooled and designated passage 1. A total of 10^5 cells were reseeded at each subculture. MEFs were not passaged for more than 2 times, kept on ice and subjected to 10 Gy IR, and were then harvested immediately for Comet Assay. Comet Assay on MEFs was performed using the CometAssay® Kit (25 × 2 well slides) (Trevigen, BioTechne ltd, United Kingdom), according to manufacturer's instructions. Briefly, the Comet Assay kit was used to perform alkali denaturing comet assays. Samples were stained with SYBR green prior to analysis by fluorescence microscopy. Pictures of individual cells were taken with a Zeiss AxioObserver Z1 inverted microscope equipped with a black-and-white CCD camera. The percentage of tail DNA was analyzed from 50 cells per sample using CaspLab software.

Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT)

GTT and ITT were performed as previously described (Sheedfar et al., 2015; Pazienza et al., 2016). Briefly, Mice were fasted for 6 h during the daytime, and given intraperitoneally (IP) injection with glucose solution or with human recombinant insulin (0.5 U kg – 1 body weight, Actrapid; Novo Nordisk[insulin tolerance test (ITT)]. Blood was collected from the tail vein and glucose levels were measured with an OneTouch Ultra glucometer (Lifescan Benelux, Belgium) before and 30, 60, and 120 after the gavage/injection.

Bioinformatic Analysis of MACROD2 SNPs in Human Cancers

Single Nucleotide Polymorphisms (SNPs) and short insertions/deletions (indels) were sought in an ad-hoc database from Wellcome Sanger Institute (i.e., COSMIC ver. 86). COSMIC includes genomic data of the following tissues: Adrenal gland, Autonomic ganglia, Biliary tract, Bone, Breast, Central nervous system, Cervix, Endometrium, Eye, Fallopian tube, Gastrointestinal tract (site indeterminate), Genital tract, Haematopoietic and lymphoid, Kidney, Large intestine, Liver, Lung, Meninges, NS, esophagus, Ovary, Pancreas, Parathyroid, Pituitary, Placenta, Pleura, Prostate, Salivary gland, Skin, Small intestine, Soft tissue, Stomach, Testis, Thymus, Thyroid, Upper aerodigestive tract, Urinary tract, and Vulva. Information on the chromosome, start and end positions of each genomic variant, together with reference and alternate alleles were downloaded from COSMIC and given in input to ANNOVAR[1]. ANNOVAR was used to retrieve information on the genomic regions hit by each variant (i.e., exon, intergenic, splicing, introns, non-coding RNA genes, UTRs), and to predict their functional consequences (i.e., non-synonymous SNV, synonymous SNV, frameshift insertion, frameshift deletion, non-frameshift insertion, non-frameshift deletion, frameshift block substitution, non-frameshift block substitution). Frequency of genomic regions and mutation types were plotted in R ver. 3.5.0 as pie-charts, using the ggplot2 package.

[1]http://annovar.openbioinformatics.org/

Statistical Methods

Data are shown as means ± standard error of the mean (SEM). Groups were compared with either Student's *t*-test or the non-parametric Mann–Whitney U-test, as appropriate, using GraphPad Prism Software (version 5.00 for Windows, San Diego, CA, United States): significance was $P \leq 0.05$. Survival analyses of mice employed the Kaplan-Meier estimator.

RESULTS

Tissue Distribution of MACROD2 mRNA and Generation of MACROD2 Knock-Out (KO) Mice

A comprehensive characterization of differential MACROD2 transcript expression in different tissues has not been performed. To this purpose, we probed a Mouse Normal Tissue qPCR Array containing first strand DNA from 36 tissues [adipose tissue, adrenal gland, bladder, bone marrow, brain, colon, embryo day7, embryo day 10, embryo day 15, embryo day 17, epididymis, esophagus, eye, heart, kidney, liver, lung, mammary gland (not pregnant), mammary gland (pregnant – 10 days from copulation plug date), mammary gland (pregnant – 15 days from copulation plug date), mammary gland (involution day 1), mammary gland (lactation day 3), muscle, ovary, placenta, prostate, skin, small intestine, spinal cord, spleen, stomach, testis, thymus, thyroid, trachea, uterus] for MACROD2 mRNA expression using specific primers (**Figure 1A**). High mRNA expression for MACROD2 was detected in the reproductive organs: the epididymis, the testis, the ovary; high MACROD2 expression levels were also detected in the late mouse embryo at day 17, as well as in the lung, in the trachea, in the eye and in the liver (**Figure 1A**). Of note, MACROD2 mRNA expression was ubiquitous as it was detected in all the 36 tissues tested (**Figure 1A**). To study the systemic effects of MACROD2 deletion, we endeavored the generation of constitutive MACROD2 knock-out (KO) mice. We obtained ES cells from the knock out mouse project (KOMP) NIH repository, where deletion of MACROD2 GENE was achieved using the VelociGene® technology (Valenzuela et al., 2003), according to the design illustrated in **Figure 1B**. VelociGene® technology uses a MACROD2 targeting vector based on bacterial artificial chromosomes (BACs), and it allowed to replace the MACROD2 with a lacZ (β-galactosidase) reporter to localize gene expression (Valenzuela et al., 2003; **Figure 1B**). MACROD2 could be easily detected in wild type (WT) mice and in protein extracts of organs such as the testis and liver by immunoblot analysis, while it was absent in KO mice, validating the MACROD2 genetic deletion strategy (**Figure 1C**).

MACROD2 Deletion in Mice Does Not Aggravate Irradiation Induced Lethality and DNA Damage

ADP-ribosylation is a dynamic post-translation modification that regulates the early phase of DNA repair pathways by recruiting repair factors to chromatin. As ADP-ribosylation can reveerted by the hydrolase activity of MACROD2, it has been

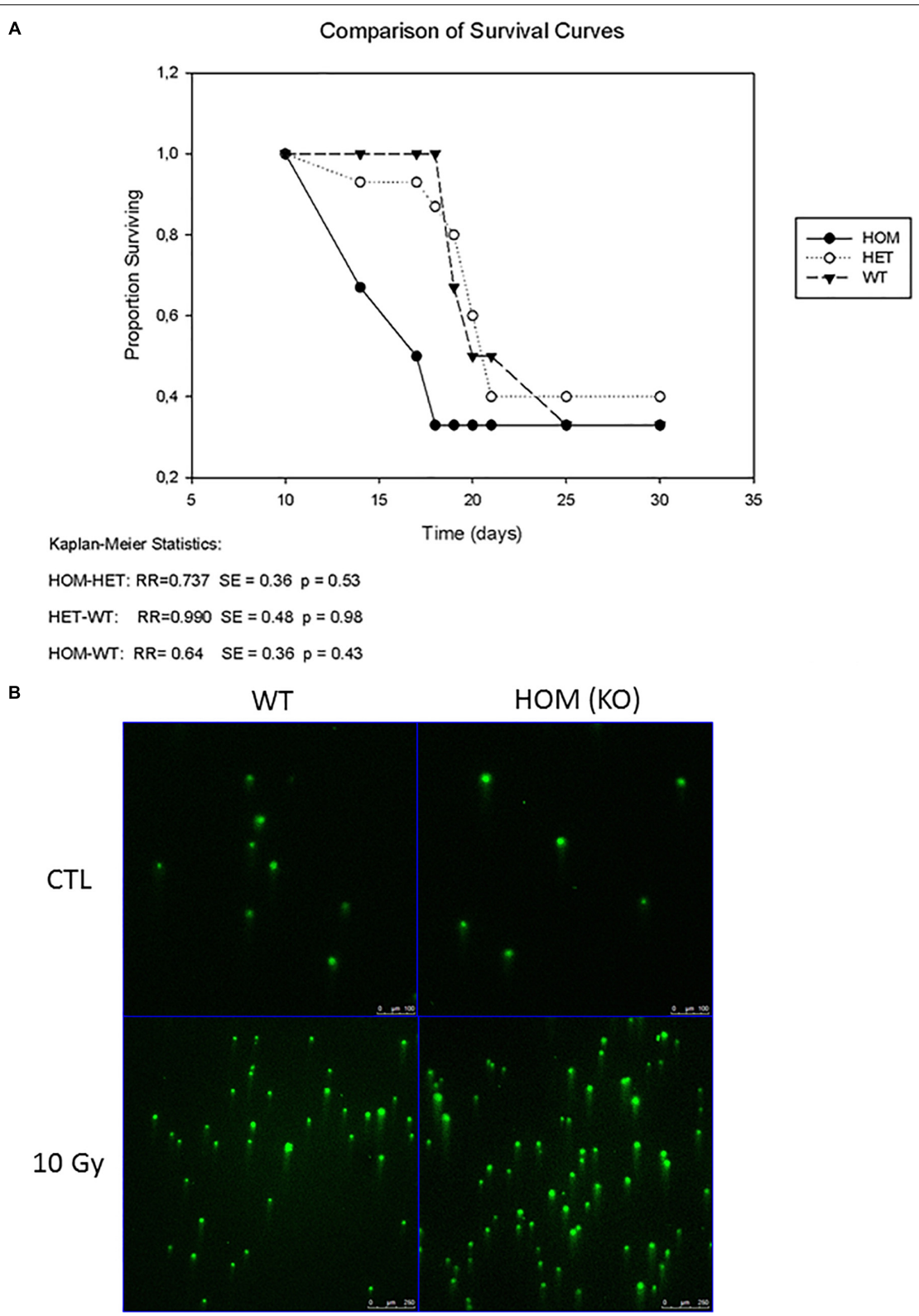

FIGURE 2 | MACROD2 deletion has no effect on γ-irradiation induced lethality and DNA damage. **(A)** A Kaplan–Meier survival curve of wild type (WT), MACROD2 HET (+/−) and MACROD2 KO (HOM; −/−) mice (n = 25–30 mice/group). Survivals of mice were closely monitored several times per day. **(B)** Comet Assay. Images of comet assay showing DNA damage mouse fibroblasts in response to γ-irradiation. The cells were processed for comet assays immediately after the irradiation.

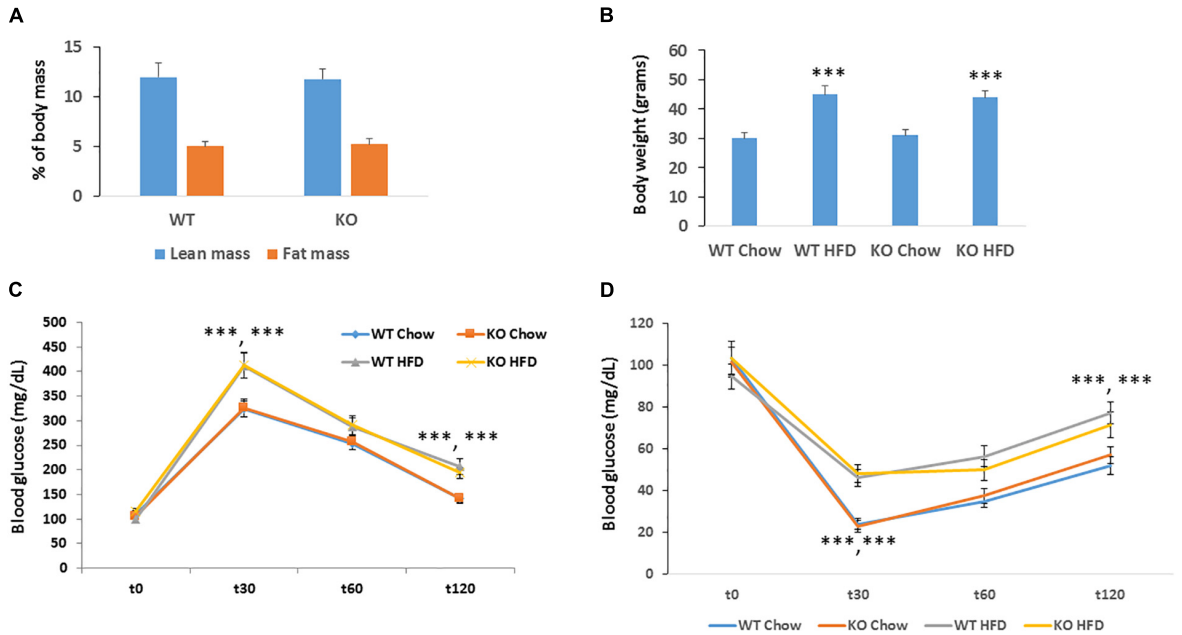

FIGURE 3 | Figure MACROD2 deletion has no effect on high-fat diet induced dysmetabolism. **(A)** lean and fat masses were determined by CT scan in WT and MACROD2 KO mice fed a HF diet (HFD). **(B)** body weight in WT and MACROD2 KO mice fed a chow or a HF diet at the experimental end point. **(C,D)** GTT and ITT were performed in WT and Tg mice fed a chow or a HFD following a 6 h fast. Mice were injected with 2 g glucose/kg of body weight intraperitoneally, and blood glucose concentrations were measured at the indicated time points (minutes). Data are expressed as means ± S.E.M. (n = 8–9 per group). ***$p < 0.001$ change versus WT fed a chow diet.

directly involved in DNA repair (Golia et al., 2017). To replicate conditions of high levels of DNA damage, we irradiated three cohorts of mice; wild type (controls) (n = 30), MACROD2 heterozygous (HET) (n = 27) and MACROD2 KO (HOM) (n = 25) with a dose of 1000 rad to the whole body. No significant changes in survival rates between group comparisons were observed (**Figure 2A**). Thus mice lacking MACROD2 do not succumb more easily to the DNA-damaging effects of lethal irradiation. To examine if the wild type, MACROD2 HET and MACROD2 KO fibroblasts experienced the same physical DNA damage in response to γ-irradiation, we subjected cells to 10 Gy of γ-irradiation and collected immediately for comet assay. All three types of cells had similarly increased tail DNA in response to γ-irradiation (**Figure 2B**), suggesting that they sustain similar levels of DNA damage. Taken together, these *in vivo* and *in vitro* results show that MACROD2 deletion does not worsen DNA damage in response to γ-irradiation.

MACROD2 Deletion in Mice Does Not Impact High Fat-Diet Induced Obesity, Insulin and Glucose Intolerance

Obesity is a major risk factor for developing cancer. Since silencing of MACROD2 significantly suppressed the expression of adipogenic genes in primary human pre-adipocytes (Chang et al., 2018), we hypothesized that MACROD2 could play a role in the development of obesity *in vivo*. We thus established a classical model of high fat diet-induced obesity (Pazienza et al., 2016). Upon feeding an obesogenic[12 weeks, 60% energy from lard (Sheedfar et al., 2015)] high fat (HF) diet, MACROD2 KO mice did not show any change in fat induced-increased adiposity as assessed by quantitative EchoMRI/CT scan (**Figure 3A**). Accordingly, body weight of age-matched MACROD2 KO mice did not differ than wild-type mice both under a chow diet and under a HF diet (**Figure 3B**). In comparison with wild-type mice, no gross changes in HF diet-induced obesity are observed in MACROD2 KO mice. Nonetheless, we sought to investigate the ability of KO mice to respond to a glucose or insulin challenge. Basal glucose levels were similar in KO versus wild-type mice, fed a chow or a HF diet (**Figures 3C,D**). In glucose tolerance tests (GTT), glucose levels remained similar in MACROD2 KO mice at every time point, compared to wild-type littermates, both upon a chow or a HF diet (**Figure 3C**). Insulin tolerance tests (ITT) showed that the insulin-mediated decrease in glycemia was also comparable in MACROD2 KO mice versus wild-type mice at every time measured upon a chow diet (**Figure 3D**). Altogether these data demonstrate that whole body deletion of MACROD2 gene is irrelevant for the insurgence and progression of obesity, and for its associated dysmetabolism.

DISCUSSION

MACROD2 is a monoADP-ribosylase that in human cells *in vitro* has been mechanistically shown to be instrumental in DNA stability and tumorigenesis (Jankevicius et al., 2013; Rosenthal et al., 2013; Barkauskaite et al., 2015; Golia et al., 2017; Sakthianandeswaren et al., 2018) and adipogenesis

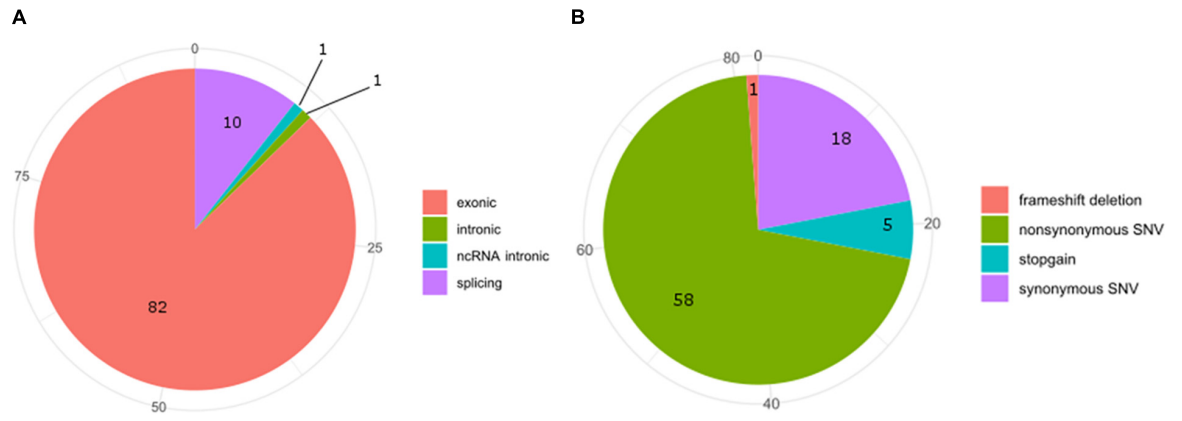

FIGURE 4 | Pie-charts of frequencies of the interested genomic regions **(A)** and functional consequences **(B)** of COSMIC variants hitting the MACROD2 genes.

(Mohseni et al., 2014; Chang et al., 2018). MACROD2 has been recently shown to be exported from the nucleus upon DNA damage, in ATM activity-dependent manner, in human U2OS cells (Golia et al., 2017). This nuclear export restricts the time that MACROD2 spend at the DNA lesions, which may decrease the net ADP-ribosylhydrolase activity at these sites of damage (Golia et al., 2017). Low expression of MACROD2 is associated with poor prognosis of colorectal cancer patients (van den Broek et al., 2018). A recent breakthrough report documented the generation of MACROD2 knock-out mice (obtained from the KOMP, Jackson Laboratory), which developed normally into adulthood and displayed significantly enhanced intestinal tumorigenesis in the ApcMin/+ susceptible genetic background (Sakthianandeswaren et al., 2018). In the latter study a phenotyping of MACROD2 KO mice was performed at the histological level in animals at 5 months of age, showing no abnormalities (Sakthianandeswaren et al., 2018). In fact, only in carriers of APC inactivating mutations MACROD2 had epistasis effects enhancing the growth of gastrointestinal tumors (Sakthianandeswaren et al., 2018). The generalization of these data pointing at MACROD2 as a tumor suppressor is thus unclear. In our study, using a newly generated mouse model we elucidated for the first time if whole body MACROD2 deletion alone is sufficient to aggravate the deleterious phenotype triggered by DNA damaging – induced by sub-lethal g-irradiation – and by metabolic stressors – obesogenic high fat diet. Our data show convincingly that MACROD2 deletion alone is dispensable in these processes. RNA and protein analyses confirmed efficient deletion of whole MACROD2 transcript and protein by the VelociGene® technology in multiple tissues examined. The MACROD2 KO mice we generated were viable and healthy, indistinguishable from wild type littermates, consistent with the study of Sakthianandeswaren et al., 2018). These authors acquired MACROD2 KO mice from the Jackson Laboratories (Maine, US), which in turn generated them from the targeted VGB6 ES cells originating from KOMP repository. We used the same cells and the same repository for the in house generation of our mice.

Exon 5 of MACROD2 gene was originally found disrupted in Japanese children affected by the Kabuki syndrome, a rare, clinically congenital mental retardation syndrome of unknown etiology, characterized by facial anomalies and mental retardation (Maas et al., 2007; Kuniba et al., 2008). Deletions or SNPs in the gene locus of MACROD2 have been often associated with tumor progression, neurological and psychiatric disorders (Anney et al., 2010; Lionel et al., 2011; Perlis et al., 2012; Cheng et al., 2013; Jahanshad et al., 2013; Linnebacher et al., 2013; Tsang et al., 2013; Jones et al., 2014; Mohseni et al., 2014; Briffa et al., 2015; van den Broek et al., 2015; Hu et al., 2016; Sakthianandeswaren et al., 2018). However, in the case of neurological and psychiatric disorders, such as for autism spectrum disorder (ASD) or for attention deficit hyperactive disorder (ADHD), the MACROD2 association failed to replicate in well-powered cohorts (Bradley et al., 2010; Chen et al., 2015). In fact, although it is known that SNPs in regulatory elements residing within intronic regions can alter silencing, enhancer, or splicing events (Chorev and Carmel, 2012; Lee et al., 2012), further work by *in silico* or multi-array analysis of all known SNPs and mutations in the MACROD2 gene region is required to narrow down on the precise mechanisms responsible for the observed phenotypic effects. For instance, SNPs within intron one of several genes have been shown to influence gene transcription events (Murani et al., 2009; Berulava and Horsthemke, 2010); however none of MACROD2 SNPs reported so far fall within the first intron. At the time of submission of this manuscript, we have interrogated the Catalogue Of Somatic Mutations In Cancer (COSMIC) and retrieved 94 SNPs hitting MACROD2 in various cancers. COSMIC is the world's largest and most comprehensive resource for exploring the impact of somatic mutations in human cancers, offered by the Wellcome Sanger Institute[2]. Additionally, it includes a catalog of genes with mutations that are causally implicated in cancer. Our bioinformatics analysis on SNPs frequencies of the interested genomic regions and functional consequences of COSMIC variants hitting the MACROD2 gene strikingly uncovered that 82 out of 94 SNPs involve exons and that most of these variants were synonymous (18%) or non-synonymous

(58%) amino acid substitutions (**Figure 4**). This could suggest that, at least for human cancers, the vast majority of reported SNPs might affect MACROD2 protein function and stability, and not non-coding regulatory regions within its gene.

Nevertheless, the mouse genetics and phenotyping data presented here argue against an independent or sufficient role of MACROD2 gene in response to metabolic and tumorigenic stresses, in absence of epistatic interactions or of a susceptible genetic background.

AUTHOR CONTRIBUTIONS

MV designed and directed the study and drafted the paper. OLR and MV performed the experiments. TM performed the bioinformatic analyses. All authors edited and approved the final version.

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[2] https://www.sanger.ac.uk/science/tools/cosmic

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Gene Expression and DNA Methylation Alterations During Non-alcoholic Steatohepatitis-Associated Liver Carcinogenesis

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Hepatocellular carcinoma (HCC) is one of the most aggressive human cancers. HCC is characterized by an acquisition of multiple abnormal phenotypes driven by genetic and epigenetic alterations, especially abnormal DNA methylation. Most of the existing clinical and experimental reports provide only a snapshot of abnormal DNA methylation patterns in HCC rather than their dynamic changes. This makes it difficult to elucidate the significance of these changes in the development of HCC. In the present study, we investigated hepatic gene expression and gene-specific DNA methylation alterations in mice using the Stelic Animal Model (STAM) of non-alcoholic steatohepatitis (NASH)-derived liver carcinogenesis. Analysis of the DNA methylation status in aberrantly expressed epigenetically regulated genes showed the accumulation of DNA methylation abnormalities during the development of HCC, with the greatest number of aberrantly methylated genes being found in full-fledged HCC. Among these genes, only one gene, tubulin, beta 2B class IIB (*Tubb2b*), was increasingly hypomethylated and over-expressed during the progression of the carcinogenic process. Furthermore, the *TUBB2B* gene was also over-expressed and hypomethylated in poorly differentiated human HepG2 cells as compared to well-differentiated HepaRG cells. The results of this study indicate that unique gene-expression alterations mediated by aberrant DNA methylation of selective genes may contribute to the development of HCC and may have diagnostic value as the disease-specific indicator.

Keywords: non-alcoholic steatohepatitis, hepatocellular carcinoma, gene specific methylation, H3K4me3, *Tubb2b*

Abbreviations: CGIs, CpG islands; ChIP, chromatin immunoprecipitation; FDR, false discovery rate; GEO, Gene Expression Omnibus; H3K4me3, histone H3 lysine 4 trimethylation; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; MeDIP, methylated DNA immunoprecipitation; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PBS, phosphate-buffered saline; qPCR, quantitative PCR; RRBS, Reduced Representation Bisulfite Sequencing; RSEM, RNA-Seq by Expectation Maximization; STAM, Stelic Animal Model; TCGA, The Cancer Genome Atlas.

INTRODUCTION

Hepatocellular carcinoma (HCC), which accounts for almost 90% of all primary liver malignancies, is one of the most aggressive and enigmatic human cancers with steadily increasing incidence in the United States and worldwide (Llovet et al., 2016; Bertuccio et al., 2017). The development of HCC is associated with well-identified etiological risk factors, including chronic hepatitis B (HBV) and C (HCV) viral infections, chemical exposure, and excessive alcohol consumption (Llovet et al., 2016); however, the contribution of specific risk factors to HCC development varies greatly by geographic location. Currently, chronic HBV infection and exposure to the fungal metabolite aflatoxin B_1 are the predominant risk factors for HCC in Southeast Asia and Africa, whereas chronic HCV infection is the main HCC risk factor in the United States and Western countries (Choo et al., 2016). The contribution of specific etiological factors to the development of HCC is dynamic and expected to change due to recent progress in the primary prevention of HCC induced by HBV and HCV infection, the arrival of a new generation of direct HCV antiviral drugs, and the fast-rising incidence of NAFLD. Recent evidence indicates that non-alcoholic steatohepatitis (NASH) is becoming a prevalent risk factor of HCC, replacing viral hepatitis and alcohol-related liver diseases as the major etiological cause of HCC (Younossi et al., 2015; Marengo et al., 2016; Younes and Bugianesi, 2018; Kim et al., 2019).

In addition to lifestyle and environmental risk factors, HCC is a disease characterized by the presence of multiple heritable abnormal cellular phenotypes driven by genetic (Zucman-Rossi et al., 2015; The Cancer Genome Atlas Research Network, 2017) and epigenetic alterations (Pogribny and Rusyn, 2014; The Cancer Genome Atlas Research Network, 2017). While the role of genetic abnormalities and sequential progression of pathomorphological lesions in liver carcinogenesis are well-characterized, the underlying epigenetic mechanisms, in general, and cancer-related cytosine DNA methylation aberrations, in particular, in the development of HCC are still poorly understood and require special attention. The primary function of cytosine DNA methylation is to defend the genome by controlling the accurate expression of genetic information (Jones, 2012; Ehrlich and Lacey, 2013; Edwards et al., 2017). Cytosine DNA methylation, mainly but not exclusively, functions as a transcriptional "ON-OFF" switch at regulatory regions: the occurrence of DNA methylation at unmethylated CpG sites inhibits transcription, whereas demethylation of methylated CpG sequences activates transcription (Bestor et al., 2015).

Numerous studies have documented profound gene-specific DNA methylation aberrations in key cancer-related pathways in full-fledged HCC (Villanueva et al., 2015; Yamada et al., 2016; Zhang et al., 2016) as well as in preneoplastic lesions (Murphy et al., 2013); however, the majority of the existing reports provide only a snapshot of DNA methylation abnormalities in HCC rather than their dynamic changes. This makes it difficult to clarify the role and functional significance of DNA methylation alterations in the development of HCC. Additionally, while DNA methylation as a mechanism of controlling gene transcription has been studied extensively, the longstanding question of the causality or consequentiality of the concordant gene expression/methylation alterations in carcinogenesis, in general (Baylin and Bestor, 2002; Long et al., 2017), and liver carcinogenesis remains unresolved. Based on these considerations, in this study we investigated the role of gene-specific epigenetic and gene expression alterations in the development of HCC associated with NAFLD using STAM non-alcoholic (NASH)-derived liver carcinogenesis, a model that resembles the development of NASH-associated HCC in humans (Fujii et al., 2013; Takakura et al., 2014).

MATERIALS AND METHODS

Animals, Experimental Design, and Treatments

In the present study, we used liver tissue samples from male mice subjected to NASH-associated hepatocarcinogenesis. This is the first mouse model that mimics the development of HCC in diabetes-associated NASH patients (Fujii et al., 2013; Takakura et al., 2014). The complete experimental design and pathomorphological description of the STAM NASH-related hepatocarcinogenesis model have been described previously by Fujii et al. (2013). Briefly, 2-day-old male C57BL/6J mice were injected with streptozotocin (200 μg/mouse) and were continuously fed a high-fat diet (CLEA Japan, Tokyo, Japan) starting from 4 weeks of age throughout the duration of the study. Control male mice were not injected with streptozotocin and were maintained on standard animal chow for the duration of the study. Liver tissue samples of STAM mice at non-alcoholic fatty liver (NAFL; 6 weeks), NASH-fibrotic (12 weeks), and full-fledged HCC (20 weeks) stages of hepatocarcinogenesis and liver samples from age-matched control mice were purchased from the SMC Laboratories., Inc. (Tokyo, Japan). All experimental procedures were performed according to the Japanese Pharmacological Society Guidelines and experimental protocols were approved by the SMC Laboratories, Inc. Research Animal Care and Use Committee.

Cells and Cell Culture

The human progenitor hepatic HepaRG cell line was obtained from the Biopredic International (Overland Park, KS, United States) and human HCC HepG2 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, United States). The cells were maintained per the manufacturer's recommendations.

In vitro Oleic Acid-Induced Model of NAFL

On the 28th day after the initial seeding, the fully differentiated HepaRG cells were continuously treated with 250 μM oleic acid (Sigma-Aldrich, St. Louis, MO, United States) in the differentiation media for an additional 14 days to induce accumulation of fatty acids (Rogue et al., 2014). The cells were then harvested by mild trypsinization, washed in PBS, and frozen immediately at −80°C for subsequent analyses.

Determination of Triglycerides Accumulation in HepaRG Cells

Triglyceride accumulation in HepaRG cells after oleic acid treatment was quantified using AdipoRed™ Assay Reagent (Lonza, Walkersville, MD, United States). Briefly, the cells were washed in PBS and AdipoRed™ Assay Reagent was added. After a 10-min incubation at room temperature, the fluorescence intensity was quantified using a Synergy™ H4 hybrid multi-mode microplate reader (BioTek, Winooski, VT, United States).

Total RNA Isolation and qRT-PCR

Total RNA was extracted from liver tissue samples of male STAM mice (n = 4/group/treatment) using miRNeasy Mini kits (Qiagen, Valencia, CA, United States). Total RNA (2 μg) was reverse transcribed using random primers and High Capacity cDNA Reverse Transcription kits (Life Technologies, Grand Island, NY, United States), and gene expression was determined by quantitative reverse-transcription PCR (qRT-PCR) using TaqMan gene expression assays and the primers listed in the **Supplementary Table 1**. TATA box binding protein (*Tbp*) was used as an endogenous control. The relative amount of each mRNA transcript was determined using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

Methylated DNA Immunoprecipitation (MeDIP)-Quantitative PCR Analysis

Genomic DNA was isolated from mouse liver tissues of control and STAM mice using DNeasy Blood and Tissue kits (Qiagen). MeDIP was performed with MethylMiner Methylated DNA Enrichment kits (Invitrogen, Carlsbad, CA, United States). Briefly, 1 μg of genomic DNA was isolated from the NAFL (6 weeks), NASH-fibrotic (12 weeks), and full-fledged HCC (20 weeks) tissue samples and from liver tissue samples of corresponding age-matched control mice. The DNA samples were randomly sheared by sonication to an average range of 0.2–1.0 kb. Ninety percent of the sheared DNA was incubated overnight at 4°C with MBD-Biotin protein coupled to M280 Streptavidin Dynabeads. The remaining 10% of the sheared DNA ("input DNA") was used to quantify the amount of DNA used for the MeDIP analysis. The captured methylated DNA was eluted as a single fraction using a high-salt elution buffer and purified by ethanol precipitation. The methylation status of the CGIs located within the 5′-UTR/first exon region of selected genes was determined by qPCR of DNA from immunoprecipitated and unbound DNA using primer sets listed in **Supplementary Table 1**. The results were normalized to the amount of input DNA. The levels of *Gapdh* gene promoter methylation and IAP repetitive element methylation were assessed for the assay performance control (**Supplementary Figure 1A**).

MeDIP-Microarray Analysis

Methylated DNA immunoprecipitation was performed with MagMeDIP kits (Diagenode, Denville, NJ, United States) using 1 μg of genomic DNA isolated from full-fledged HCC (20 weeks) tissue samples and from liver tissue samples of corresponding age-matched control mice. The immunoprecipitated DNA and input DNA pellets were labeled with cyanine 5-dUTP and cyanine 3-dUTP, respectively, using Agilent SureTag DNA Labeling kits (Agilent Technologies, Santa Clara, CA, United States). The data acquisition and analysis was performed as described in Tryndyak et al. (2016). A list of differentially methylated CGIs was generated by calculation of Benjamini–Hochberg adjusted p-values (Benjamini and Hochberg, 1995) to control the FDR in multiple testing data, with an adjusted p-value cut-off 0.05, and a Z-score fold-change threshold of 1.5.

Reduced Representation Bisulfite Sequencing (RRBS) Analysis of Genome-Wide DNA Methylation

Genomic DNA from HepaRG and HepG2 cells was isolated with DNeasy Blood and Tissue Kit (Qiagen) and RRBS libraries were prepared with NEBNext® Ultra™ DNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA, United States) per the manufacturer's protocol. The RRBS library preparation, next-generation sequencing, data analysis, and digital methylation quantitation were performed as described in Tryndyak et al. (2017).

Chromatin Immunoprecipitation Assay

Formaldehyde cross-linking and the ChIP assay, with primary antibody against trimethylated histone H3 lysine 4 (Abcam, Cambridge, MA, United States), were performed using Magna ChIP™ A – Chromatin Immunoprecipitation kits (Millipore Corporation, Burlington, MA, United States). Purified DNA from immunoprecipitated and input DNA was analyzed by qPCR with the same primers used in the MeDIP assay (**Supplementary Table 1**). The results were normalized to the amount of input DNA and presented as fold change calculated from the difference between the DNA from livers of mice from the experimental groups relative to that in control mice. The levels of H3K4me3 enrichment in the gene desert region of chromosome 6 (Mouse Negative Control Primer Set 1; Active Motif, Carlsbad, CA, United States) and in the promoter region of *Gapdh* gene were assessed for the assay performance control (**Supplementary Figure 1B**).

Retrieval of Gene Expression Data From Online Databases

The high-throughput whole genome microarray analyses and the gene expression profiles in the livers of control mice and mice subjected to the STAM model of liver carcinogenesis are detailed in de Conti et al. (2017) (NCBI's GEO database; accession number GSE83596). The gene expression data in HepaRG and HepG2 cells were downloaded from the publicly available GEO dataset (accession number GSE40117). The gene expression in human HCC and tumor pathological data were extracted as .txt files from The Cancer Genome Atlas database (TCGA[1]). The RSEM software package was used for TCGA RNA-Seq gene expression

[1]http://cancergenome.nih.gov

quantitation and normalization (Li and Dewey, 2011). RSEM-normalized expression values were used for differential gene expression analysis.

Statistical Analyses

Results are presented as mean ± SD. Student's *t*-test was used to evaluate significant differences between STAM mice and age-matched control mice at the same time point. One-way analysis of variance (ANOVA), followed by Tukey *post hoc* analysis, was used to evaluate significant differences between the stages during the progression to HCC. When necessary, the data were natural log transformed before conducting the analyses to maintain an equal variance or normal data distribution. Simple linear regression was applied to calculate the trends. Pearson product-moment correlation coefficients were used to determine the relationship between gene expression and gene-specific methylation or level of histone modification. Values of $P < 0.05$ were considered significant.

RESULTS

Methylation Status of Common Differentially Expressed Genes in the Livers of STAM Mice

Previously, we demonstrated that the development of NASH-derived HCC in male STAM mice was characterized by substantial stage-dependent alterations in gene expression as compared to the age-matched control mice (de Conti et al., 2017). Among differentially expressed genes (a twofold change in gene expression and Benjamini-Hochberg adjusted *p*-value <0.05), 60 genes exhibited the same trend of the expression changes at each stage of NASH-associated liver carcinogenesis, at NAFL (6 weeks), NASH-fibrotic (12 weeks), and full-fledged HCC (20 weeks) stages. Analysis of the promoter region of these 60 genes identified 35 CpG island-containing genes that could be epigenetically regulated. This was indicated by the presence of a strong CpG island in the gene promoter regions, based on well-established criteria: greater than 500 bp in length, G+C greater than 55%, and an observed CpG/expected CpG ratio of >0.65 (Takai and Jones, 2002).

Figure 1A shows that in the livers of STAM mice, 32 epigenetically regulated genes were over-expressed and 3 were down-regulated as compared to the age-matched control mice. Among these genes the expression of the *Tubb2b*, *Bmp8b*, *Nusap1*, *Plekhh1*, *Cd24a*, *Smox*, *Eid2*, and *Lect1* genes was altered in a stage-dependent manner.

To determine the role of epigenetic mechanisms in the altered expression of these genes, the status of the promoter region cytosine DNA methylation was investigated. **Figure 1B** shows that the extent of promoter methylation in these 35 genes varied greatly in the livers of control mice ranging from 4 to 90%. To evaluate the functional significance of the DNA methylation changes, a threshold level of ≥20% cytosine methylation was applied. Based on this criterion, eight genes, *Btg2*, *Bmp8b*, *Gipc2*, *Mest*, *Lect1*, *Cadm4*, *Uap1/1*, and *Tubb2*, were detected, and the status of their gene-specific cytosine DNA methylation was investigated (**Figure 1C**). Among these genes, only one, *Tubb2b*, an important microtubule cytoskeleton gene (Gadadhar et al., 2017), was differentially methylated ($P < 0.05$) in NAFL, NASH-fibrotic, and HCC tissue samples as compared to that in control mice, exhibiting a 23, 31, and 45% decrease in the promoter region methylation, respectively (**Figure 1C** and **Supplementary Table 2**). A correlation analysis between gene expression and gene-specific methylation revealed that the methylation status of only the *Tubb2b* gene was inversely correlated ($P < 0.05$) with its expression at each stage of liver carcinogenesis (**Figure 1D**).

Status of Gene-Specific Histone H3K4me3 in NASH-Related Hepatocarcinogenesis

To elucidate the role of an additional epigenetic mechanism in gene expression alterations in NASH-related hepatocarcinogenesis, the status of H3K4me3, a well-established transcription activating modification (Santos-Rosa et al., 2002), was investigated in the promoter region of the 35 genes shown in **Figure 1B**. In contrast to gene-specific cytosine DNA methylation changes, the most prominent histone H3K4me3 alterations, in terms of significance, fold-change, and number of genes, were found in the NAFL, with the extent of histone H3K4me3 gradually diminishing during the progression of hepatocarcinogenesis and the development of HCC (**Figure 2A** and **Supplementary Table 3**). This was evidenced by the fact that the number of genes, especially those that exhibited a ≥2.0-fold change in gene-specific histone H3K4me3, decreased from 27 in NAFL (6 weeks) to nine in NASH-fibrotic (12 weeks) livers and five in full-fledged HCC (20 weeks). Likewise, the number of genes, the expression of which correlated with the level of gene-specific histone H3K4me3 enrichment, decreased with the progression of liver carcinogenesis (**Figure 2B**).

Alterations of the *TUBB2B* Gene in Human Liver HepG2 and HepaRG Cells and in Human HCC

To investigate the role of the *Tubb2b* gene over-expression in the pathogenesis of NASH and NASH-associated liver carcinogenesis, we performed a gene network interaction analysis and identified several co-regulated genes involved in microtubule dynamics and cytoskeleton organization (**Figure 3A**) that were up-regulated in NASH-derived HCC in STAM mice at 20 weeks (**Supplementary Table 4**).

Over-expression of the *TUBB2B* gene was also found in the human HCC HepG2 cell line as compared to non-tumorigenic HepaRG cells (**Figure 3B**) and in human HCC tissue samples (**Figure 3C**). In addition to the over-expression of *TUBB2B*, several other members of the tubulin family of genes were up-regulated in human HCC (**Supplementary Figure 2**). **Figure 3D** shows that over-expression of the *TUBB2B* gene in HepG2 hepatocarcinoma cells was accompanied by the promoter region demethylation as compared to that in HepaRG cells.

To confirm our *in vivo* findings of early up-regulation and demethylation of *Tubb2b* during NAFLD-associated liver

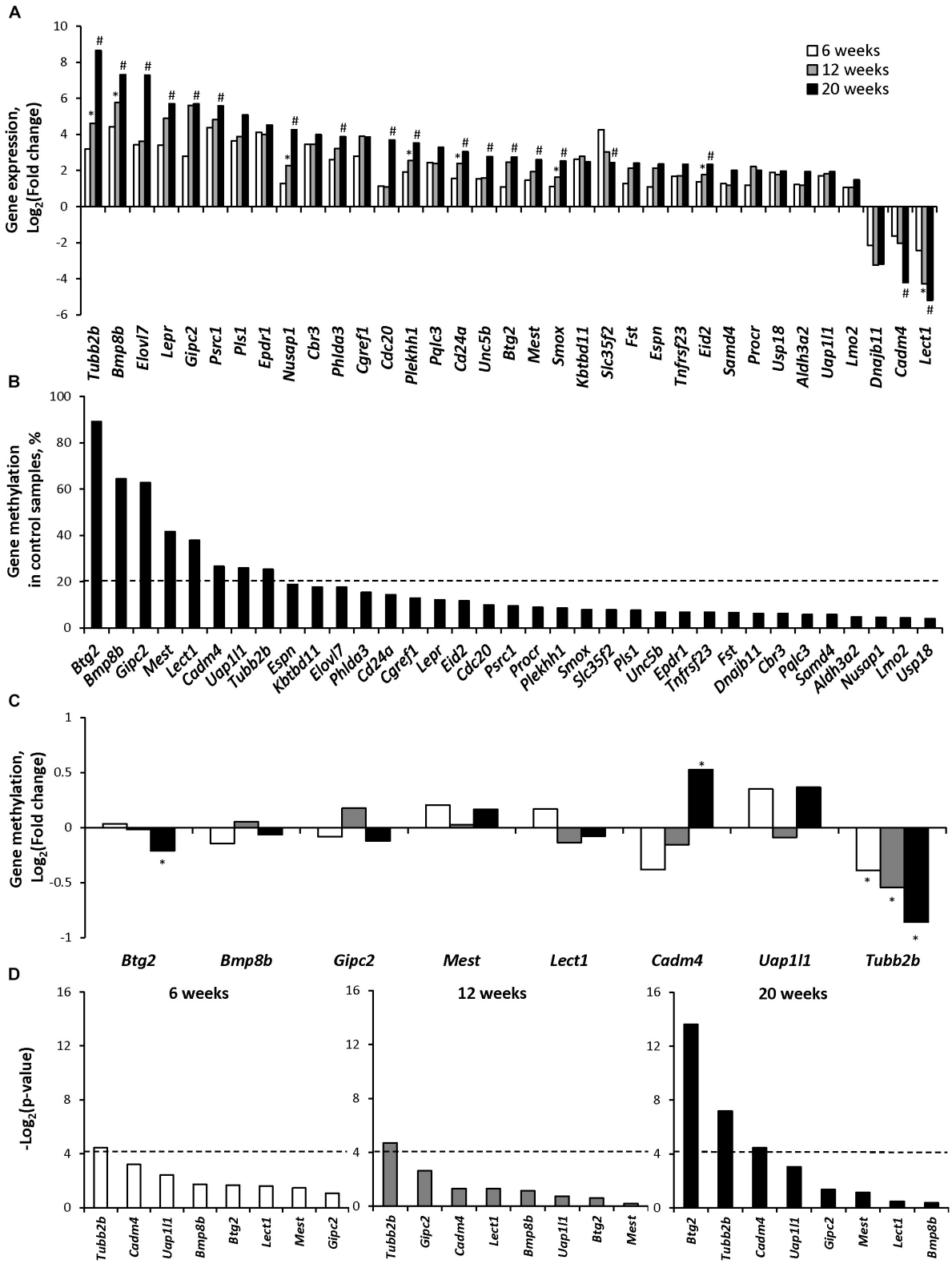

FIGURE 1 | Gene expression and gene-specific methylation of epigenetically regulated differentially expressed genes during NASH-associated liver carcinogenesis in STAM mice. **(A)** Expression of the selected epigenetically regulated genes at NAFL (6 weeks), NASH-fibrotic (12 weeks), and full-fledged HCC (20 weeks) stages of NASH-associated liver carcinogenesis. *Denotes significantly expressed genes ($P < 0.05$) in the livers of STAM mice at 12 weeks as compared to their expression at

(Continued)

FIGURE 1 | Continued
6 weeks. #Denotes significantly expressed genes ($P < 0.05$) in the livers of STAM mice at 20 weeks as compared to their expression at 6 and 12 weeks. **(B)** Gene-specific methylation of selected genes in the livers of control mice. Dashed line indicates a 20% threshold methylation level. **(C)** Gene-specific DNA methylation changes in the promoter region of the upregulated genes that passed 20% threshold DNA methylation level in control livers or genes that were down-regulated during the development of HCC. *Denotes statistically a significant ($P < 0.05$) difference in the promoter region DNA methylation in the livers of STAM mice as compared to that in the livers of age-matched control mice. **(D)** Pearson correlation *P*-value between gene expression and promoter DNA methylation changes in the livers of mice subjected to STAM hepatocarcinogenesis at 6, 12, and 20 weeks. Dashed line indicates the threshold level, which was selected as $P = 0.05$ (Log_2 $P = -4.13$).

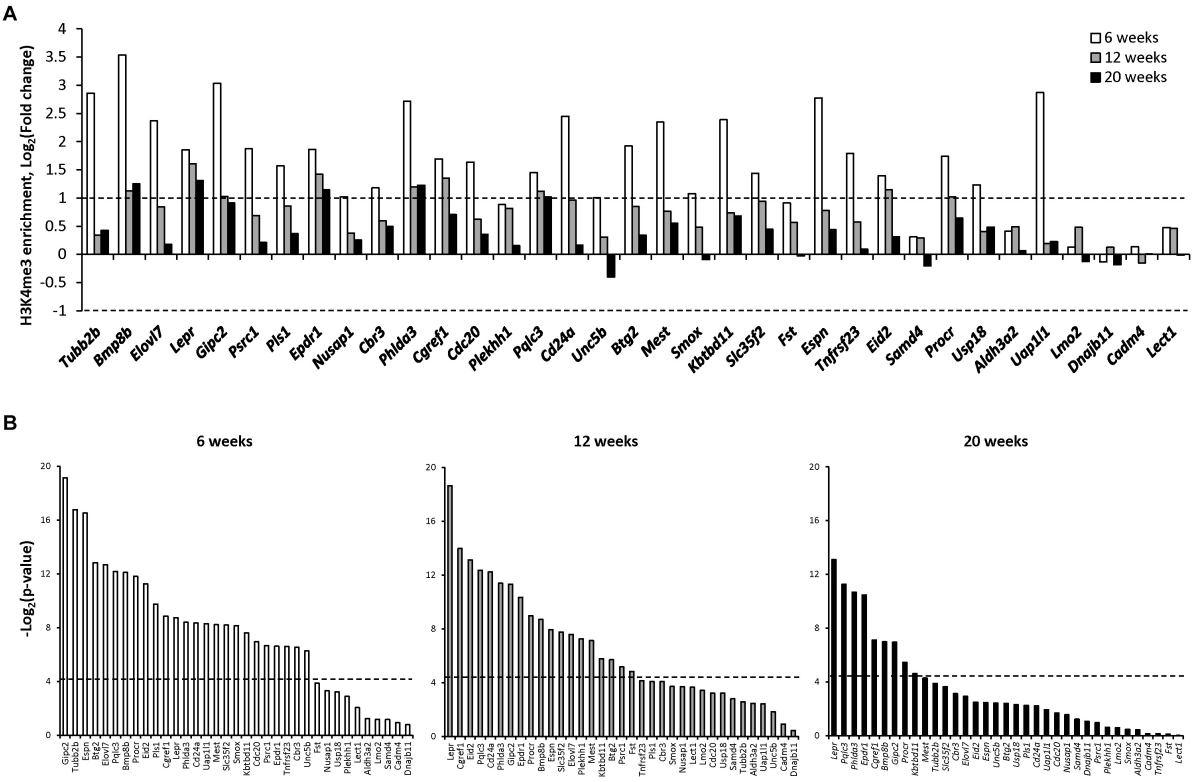

FIGURE 2 | Gene-specific histone H3K4 trimethylation in the livers of STAM mice during hepatocarcinogenesis. **(A)** The changes in the level of histone H3 lysine 4 trimethylation in the promoter region of selected genes at NAFL (6 weeks), NASH-fibrotic (12 weeks), and full-fledged HCC (20 weeks) stages of NASH-associated liver carcinogenesis. Dashed line indicates threshold level, which was selected as a twofold change comparing to control age-matched mice (Log_2 fold change = +1 and −1). **(B)** Pearson correlation *P*-value between gene expression and change in the promoter region histone H3 lysine 4 trimethylation in the livers of mice subjected to STAM hepatocarcinogenesis at 6, 12, and 20 weeks. Dashed line indicates the threshold level, which was selected as $P = 0.05$ (Log_2 $P = -4.13$).

carcinogenesis, we cultured human liver HepaRG cells in the presence of 250 μM oleic acid in the differentiation media for 14 days. We found that accumulation of triglycerides in HepaRG cells (**Figure 3E**) was accompanied by increased expression of *TUBB2B* (**Figure 3F**) and decreased methylation (by 21%) of the *TUBB2B* promoter region (**Figure 3G**). These findings provided independent evidence of the involvement of promoter DNA hypomethylation-associated over-expression of *TUBB2B* in the pathogenesis of NAFLD.

Gene-Specific Methylation of Uniquely Differentially Expressed Genes in HCC

Considering the minimal association between gene expression and epigenetic alterations in the genes expressed in common during the hepatocarcinogenic process, the status of cytosine DNA methylation was investigated in genes differentially expressed only in HCC tissue samples. The results of high-throughput whole genome microarray gene expression and MeDIP microarray analyses demonstrated extensive gene expression and cytosine DNA methylation changes in the HCC samples. A total of 1563 genes was differentially expressed and 855 promoter CpG island-containing genes were differentially methylated; however, a combined analysis of differentially expressed and differentially methylated genes revealed a limited overlap in the number of differentially expressed and differentially methylated genes (**Figure 4A**). Specifically, only 34 genes exhibited an inverse correlation between expression and methylation (**Supplementary Figure 3**), among which only eight genes had a methylation level in HCC-tissues greater than

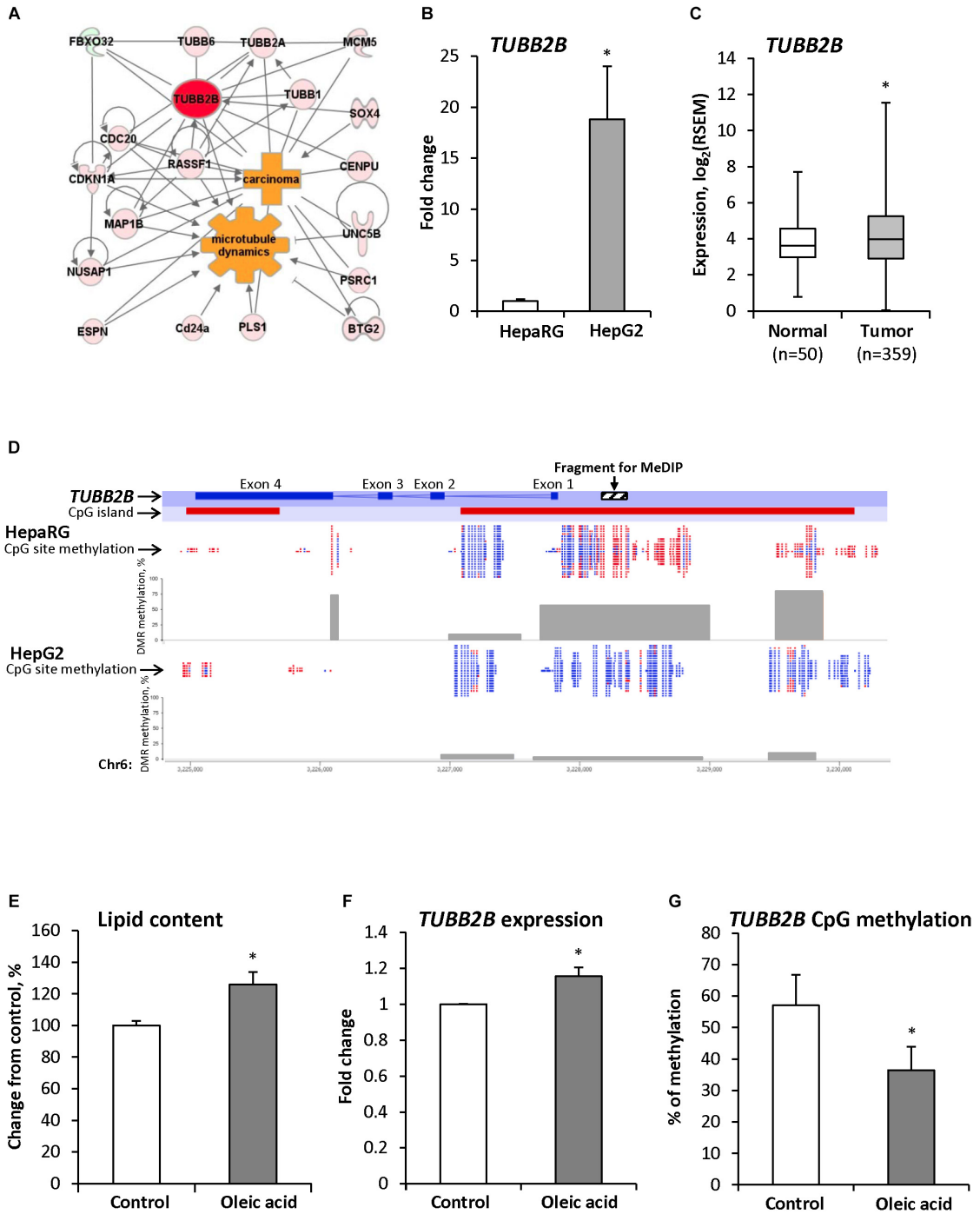

FIGURE 3 | *TUBB2B* gene expression and methylation aberrations during hepatocarcinogenesis. **(A)** Molecular network of differentially expressed genes associated with *Tubb2b* and microtubule dynamics in HCC of mice subjected to STAM hepatocarcinogenesis. Red color indicates up-regulated genes and green color indicates down-regulated genes. **(B)** Expression of the *TUBB2B* gene in non-tumorigenic liver HepaRG cells and human HepG2 hepatocarcinoma cells. The *TUBB2B* gene expression is presented as an average fold change in HepG2 cells relative to that in the fully differentiated HepaRG cells, which was assigned value 1. **(C)** Expression of the *TUBB2B* gene in human HCC samples. **(D)** RRBS analysis of DNA methylation in the *TUBB2B* gene promoter region in HepaRG and HepG2 cells. Red color indicates methylated CpG sites and blue color indicates unmethylated CpG sites. Gray boxes indicate percent of methylated CpG sites from total number of CpG sites in the differentially methylated regions (DMR). **(E)** The level of triglycerides in the fully differentiated human hepatic HepaRG cells after culturing cells the presence of 250 μM oleic acid for 14 days. **(F,G)** Expression and promoter DNA methylation of the *TUBB2B* gene in fully differentiated HepaRG cells after culturing cells in the presence of 250 μM oleic acid for 14 days. The *TUBB2B* gene expression is presented as an average fold change in the HepaRG cells treated with oleic acid relative to the gene expression in non-treated HepaRG cells, which was assigned value 1. Values are mean ± SD, $n = 3$. *Denotes statistically a significant ($P < 0.05$) difference of gene expression and promoter DNA methylation.

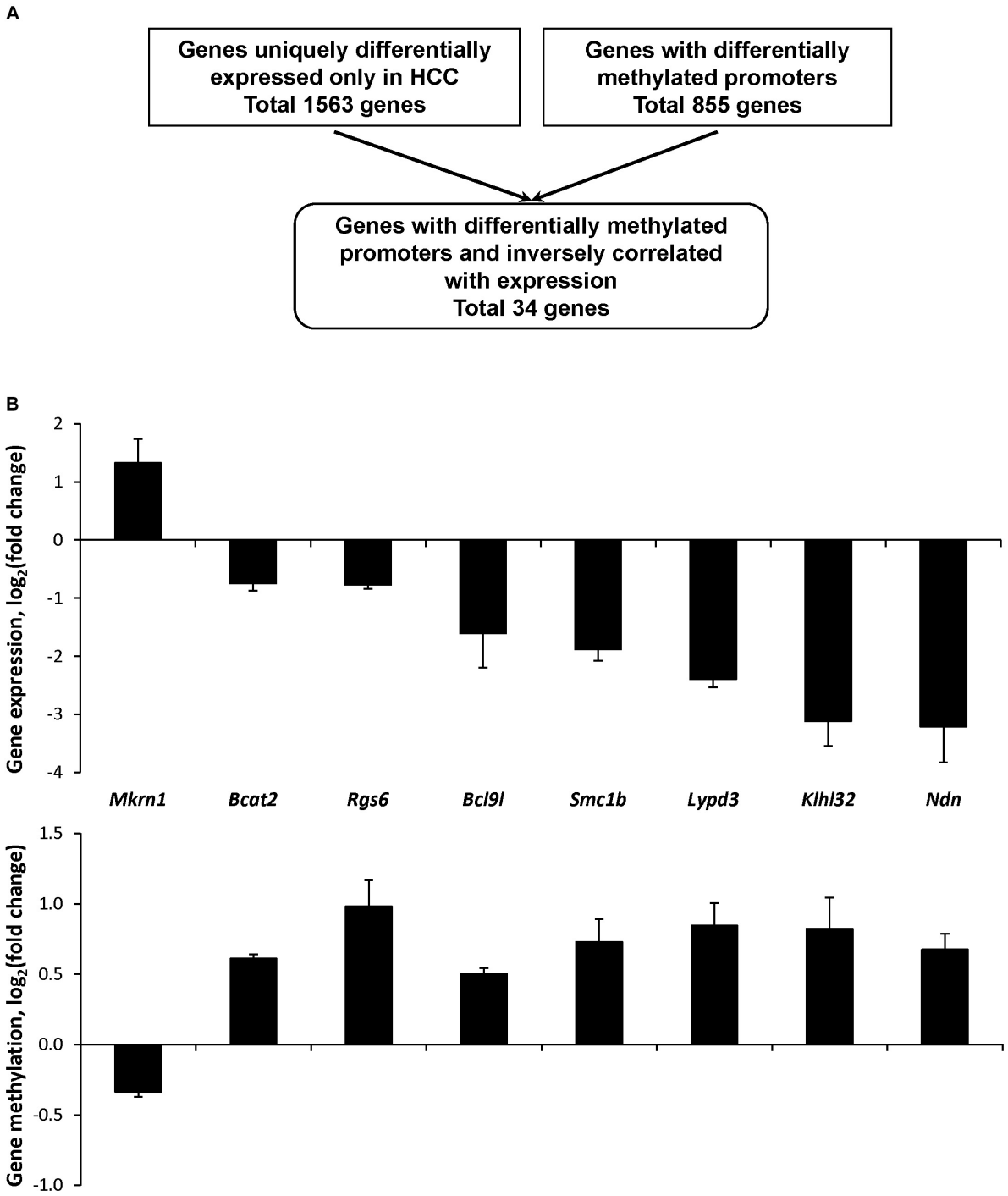

FIGURE 4 | Expression and promoter DNA methylation of uniquely differentially expressed CpG island-containing genes in HCC in STAM mice at 20 weeks. **(A)** Algorithm for the selection of genes in HCC with differentially methylated promoters and inversely correlated with gene expression. **(B)** Gene expression and promoter DNA methylation of the selected genes differentially expressed in HCC. The results of gene expression and DNA methylation are presented as an average $\log_2$ fold change in the livers of mice with full-fledged HCC relative to respective values of age-matching control mice, which were assigned a value 1.

20% (**Supplementary Table 5**). Of these eight genes, seven were hypermethylated and down-regulated (**Figure 4B**).

To investigate the role of these genes uniquely expressed in HCC during the process of liver carcinogenesis, their expression and gene-specific methylation were investigated in NAFL and NASH-fibrotic tissues. No significant changes in the expression and cytosine methylation were found at the pre-HCC stages of liver carcinogenesis (**Supplementary Table 5**).

Expression of Chromatin Modifying Genes in NASH-Related Hepatocarcinogenesis

To investigate the mechanism of the observed cytosine DNA methylation and histone H3K4me3 alterations in the livers of STAM mice, the expression of chromatin modifying genes was investigated. The expression of the DNA methyltransferase genes *Dnmt1*, *Dnmt3a*, and *Dnmt3b* and the DNA demethylase genes *Tet1* and *Tet2* was significantly up-regulated in HCC samples only, while the expression of *Uhrf1* gradually increased throughout liver carcinogenesis (**Figure 5**). In contrast, the expression of histone H3K4 methylase genes, *Kmt2a*, and *Setd7*, gradually decreased during carcinogenesis, whereas the expression of the histone H3K4 demethylase gene *Kdm1a* increased, reflecting the dynamics of histone H3K4me3 alterations (**Figure 5**).

DISCUSSION

Profound alterations have been shown in HCC in the pattern of cytosine DNA methylation for multiple cancer-related genes, a well-recognized common mechanism of gene transcription regulation (Zhang et al., 2016; The Cancer Genome Atlas Research Network, 2017). This has led to the suggestion that aberrant cytosine DNA methylation may contribute to the hepatocarcinogenic process. Nonetheless, most human studies have provided a static snapshot of gene expression and gene-specific epigenetic alterations in HCC that does not necessarily clarify the relative contribution of these aberrations in the hepatocarcinogenic process.

In this study, using the STAM mouse model of NASH-derived liver carcinogenesis that depicts the sequential development of clinical and pathomorphological characteristics of NASH in diabetic patients, we demonstrated that NASH-related liver carcinogenesis is characterized by progressive accumulation of gene expression and gene-specific DNA methylation changes, with the greatest magnitude being found in full-fledged HCC; however, it is highly unlikely that all transcriptomic and DNA methylation aberrations found in full-fledged HCC may have significance in the development of HCC and its progression. To uncover alterations that may drive NASH-related liver carcinogenesis, we focused our investigation on 60 differentially expressed genes exhibiting the same trend of the expression changes at each stage of NASH-associated liver carcinogenesis. The results of our study showed that 35 out of 60 differentially expressed genes can be epigenetically regulated, as evidenced by the presence of CGIs in the promoter regions. Analysis of the promoter methylation status of these genes revealed that only the *Tubb2b* gene exhibited simultaneous methylation and gene expression changes during the carcinogenic process. This indicates that the gene expression changes in most of the differentially expressed genes are independent of promoter cytosine methylation and preceded the appearance and accumulation of gene-specific DNA methylation alterations in this model of liver carcinogenesis. This was further evidenced by a greater occurrence of gene promoter DNA methylation changes in full-fledged HCC compared to NAFL and NASH-fibrotic stages.

This finding is supported by growing evidence of the independence of gene expression changes from the gene promoter DNA methylation, especially at early stages of disease development or intervention. For instance, McKay et al. (2016) reported that feeding female C57BL/6J mice a low-folate (400 μg folic acid/kg) diet resulted in altered expression of 989 genes in male fetal liver, among which only 16 genes exhibited DNA methylation changes. Furthermore, several studies have demonstrated the existence of DNA methylation-independent changes in the expression of epigenetically regulated genes (Diesel et al., 2011; Espada et al., 2011; Navasa et al., 2015). Additionally, this finding is in good agreement with the suggestion that not all epigenetic alterations are equally important for the carcinogenic process; some may be drivers and trigger other molecular processes leading to neoplastic cell transformation, whereas others may be passenger events accompanying the transformation process and be a feature of transformed phenotype. In this respect, our finding of concurrent over-expression and promoter hypomethylation of the *Tubb2b* gene in this model of liver carcinogenesis is of special interest.

The *Tubb2* gene belongs to a gene family encoding several microtubule cytoskeleton α- and β-tubulin protein isotypes. Tubulins are essential for every eukaryotic cell, controlling cell shape, division, motility, and differentiation (Gadadhar et al., 2017). The fundamental mechanism that underlies a proper microtubule organization is the regulation of the levels of α- and β-tubulin isotypes. In normal cells, the composition of tubulin isotypes is tightly controlled and maintained, while in a broad range of cancer cells, including HCC cells, this composition is perturbed, and the expression of several tubulin isotypes is up-regulated. For example, Sun et al. (2007) demonstrated an over-expression of the *Tubb2*, *Tubb3*, and *Tubb6* genes and other cytoskeletal genes in HBV-related HCC in *Hbx* transgenic mice. Up-regulation of TUBA6, TUBA8, and TUBB3 has been reported in human liver cancer (Kuramitsu et al., 2011; Zen et al., 2014; Rein-Fischboeck et al., 2017). An increased expression of tubulin isotypes has been found not only in full-fledged HCC, but also in preneoplastic livers (Sun et al., 2007; Rein-Fischboeck et al., 2017). These findings are in good correspondence with the results of our study that showed a stage-dependent *Tubb2b* over-expression during NASH-associated liver carcinogenesis and the up-regulation of the *TUBB2B* gene in HepaRG cells subjected to the oleic acid-induced model of NAFL.

It is well-established that the regulation of the tubulin isotype composition, the "tubulin code," is mediated by (i) expression of different α- and β-tubulin isotypes and (ii) post-transcriptional modifications of tubulins (Gadadhar et al., 2017). While the mechanisms of post-translational tubulin modifications are well-investigated, mechanism of transcriptional regulation of tubulin gene expression is less well studied. In view of this, the result of our study showing the involvement of epigenetic mechanisms in the regulation of *Tubb2b* expression, which

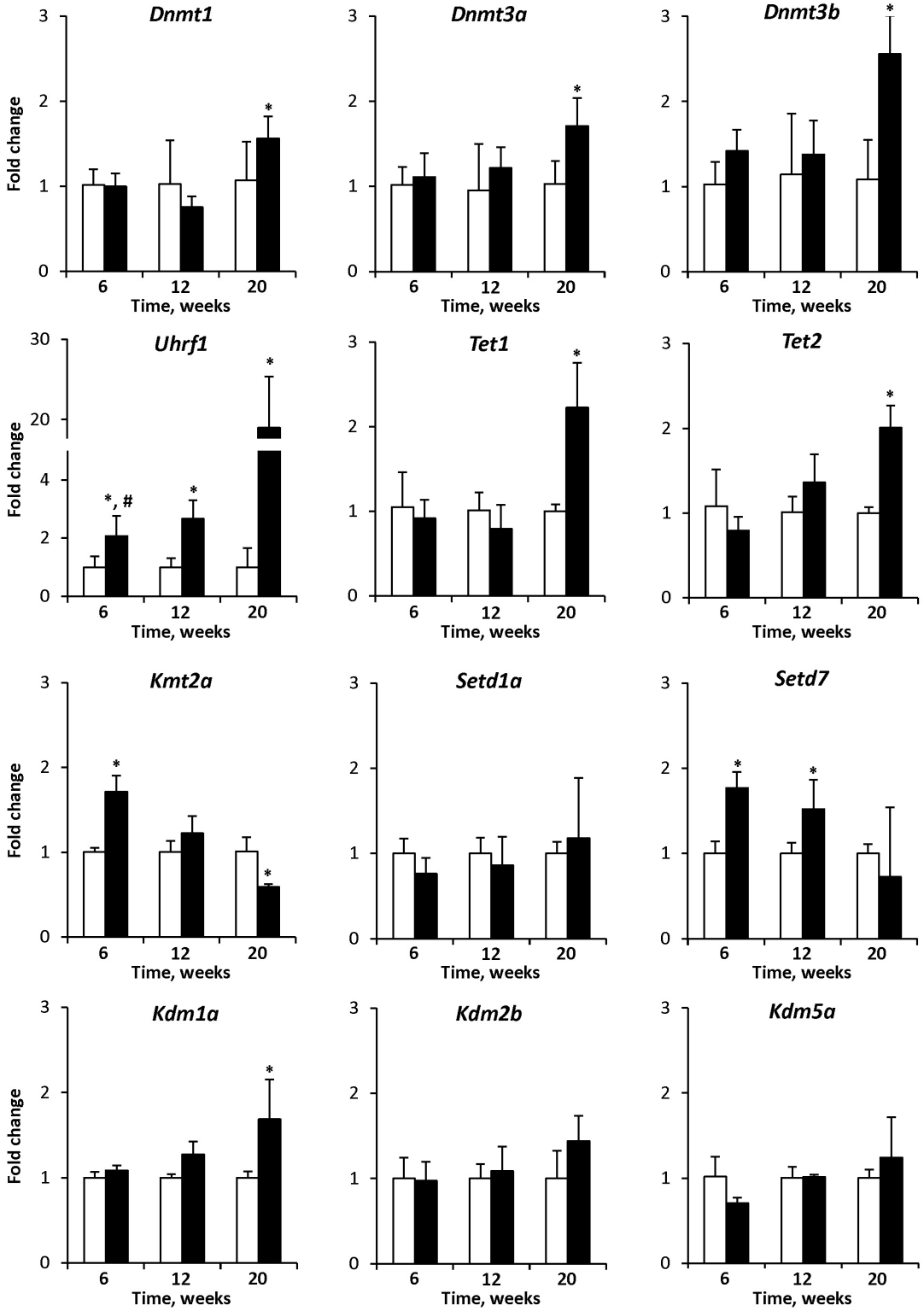

FIGURE 5 | The expression of chromatin modifying genes in the livers of control mice and STAM mice subjected to NASH-derived hepatocarcinogenesis. The results are presented as an average fold change in the expression of each gene in the livers of STAM mice at 6, 12, and 20 weeks relative to that in control age-matching mice. The data are presented as the mean ± SD; $n = 4$. *Denotes a significant ($P < 0.05$) difference from the control age-matching group; #denotes significant ($P < 0.05$) trend.

was evidenced by simultaneous and progressive *Tubb2b* overexpression and gene promoter DNA hypomethylation during hepatocarcinogenesis and in HepaRG cells subjected to the oleic acid-induced model of NAFL, are of special interest. This finding is in good agreement with growing evidence on the role of DNA demethylation, including genome-wide (Yamada et al., 2005), global enhancer (Xiong et al., 2019), microRNA (Nojima et al., 2016), and gene-specific (Li et al., 2019) hypomethylation in the pathogenesis of HCC.

In summary, this report shows that the development of NASH-derived HCC is characterized by progressive accumulation of DNA methylation and gene expression alterations, with the greatest level of these abnormalities being found in full-fledged HCC. The results of the study illustrate that not all DNA methylation changes have an equal importance in the carcinogenic process. This was evidenced by the fact that majority of gene-specific DNA methylation changes occurred at the later stages of hepatocarcinogenesis, especially in full-fledged HCC, and were preceded by gene expression changes. In this respect, the observed concurrent and progressive *Tubb2b* expression and promoter methylation changes during the development of NASH-associated liver carcinogenesis are of great importance and indicate that unique *Tubb2b* gene-expression alterations mediated by aberrant DNA methylation may contribute to the development of HCC and may be used as the disease-specific indicator. Nevertheless, this study, as well as other studies using only male mouse models of NASH-associated liver carcinogenesis only (Asgharpour et al., 2016; Tsuchida et al., 2018), does not provide the answer whether similar alterations exist in female mice. Therefore, future studies are needed to address this question. The results of these studies may identify early sex-independent diagnostic biomarkers of NASH that may be useful for monitoring disease stratification.

ETHICS STATEMENT

All experimental procedures were performed according to the Japanese Pharmacological Society Guidelines and experimental protocols were approved by the SMC Laboratories, Inc., Research Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

AC, FB, and IP designed the study. KD, VT, and AC performed the experiments. All authors participated in analyzing and interpretation of the data, discussion of the results, and writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2019.00486/full#supplementary-material

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Preclinical and Clinical Evidence for a Distinct Regulation of Mu Opioid and Type 1 Cannabinoid Receptor Genes Expression in Obesity

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Among endogenous signaling networks involved in both rewarding and homeostatic mechanisms of obesity, a relevant role is played by the endocannabinoid (ECS) and the opioid (EOS) systems. We here studied the transcriptional regulation of ECS and EOS genes in the hypothalamus of Diet-induced obesity rats, a preclinical model of obesity, as well as in humans with obesity and healthy controls. A significant and selective increase in type 1 cannabinoid receptor gene (*Cnr1*) expression was observed at the beginning of obesity development (5 weeks on high fat diet) as well as after 21 weeks of high diet exposure. After 5 weeks on high fat diet, selective up-regulation of mu opioid receptor gene (*Oprm1*) expression was also observed. Consistently, epigenetic studies showed a selective and significant decrease in DNA methylation at specific CpG sites at both gene promoters in overweight rats, but only after 5 weeks on high fat diet. Moreover, significantly lower levels of DNA methylation were observed at selected CpG sites of both receptor gene promoters, analyzed in peripheral blood mononuclear cells from younger (<30 years old) humans with obesity, as well as in those with shorter time length from disease onset. Taken together, we here provide evidence of selective, synergistic and time-dependent transcriptional regulation of *CNR1* and *OPRM1* genes in overweight rats, as well as in human subjects. These alterations in genes regulation could contribute to the development of the obese phenotype, and we thus suggest *CNR1* and *OPRM1* epigenetic modulation as possible biomarkers of obesity development. Due to the reversible nature of the epigenetic hallmark, our data might also open new avenue to early environmental strategies of intervention.

Keywords: obesity, endocannabinoid system, opioid system, DNA methylation, biomarker

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INTRODUCTION

Obesity is a growing public health threat, potentially affecting emotional, and physical health with a relevant mortality rate and a high burden of disease for Western societies. Core symptoms of this disease are disturbance of eating habits and inability to control body weight, thus leading to an imbalance between energy intake and expenditure. In recent years several approaches have been used to treat obesity, yet the efficacy of the few medications proven useful remains a matter of debate. Thus, effective and safe treatments are urgently needed (Patel and Stanford, 2018). Efforts should be made to disclose the causes that make individuals more vulnerable to the development of obesity, in order to gain useful information for alleviating or even preventing occurrence of this condition. Genetic predisposition has been implicated in disease susceptibility (Stice, 2002; Striegel-Moore and Bulik, 2007), however, differences in phenotype heterogeneity point also to the relevant role of the environment and lifestyle (Hill et al., 2003). Molecular research has formed the basis to understand how environmental factors (i.e., dieting) may facilitate disease progression by engaging epigenetic mechanisms (van Dijk et al., 2015), helping also to understand the increasing epidemic of the disease in the last years within the same genome. It is in fact well known that the latter mechanisms can evoke transient changes (although how long they actually last is unclear) in gene expression, involving chemical modifications of DNA that do not affect the actual DNA sequence of the organism. The understanding of how transcriptional regulation might affect individual risk of developing obesity represents a major challenge in research and may provide invaluable help for the development of preventive strategies, or of more effective therapeutics. Central regulation of food intake is rather complex, and thus different endogenous key players should be considered. For instance, it is clear that rewarding properties of food, possibly leading to drug-like food addiction (D'Addario et al., 2014; Mancino et al., 2015), are responsible for disease development and share neuronal pathways that seem to overlap with drug addiction (Volkow and Wise, 2005). These neural circuitries, driving eating behaviors in the brain to ensure food-intake and to regulate caloric balance, are thus controlled not only by homeostatic mechanisms but also by reward systems to promote motivational, hedonically driven feeding (Coccurello and Maccarrone, 2018). Among the endogenous systems involved in both rewarding and homeostatic mechanisms, a relevant role is played by both the endocannabinoid (ECS) and the opioid (EOS) systems (Cota et al., 2003a; Tanda and Goldberg, 2003), which functionally interact with each other in mediating neurological functions (Manzanares et al., 1999; Tanda and Goldberg, 2003). In feeding regulation, both ECS and EOS seem to contribute to the reward aspect of eating (Saper et al., 2002; Yeomans and Gray, 2002; Cota et al., 2003b). Of note, several preclinical studies reported that both opioid and cannabinoid receptor agonists stimulate food intake (Reid, 1985; Foltin et al., 1988; Williams et al., 1998; Glass et al., 1999; Williams and Kirkham, 1999), that is instead reduced by antagonists (Recant et al., 1980; Marks-Kaufman et al., 1984; Colombo et al., 1998; Di Marzo et al., 2001; Glass et al., 2002; Hildebrandt et al., 2003; Ravinet Trillou et al., 2003; Statnick et al., 2003; Vickers et al., 2003) with suppression of body weight gain in rodents. It has been also observed in human trials that opioid antagonists (naloxone, naltrexone, or nalmefene) may be helpful in the short term to suppress appetite, even though caution should be taken for possible long term use because of side effects and limited weight loss (Nathan and Bullmore, 2009). In this context, it should be recalled that rimonabant, a selective type 1 cannabinoid receptor (CB_1) antagonist able to reduce body weight (Rinaldi-Carmona et al., 1995; Di Marzo and Despres, 2009), was an approved drug on the European market, but was withdrawn because of an increased risk of psychiatric disorders (Johansson et al., 2009).

Moreover, combination therapies [i.e., *Contrave* (Apovian et al., 2013) and *Qysmia* (Gadde et al., 2011)] also held promise for obesity treatment, and preclinical studies performed using a combination of opioid and cannabinoid antagonists (naloxone and nor-BNI with rimonabant) demonstrated enhanced feeding reduction when compared to that evoked by rimonabant alone (Lockie et al., 2011, 2015).

Against this background, the aim of this study was to advance our knowledge about the molecular mechanisms engaging ECS and EOS in the development of obesity, in order to identify disease biomarkers and disclose new molecular clues to be targeted by innovative strategies of pharmacological intervention. By using a well-established rat model of Diet-Induced Obesity (DIO), we first investigated regulation of ECS and EOS genes expression during disease progression in the hypothalamus, a brain region involved in appetite regulation (Bazhan and Zelena, 2013), where the role of ECS (Berthoud, 2012; Zeltser et al., 2012; Ramírez-López et al., 2015) and EOC (Dum et al., 1983; Nogueiras et al., 2012; Romero-Picó et al., 2018) in feeding response modulation has been deeply investigated and already reported. We followed gene expression regulation at the beginning of obesity development (i.e., after 5 weeks on high fat diet), as well as when the phenotype was well-established (i.e., after 21 weeks on high fat diet). Among the epigenetic mechanisms possibly responsible for altered gene expression, we focused on DNA methylation, that consists of a methyl group transfer to the position 5 of the cytosine pyrimidine ring in a cytosine guanine dinucleotide (CpG), which ultimately blocks the binding of transcription factors thus causing chromatin compaction and gene silencing (Zhu et al., 2016). Of note, by using the same DIO animal model considered here, we already showed the epigenetic regulation of other obesity genes in the progression of the disorder (Cifani et al., 2015).

In addition, in this work, we also analyzed the DNA methylation status of key ECS and EOS genes in peripheral blood mononuclear cells (PBMCs) from a subset of humans with obesity. It is important to recall that PBMCs contain the complete epigenetic machinery present in neurons (Arosio et al., 2014), and are considered a convenient substitute for cerebral markers (Woelk et al., 2011) that are readily accessible and reflects the molecular processes occurring in the central nervous system (Gladkevich et al., 2004). PBMCs have been already proposed in obesity studies as a valuable tool to monitor metabolic recovery in weight loss strategies (Reynés et al., 2015), and thus their study in this disorder is of clear relevance to detect altered pathways

(metabolic or signaling) that could be transferred to the brain. Moreover, it has been well-documented since many years the activation of the ECS in human obese subjects and the regulation of EOS components in obesity and related eating disorders in the periphery (Jimerson and Wolfe, 2004).

MATERIALS AND METHODS

Animals and Diet Composition

Male Sprague Dawley rats (Charles River; total $n = 38$; 225–250 g, 7 weeks old at the beginning of the experiments) were used. Rats were housed in individual cage sunder 12:12 h light/dark cycle (lights on at 9:00 a.m.) with access to food and water *ad libitum* for 2 weeks before the experiments. They were kept in a room at constant temperature (20–22°C) and humidity (45–55%). All procedures involving rats were carried out in accordance with the Institutional Guidelines and complied with the Italian Ministry of Health and associated guidelines from European Communities Council Directive. Rats were randomly divided into two groups with comparable mean body weight (no significant difference). The first group ($n = 16$) was the control group and was fed with standard laboratory chow *ad libitum* (4RF18, Mucedola, Settimo Milanese, Italy; 2.6 kcal/g); the second group ($n = 22$), was fed with high energy diet (45% fat, 35% Carbohydrate, 20% Protein) *ad libitum* (D12451, Research Diets, Inc., New Brunswick, NJ, United States; 5.24 kcal/g). After 5 weeks, 6 of the 22 rats fed with high fat diet did not significantly increase body weight in comparison to rats fed with chow. These resistant rats were excluded from the study (Cifani et al., 2015) because they did not develop obese phenotype. At the end of the 5 weeks, eight Chow and eight DIO rats were sacrificed by decapitation. The remaining animals (additional eight Chow and eight DIO rats) were maintained on their respective diets for 21 weeks, and then were sacrificed. Brains were quickly removed and the whole hypothalamus was manually dissected (from Bregma level −0.26 to Bregma level −4.20) (Paxinos and Watson, 1998). The tissues were collected and stored at −80°C until further analysis. Body weight and food intake were daily recorded (**Figure 1**).

Human Subjects

Patients and Methods

Editorial policies and ethical considerations

The study, accomplishing the Declaration of Helsinki, was approved by the Ethic Committee of IRCCS – Istituto Auxologico Italiano (RBFR12DELS_004/05C302_2013), and all patients or their tutors gave a written informed consent.

Study population

The entire cohort consists of 63 humans with obesity recruited from the San Giuseppe Hospital's and San Luca Hospital's (Istituto Auxologico Italiano) since 2012. Clinical characteristics of our cohort are reported in **Table 1**. Anonymous patient data, referred to the time of diagnosis, were collected either prospectively or retrospectively and a clinical database was created. We performed the same epigenetic analysis in 58 non-obese blood donors matching for age and sex (Males: No. = 32, age = 42.27 ± 2.97; Females: No. = 26, age 40.87 ± 4.12).

Data collection

In this study all humans with obesity had physical examination and biochemistry analyses. To perform the body weight and height measurements, subjects were dressed in light clothing without socks and shoes. Weight was measured (in kg to two decimal places) with a digital balance scale. Height was measured with a wall-mounted stadiometer (to the nearest millimeter) using the stretch technique. Body mass index (BMI) was calculated using the Quetelet's formula as weight (in kg) divided by height (in m^2). Systolic (SBP) and diastolic (DBP) blood pressure were measured in the seated position with a Tema Certus sphygmomanometer and an appropriately sized cuff on the right arm. These values were obtained as the average of three measurements. Blood samples were separated by centrifugation after clotting, and aliquots of serum or plasma supernatants were processed for routine measurements and stored at −80°C until assay. Routine laboratory data included levels of glucose, total cholesterol, high-density (HDL) and low-density lipoprotein (LDL) cholesterol, triglycerides (TG) determined by enzymatic assays (Roche Diagnostics GmbH, Mannheim, Germany). Anthropometrical and biochemical measurements were made once for each participant. According to the World Health Organization guidelines, obesity was defined as BMI $\geq$ 30 kg/m^2 (WHO, 2000).

Non-obese blood donors were selected in accordance with National mandatory standards for blood donor selection. They were known to be free from chronic disease (including diabetes, hypertension, dyslipidemia, and cardiovascular disease), not taking any drugs and with BMI $<$ 29.9 kg/m^2.

Molecular Biology Studies

Real-Time Quantitative PCR

Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific) according to the specifications of the instructions, from rat dissected Hypothalamus and from PBMCs, separated by density gradient using the Lympholyte-H kit (Cedarlane Laboratories, Canada). Each 0.5 μg of total RNA was reverse-transcribed into complementary DNA using a RevertAid RT Reverse Transcription Kit (Thermo Scientific). Random hexamers and oligo-dT primers were used in the RT reaction in an unbiased manner Quantitative PCR was performed using an Applied Biosystems 7500 Real-time PCR system (Thermo Fisher Scientific) with SensiFAST SYBR Low-ROX kit (Bioline) in a total reaction volume of 10 μl according to the manufacturer's instructions. The thermal cycles were as follows: initial denaturation at 95°C for 2 min; 45 cycles of denaturation at 95°C for 15 s; annealing and extension at 60°C for 15 s. Relative mRNA expression levels were calculated using the 2^{-DDCt} method and normalized to two internal control, β-actin and GAPDH. The primers used for the amplification of ECS and EOS genes are reported in **Supplementary Table S1**.

DNA Methylation Analysis by Pyrosequencing

Genomic DNA was extracted from hypothalamic region and PBMC by using TRIzol Reagent (Life Technologies)

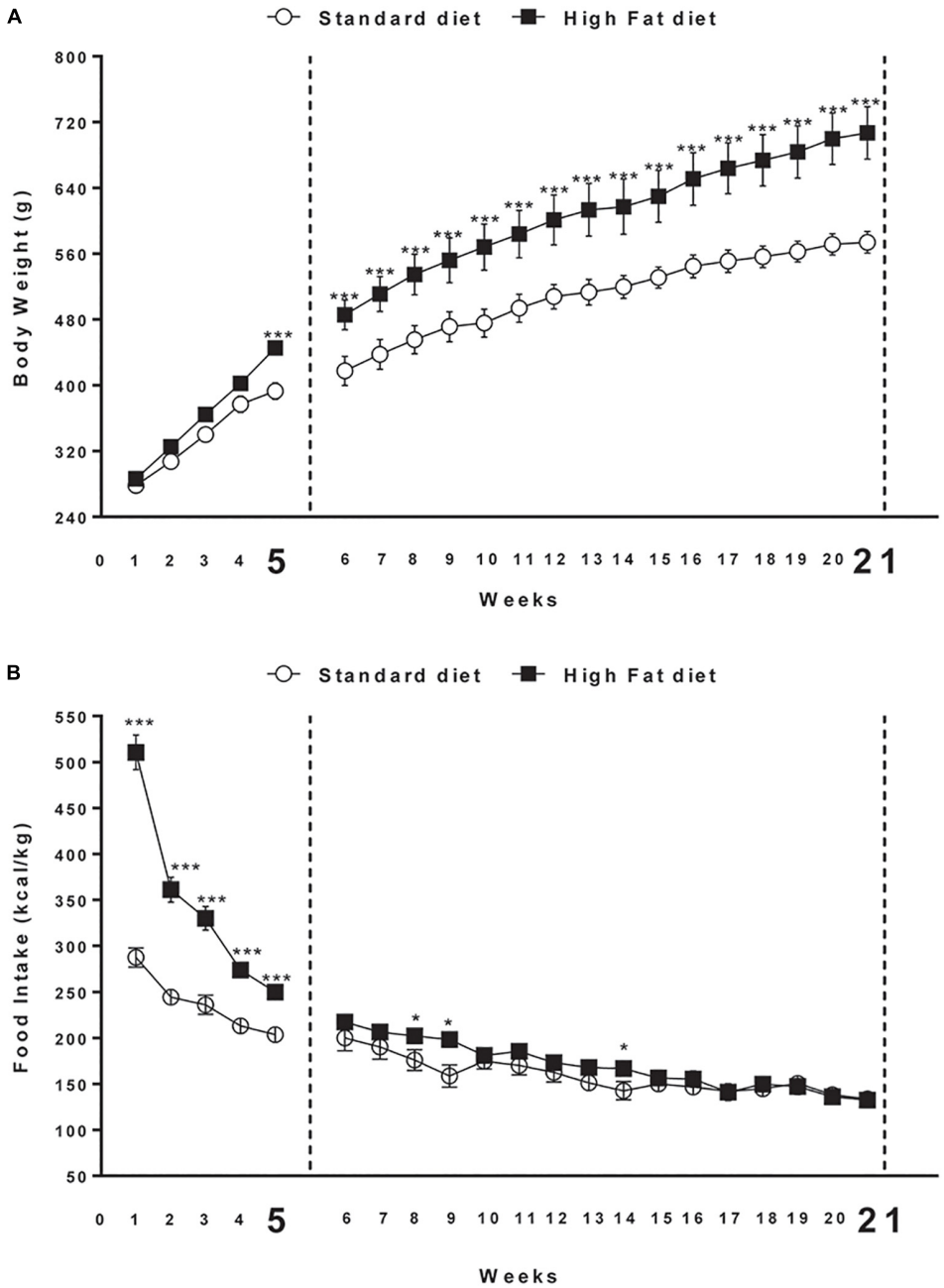

FIGURE 1 | (A) Body weight and **(B)** cumulative food intake (kcal) measured weekly in rats exposed for 5 and 21 weeks to high fat (HFD) or standard diet (STD). Significant differences are indicated: ***$P < 0.001$, *$P < 0.05$ vs. STD.

with the concentration and purity detected by NanoDrop spectrophotometer (NanoDrop Technologies, United States). DNA was subjected to bisulfite modification by means of a commercially available modification kit (Zymo Research). Pyrosequencing was used to quantify the methylation levels of individual CpG sites. The sequencing was performed for all the study samples on a PyroMark Q24 ID using Pyro Mark Gold reagents (Qiagen). Primers for rat and human *CNR1,* the gene coding for the cannabinoid receptor type 1 (CB_1) (targeting in rats eight CpG sites and in humans five CpGs) and *OPRM1*, the gene coding for mu opioid receptor (MOP) (targeting in rats four CpG sites and in humans five CpGs), were generated according to Pyro Mark Assay Design software version 2.0 (Qiagen). The schematic representation of CpG island at *CNR1* and *OPRM1* promoter regions and the details of the pyrosequencing assay are illustrated in **Figure 2** and **Supplementary Table S2**. Bisulfite treated DNA was amplified by PyroMark PCR Kit (Qiagen) according to the manufacturer's protocol. Polymerase chain

TABLE 1 | Clinical characteristics and biochemical parameters of subjects enrolled for the DNA methylation study.

Obese subjects	Males	Females
No.	27	36
Age, years ± SD	44.15 ± 4.45	41.62 ± 4.135
BMI, kg/m^2 ± SD	40.69 ± 1.52	40.23 ± 1.17
Glicemia ± SD	115.30 ± 9.18	98.89 ± 4.29
Cholesterol-total, mg/dL ± SD	172.70 ± 8.74	175.20 ± 7.24
Cholesterol-HDL, mg/dL ± SD	93.85 ± 9.93	104.40 ± 11.98
Cholesterol-LDL, mg/dL ± SD	60.70 ± 6.46	65.47 ± 4.67
Triglycerides, mg/dL ± SD	135.00 ± 11.11	116.80 ± 7.90
Systolic blood pressure, mmHg ± SD	125.20 ± 1.88	123.10 ± 1.25
Diastolic blood pressure, mmHg ± SD	77.22 ± 1.11	78.06 ± 1.34

reaction conditions were as follows: 95°C for 15 min, followed by 45 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30 s, and finally, 72°C for 10 min. Polymerase chain reaction products were verified by agarose electrophoresis. Pyromark Q24 ID version 1.0.9 software, which generates and automatically analyzes the resulting pyrograms, was used to calculate the methylation percentage mC/(mC + C) (where mC is methylated cytosine and C is unmethylated cytosine), for each CpG site, allowing quantitative comparisons. Quantitative methylation results were considered both as a percentage of individual CpG sites and as an average of the methylation percentage of the all the investigated CpGs.

Statistical Analysis

All results are expressed as mean ± SEM. Statistical differences of genes expression and DNA methylation changes at gene promoters in both human and animal samples were determined using Prism version 6 (Graph-Pad Software, San Diego, CA, United States). In behavioral experiments, data were analyzed by two-way ANOVA with the animal group as the between-subject variable and time as the within-subject variable followed by *post hoc* comparison carried out by the Bonferroni test. In molecular biology studies, data were analyzed by two-way ANOVA with the animal group as the between-subject variable and CpG sites as the within-subject variable. Significant differences induced by diet were analyzed using the Mann–Whitney *U* test. DNA methylation at each CpG site was analyzed using the Mann–Whitney test and Sidak–Bonferroni correction was used for the multiple comparisons. The *P*-values < 0.05 were considered to be statistically significant.

RESULTS

High Fat Diet Effect on Body Weight and Food Intake

At the beginning of the study, body weight of rats in the high fat diet group (278.3 ± 7.0 g) did not differ significantly from that of the rats in the control group (286.2 ± 6.3 g) [$F_{(1,30)} = 0.7$; $P > 0.05$]. At the end of the fifth week, two-way ANOVA showed a significant difference in body weight between the groups (Diet: [$F_{(1,30)} = 7.2$; $P < 0.05$]; Time: [$F_{(4,120)} = 297.5$; $P < 0.01$]; Interaction: [$F_{(4,120)} = 6.7$; $P < 0.01$]). At the fifth week time point, *post hoc* test showed that body weight of DIO rats began to be significantly higher compared to that of control group ($P < 0.05$).

At the end of the 21st week, two-way ANOVA showed a significant difference in body weight between the groups (Diet: [$F_{(1,14)} = 10.0$; $P < 0.01$]; Time: [$F_{(20,280)} = 230.2$; $P < 0.001$]; Interaction: [$F_{(20,280)} = 5.3$; $P < 0.001$]). At the same time point (21st week) of free access to high fat diet, *post hoc* test

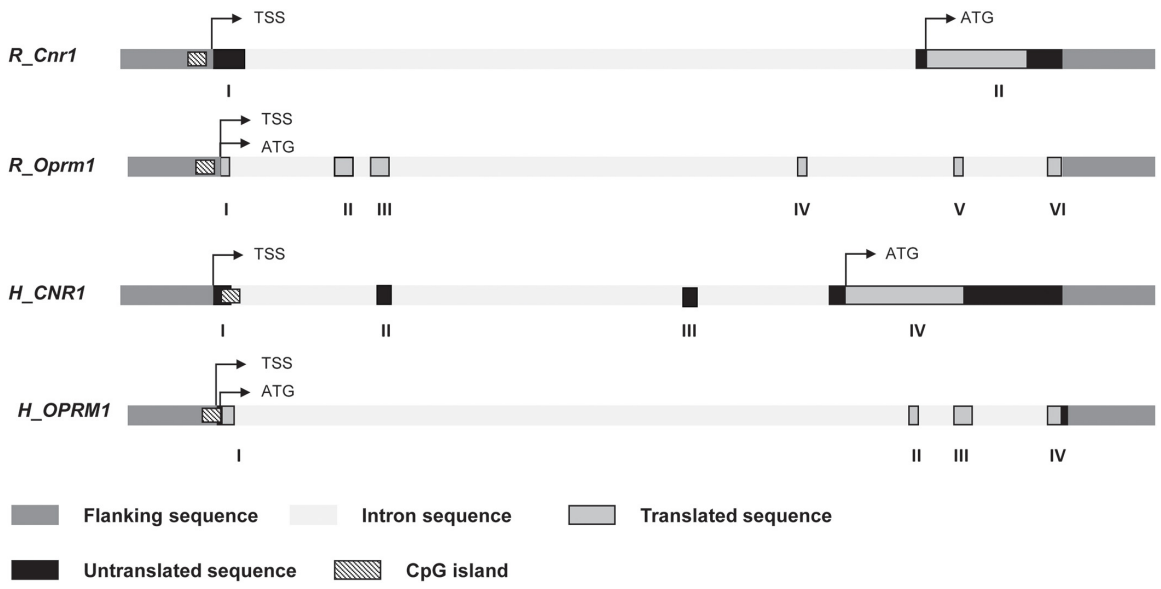

FIGURE 2 | Schematic representation of rat and human *Cnr1* and *Oprm1* genes. Position of transcription start site (TSS), translation start code (ATG), exons and introns, CpG island are depicted. Details of the sequences under study for DNA methylation are shown in **Supplementary Table S2**.

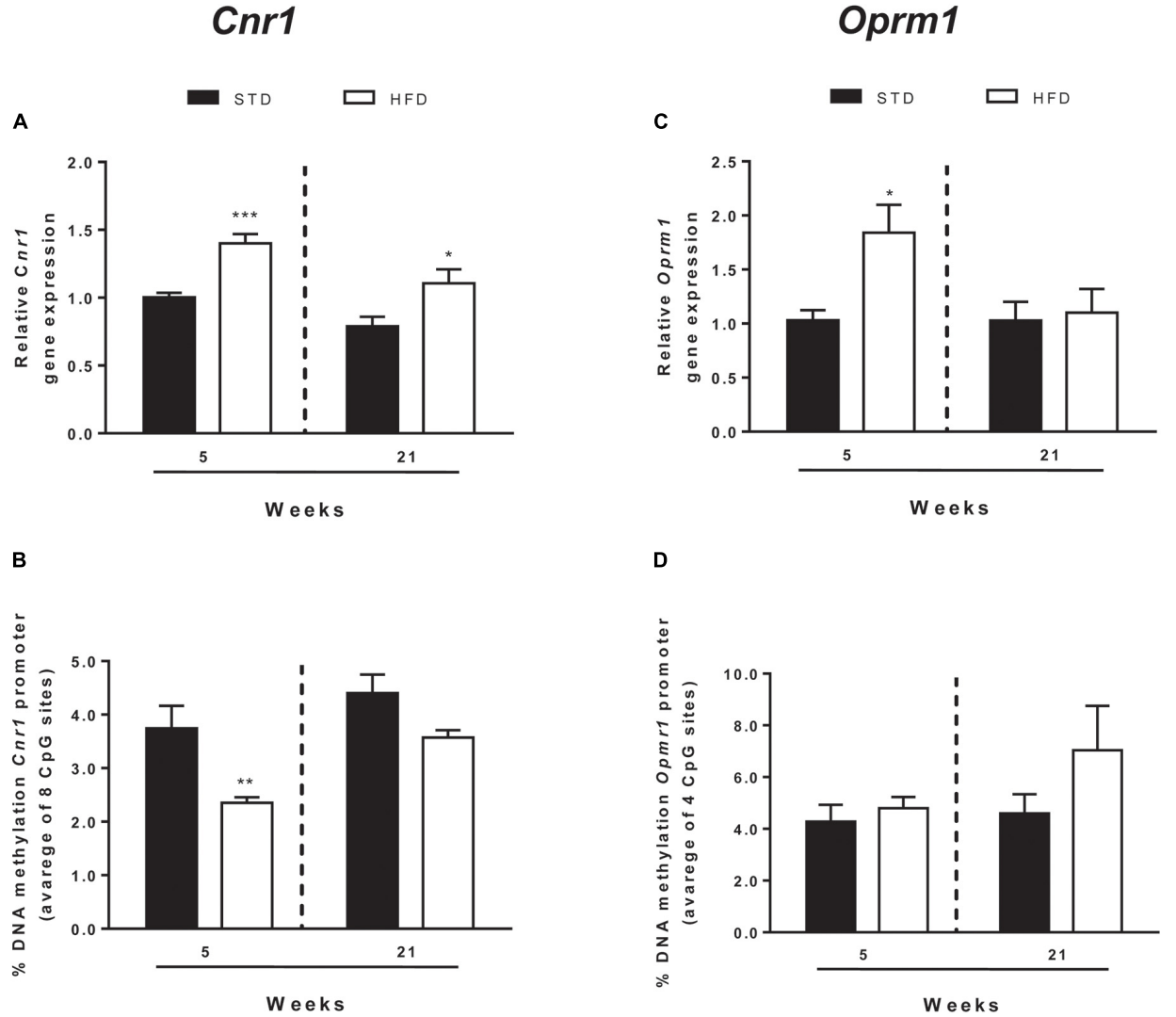

FIGURE 3 | *Cnr1* and *Oprm1* transcriptional regulation. *Cnr1* relative gene expression **(A)** and DNA methylation at gene promoter **(B)** and *Oprm1* relative gene expression **(C)**, and DNA methylation at gene promoter **(D)** analyzed in the hypothalamus of rats exposed for 5 and 21 HFD and/or STD Gene expression data are reported as 2^{-DDCt} values calculated by Delta–Delta Ct (DDCt) method vs. STD (5 weeks) posed equal to 1. DNA methylation data are presented as the mean of the methylation % values of individual CpG sites under study as well as of the average (Ave) of the eight CpG sites ± SEM. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ vs. STD.

showed that body weight of DIO rats was significantly higher in comparison to body weight of rats fed with standard diet (591.0 ± 2.2 g) ($P < 0.001$; **Figure 1A**).

Overall ANOVA showed a significant difference in energy intake (kcal/kg) between the groups in the first 5 weeks (Diet: $[F_{(1,30)} = 110.9; P < 0.001]$; Time: $[F_{(4,120)} = 117.7; P < 0.001]$; Interaction: $[F_{(4,120)} = 29.8; P < 0.001]$). At the end of the 21st week, two-way ANOVA showed a significant interaction between diet and time $[F_{(4,120)} = 29.9; P < 0.001]$. Significant differences at each time point are shown in **Figure 1B**. Open field test showed significant difference between the groups in Distance travel, Vertical count, Grooming and Zone Entries only after 21 weeks of diet exposure (**Supplementary Table S3**).

Regulation of ECS and EOS Genes Transcription in the Hypothalamus

ECS

In addition to CB_1, endocannabinoid signaling is based on a rather complex array of proteins that form the so-called ECS. These include additional receptor targets, like type-2 cannabinoid receptor (CB_2) and transient receptor potential vanilloid-1 (TRPV1) channels, and metabolic enzymes involved in biosynthesis and degradation of endocannabinoids (Chiurchiù et al., 2018). All these elements of ECS were analyzed by qRT-PCR (**Figure 3** and **Table 2**).

After 5 and 21 weeks, statistical analysis showed no significant changes of ECS elements, except for *Cnr1*, the

TABLE 2 | Gene expression of ECS and EOS elements in the hypothalamus of normal weight and overweight rats exposed for 5 and 21 weeks to high fat and/or standard diet, reported as 2^{-DDCt} values calculated by Delta–Delta Ct (DDCt) method vs. normal weight (5 weeks) posed equal to 1.

HYP		5 weeks		21 weeks	
Gene		STD	HFD	STD	HFD
ECS	*Cnr1*	1.03 ± 0.03	1.42 ± 0.09[a]	0.79 ± 0.07	1.18 ± 0.16[c]
	Cnr2	1.21 ± 0.25	1.16 ± 0.70	1.98 ± 0.57	1.90 ± 0.59
	Gpr55	1.22 ± 0.29	1.10 ± 0.21	1.44 ± 0.14	1.54 ± 0.27
	Trpv1	1.24 ± 0.30	1.34 ± 0.45	1.22 ± 0.29	0.78 ± 0.06
	Nape-Pld	1.06 ± 0.10	1.08 ± 0.15	1.10 ± 0.13	1.08 ± 0.18
	Faah	1.09 ± 0.17	0.87 ± 0.12	1.36 ± 0.12	1.48 ± 0.10
	Dagl	1.05 ± 0.13	1.49 ± 0.26	0.74 ± 0.07	0.58 ± 0.05
	Magl	1.04 ± 0.11	1.14 ± 0.14	0.94 ± 0.08	0.88 ± 0.13
Opiod system	*Oprl1*	0.98 ± 0.05	1.23 ± 0.08	1.11 ± 0.12	1.15 ± 0.02
	Oprm1	1.03 ± 0.09	1.84 ± 0.26[b]	1.03 ± 0.17	1.10 ± 0.22
	Oprd1	1.03 ± 0.11	1.36 ± 0.22	0.73 ± 0.09	0.71 ± 0.07
	Oprk1	1.05 ± 0.17	1.23 ± 0.30	1.29 ± 0.23	1.19 ± 0.30
	Pomc	1.18 ± 0.33	0.89 ± 0.22	1.19 ± 0.30	0.73 ± 1.19
	Pnoc	1.03 ± 0.10	1.48 ± 0.17	1.00 ± 0.13	0.89 ± 0.11
	Pdyn	1.04 ± 0.11	1.30 ± 0.23	1.02 ± 0.12	0.97 ± 0.08
	Penk	1.01 ± 0.04	0.99 ± 0.15	1.06 ± 0.13	1.15 ± 0.17

[a] $p < 0.05$ vs. STD; [b] $p < 0.01$ vs. STD; [c] $p < 0.001$ vs. STD.

TABLE 3 | DNA methylation changes at *Cnr1* gene promoter in the hypothalamus of normal weight and overweight rats exposed for 5 weeks to high fat and/or standard diet.

Cnr1	5 weeks		21 weeks	
CpG sites	STD	HFD	STD	HFD
1	4.55 ± 0.48	4.24 ± 0.42	5.11 ± 0.31	5.28 ± 0.67
2	4.76 ± 0.64	2.30 ± 0.28[b]	5.49 ± 0.63	4.64 ± 0.29
3	3.37 ± 0.45	1.37 ± 0.10[c]	3.83 ± 0.35	2.77 ± 0.26
4	6.36 ± 0.87	3.81 ± 0.28[a]	8.17 ± 0.73	6.15 ± 028
5	1.31 ± 0.21	1.01 ± 0.20	1.69 ± 0.14	1.43 ± 0.18
6	2.22 ± 0.23	1.69 ± 0.13	2.35 ± 0.25	2.14 ± 0.20
7	3.13 ± 0.45	2.23 ± 0.28	3.64 ± 0.29	2.67 ± 0.28
8	3.77 ± 0.35	2.60 ± 0.25[a]	4.30 ± 0.48	3.21 ± 0.23
Average	3.72 ± 0.43	2.46 ± 0.11[a]	4.33 ± 0.32	3.54 ± 0.17

[a] $p < 0.05$ vs. STD; [b] $p < 0.01$ vs. STD; [c] $p < 0.001$ vs. STD.

gene encoding for CB_1 (**Table 2**). Two-way ANOVA showed that mRNA levels were affected by time [$F_{(1,25)}$ = 10.50, P = 0.003] and diet [$F_{(1,25)}$ = 20.72, P = 0.0001], without a significant interaction between these two factors [$F_{(1,25)}$ = 0.27, P = 0.607]. *T*-test revealed a selective and significant increase in *Cnr1* mRNA levels in HFD rats with respect to STD animals at both time points analyzed (5 weeks = $P < 0.0006$; 21 weeks = $P < 0.036$; **Figure 3A**).

DNA methylation analysis of each CpG site, and as an average of all eight CpG sites analyzed at the *Cnr1* promoter, showed significant changes in rats after 5 weeks on high fat diet (*t*-test: $P = < 0.006$; **Figure 3B** and **Table 3**). Two-way ANOVA showed that DNA methylation was affected by time [$F_{(1,26)}$ = 6.62, $P = 0.016$] and diet [$F_{(1,26)} = 5.34$, $P = 0.029$], without a significant interaction between these two factors [$F_{(1,26)}$ = 0.024, P = 0.876].

Moreover, a *t*-test corrected by Sidak–Bonferroni multiple comparisons indicated a significant decrease of *Cnr1* methylation levels at sites 2, 3, 4, and 8, as well as in the average of the eight sites analyzed, when compared to the STD group. No changes in DNA methylation were observed after 21 weeks (**Table 3** and **Figure 3B**). Consistently, a correlation between gene expression and average DNA methylation was also observed in overweight and normal weight animals after 5 weeks of diet (Spearman $r = 0.609$; $P = 0.018$; **Figure 4A**), but not after 21 weeks (Spearman $r = 0.316$; $P = 0.271$; **Figure 4B**).

EOS

EOS consists of four receptors, mu (OPRM1), delta (OPRD1), kappa (OPRK1), and Opioid related nociceptin receptor 1 (OPRL1) which are activated by endogenous opioid peptides processed from four protein precursors, proopiomelanocortin (POMC), proenkephalin (PENK), prodynorphin (PDYN) and pronociceptin (PNOC) (Corbett et al., 2006; Bodnar, 2010). Among EOS elements, only the expression of genes encoding for the receptors *Oprm1* and *Oprd1* were altered (**Figure 3** and **Table 4**). Two-way ANOVA showed that *Oprm1* mRNA levels were affected by diet [$F_{(1,25)}$ = 4.90, P = 0.036] but not by time [$F_{(1,25)}$ = 3.45, P = 0.07], and there was no significant interaction between these two factors [$F_{(1,25)}$ = 3.43, P = 0.07]. *T*-test revealed a selective and significant increase in *Oprm1* mRNA levels in rats after 5 weeks on high fat diet with respect to STD animals (5 weeks = $P < 0.05$; **Figure 3C** and **Table 4**).

DNA methylation analysis of each CpG site, and as an average of all four CpG sites analyzed at the *Oprm1* promoter, did not show any significant change in rats exposed for 5 and 21 weeks to high fat diet (**Table 4** and **Figure 3D**), nor any correlation between gene expression and average DNA methylation (at 5 weeks: Spearman $r = -0.001$; $P = 0.961$; at 21 weeks: Spearman $r = -0.304$; $P = 0.282$; **Figures 4C,D**).

DNA Methylation at *CNR1* and *OPRM1* Promoter Regions in Clinical Samples

We sought to extend the analysis of DNA methylation at *CNR1* and *OPRM1* promoters in the preclinical animal model to a group of humans with obesity and matched healthy controls (CTRLs). In these samples, we failed to observe any alteration of the epigenetic hallmark at both gene promoters in the overall population (**Table 5**). Yet, data stratification based on subject age showed significant differences at both gene promoters in individuals younger than 30 years (**Figure 5**). Namely, DNA methylation at *CNR1* promoter was lower in humans with obesity at CpG 5 (CTRLs = 2.62 ± 0.17, obese = 2.16 ± 0.06; $p = 0.006$), as well as at the average of the five CpG sites under study (CTRLs = 4.97 ± 0.11, obese = 5.58 ± 0.10; $p = 0.008$), when compared to age-matched controls (**Figure 5A**). Instead, DNA methylation at *OPRM1* promoter was significantly lower in obese subjects at CpG 1 (CTRLs = 6.57 ± 0.31, obese = 5.28 ± 0.30; $p < 0.01$), CpG 2 (CTRLs = 12.37 ± 0.44, obese = 9.85 ± 0.40;

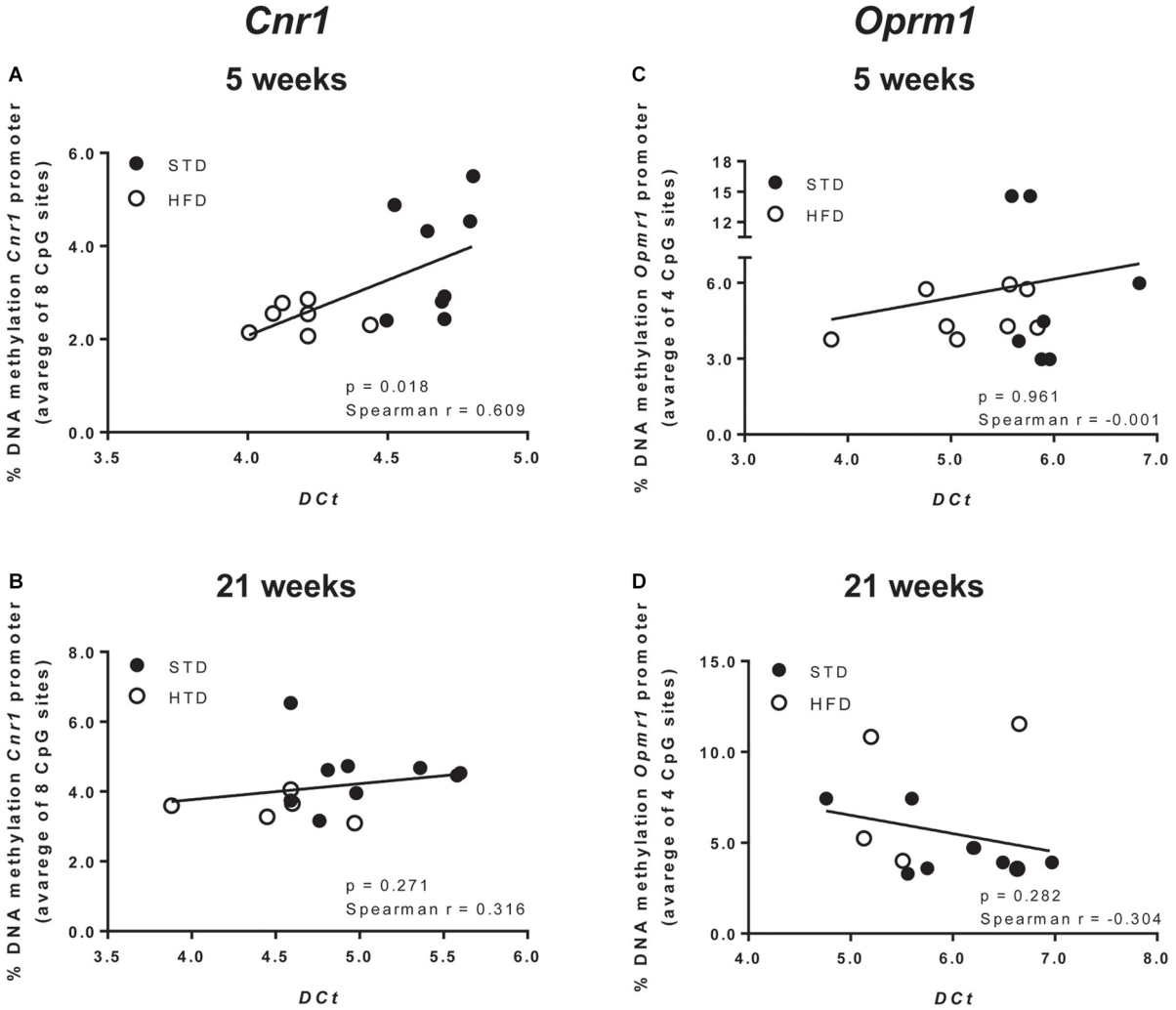

FIGURE 4 | Correlation between Cnr1 expression and % change of DNA methylation after 5 **(A)** and 21 weeks of diet **(B)**. Correlation between *Oprm1* expression and % change of DNA methylation after 5 **(C)** and 21 weeks of diet **(D)**. Data were analyzed by Spearman's rank correlation coefficient.

$p < 0.001$), CpG 3 (CTRLs = 11.10 ± 0.76, obese = 8.31 ± 0.27; $p < 0.001$) and CpG 4 (CTRLs = 8.22 ± 0.47, obese = 6.61 ± 0.25; $p < 0.01$), when compared to age-matched controls (**Figure 5A**) No differences in DNA methylation were observed between obese and healthy controls in subjects older than 30 years (**Figure 5B**). We also observed the DNA methylation levels on *CNR1* and *OPRM1* gene promoters in humans with obesity and Ctrl stratified for gender (**Supplementary Figure S1**).

TABLE 4 | DNA methylation changes at *Oprm1* gene promoter in the hypothalamus of normal weight and overweight rats exposed for 5 and 21 weeks to high fat and/or standard diet.

Oprm1	5 weeks		21 weeks	
CpG sites	**STD**	**HFD**	**STD**	**HFD**
1	3.56 ± 0.62	4.16 ± 0.40	3.05 ± 1.14	4.64 ± 1.00
2	7. 30 ± 1.13	8.55 ± 0.57	8. 05 ± 1.16	7.31 ± 0.52
3	2. 81 ± 0.45	2.92 ± 0.67	3. 38 ± 0.59	5.15 ± 1.40
4	3.46 ± 0.43	3.54 ± 0.34	3.90 ± 0.68	4.82 ± 1.12
Average	4.28 ± 0.64	4.80 ± 0.44	4.60 ± 0.75	7.04 ± 1.72

TABLE 5 | DNA methylation changes at human *CNR1* and *OPRM1* gene promoters in controls (CTRL) and humans with obesity.

Gene	*CNR1*		*OPRM1*	
CpG sites	**CTRL**	**Obese**	**CTRL**	**Obese**
1	2.36 ± 0.14	2.27 ± 0.07	7.87 ± 0.37	7.11 ± 0.32
2	10.41 ± 0.28	10.39 ± 0.25	15.62 ± 1.20	14.76 ± 0.83
3	5.84 ± 0.24	5.72 ± 0.15	12.90 ± 0.56	12.04 ± 0.57
4	3.03 ± 0.22	2.93 ± 0.10	9.55 ± 0.38	9.03 ± 0.43
5	2.70 ± 0.13	2.52 ± 0.09	7.65 ± 0.35	8.03 ± 0.95
Average	4.87 ± 0.17	4.74 ± 0.13	10.72 ± 0.50	10.19 ± 0.47

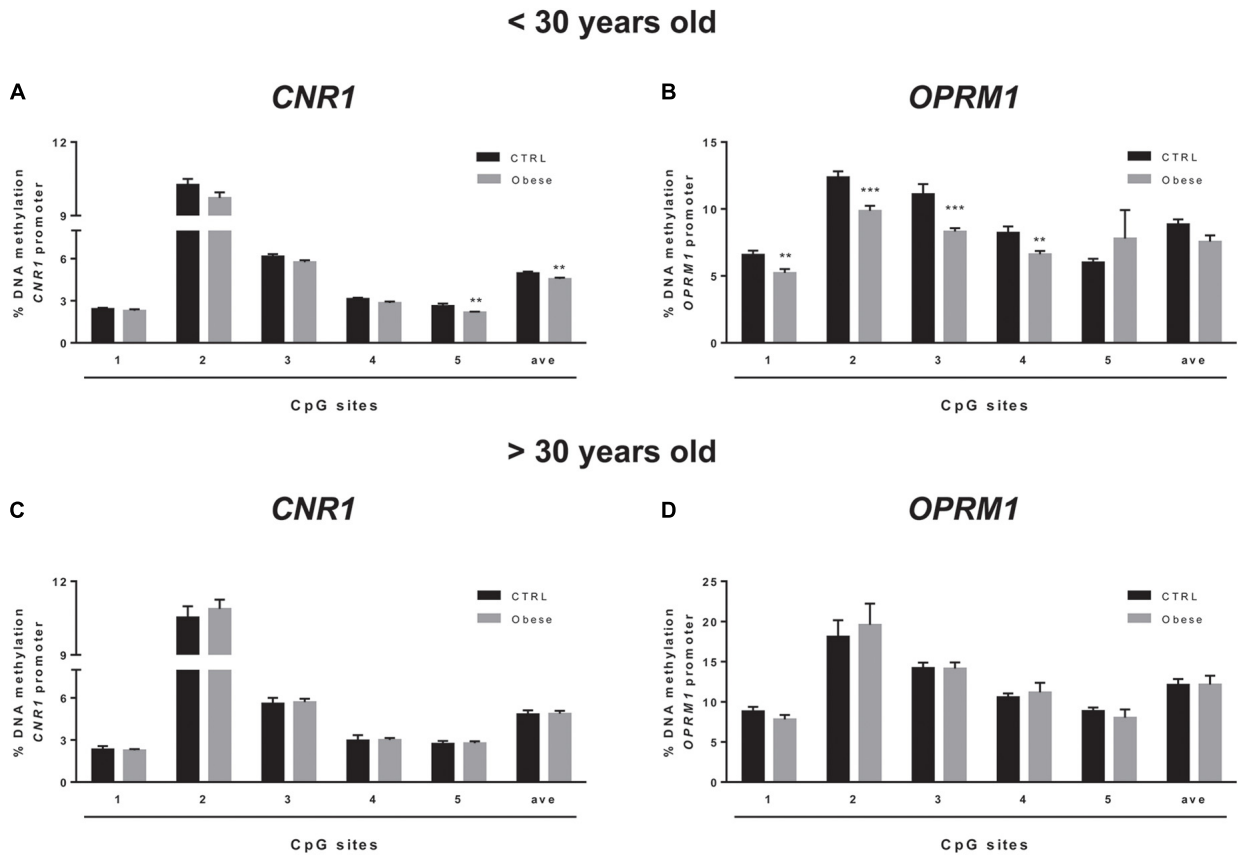

FIGURE 5 | Comparison of DNA methylation status at human *CNR1* **(A,C)** and *OPRM1* **(B,D)** promoters in the obese population and control (CTRL) subjects stratified based on age (**A,B** = < 30 years old; **C,D** > 30 years old). The bars represent the mean of the % of methylation values of individual CpG sites under study as well as of the average (ave) of the CpG sites ± the SEM. Significant differences are indicated: $^{**}P < 0.01$; $^{***}P < 0.001$ vs. CTRL.

No differences in the % of DNA methylation of *CNR1* were observed in female and male subjects (**Supplementary Figures S1A,B**). Lower levels in DNA methylation at *OPMR1* were observed in human males with obesity at CpG site 3 (CTRLs = 13.45 ± 0.65, obese = 10.13 ± 0.79, $P < 0.004$), CpG site 4 (CTRLs = 9.94 ± 0.45, obese = 7.61 ± 0.52, $P < 0.003$), as well as at the average of the five CpG sites under study (CTRLs = 10.96 ± 0.49, obese = 8.72 ± 0.64, $P < 0.010$), when compared to CTRLs (**Supplementary Figure S1A**). No differences in the % of DNA methylation of *OPRM1* were observed in female subjects (**Supplementary Figure S1B**). Considering just the humans with obesity, DNA methylation differences were not observed after data stratification based on gender (**Supplementary Figure S2**). Focusing on the obese population only, DNA methylation differences were observed after data stratification based on the time length from obesity onset (**Figure 6**). Additionally, methylation at *CNR1* promoter of subjects that were obese for more than 5 years was higher than that of humans with obesity for a shorter time. In particular, we observed a significant increase at two of the five sites analyzed, namely at the second (CTRLs = 9.78 ± 0.23, obese = 11.32 ± 0.37; $p < 0.008$) and at the fifth (CTRLs = 2.23 ± 0.06, obese = 2.80 ± 0.13; $p < 0.14$) CpG site, respectively (**Figure 6A**). Methylation of the combined five CpG sites analyzed in the promoter region of *OPRM1* showed a significant increase in DNA methylation of humans with obesity for a long time (>5 years from onset) (CTRLs = 7.36 ± 0.51, obese = 11.51 ± 0.95; $p < 0.001$). Moreover, we observed a significant increase in the first (CTRLs = 4.91 ± 0.24, obese = 7.73 ± 0.50; $p < 0.001$), second (CTRLs = 9.49 ± 0.34, obese = 18.18 ± 2.23; $p < 0.001$), third (CTRLs = 8.00 ± 0.21, obese = 13.42 ± 0.73; $p < 0.001$), and fourth (CTRLs = 6.45 ± 0.25, obese = 10.50 ± 1.01; $p < 0.001$) CpG site analyzed (**Figure 6B**). Finally, differences were also observed when considering the BMI of humans with obesity, and *post hoc* group differences are reported in **Supplementary Figure S3**. Focusing on humans with obesity younger than 30 years, we compared the DNA methylation status at *CNR1* and *OPRM1* promoters in Preadolescents (8–12 years old), Adolescents (13–17), Young adults (18–30) (**Supplementary Figure S4**). A *t*-test corrected by Sidak–Bonferroni multiple comparisons indicated a significant increase of *OPRM1* methylation levels at CPG site 3 of young adults, when compared to the adolescents (Adolescent = 7.96 ± 0.26, Young adult = 9.85 ± 0.78, $P < 0.008$). Association remained for all stratified data, after Sidak–Bonferroni correction for multiple testing.

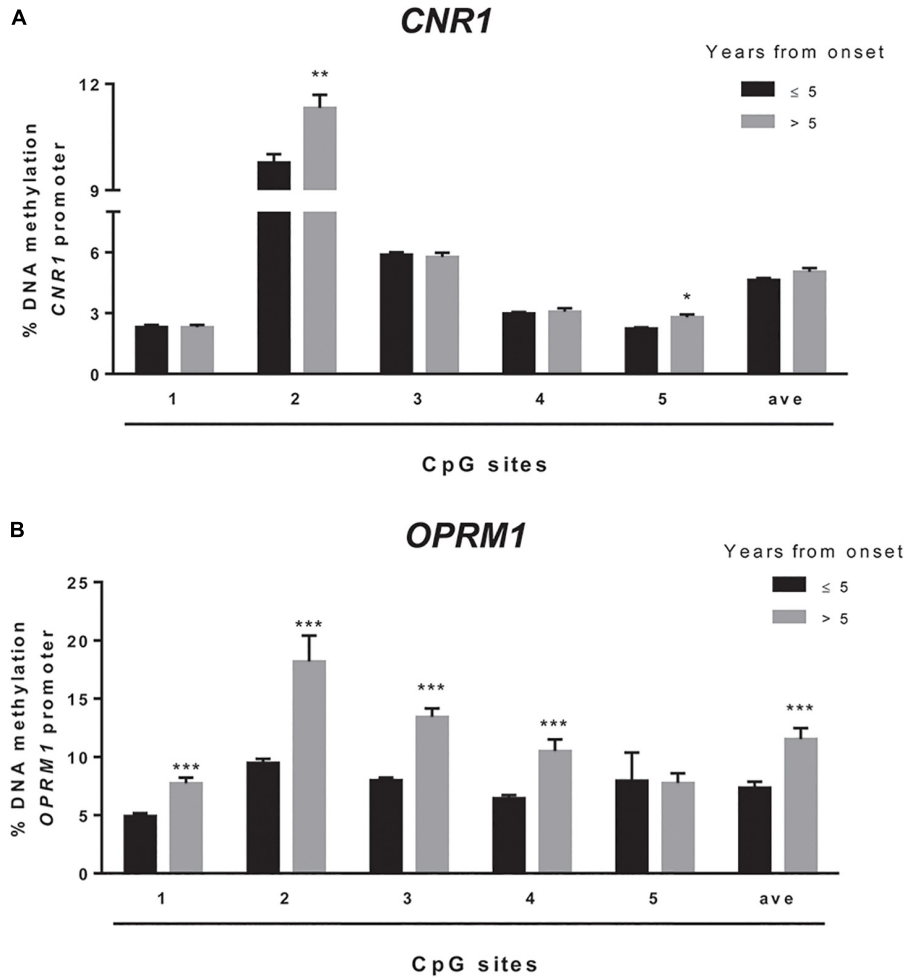

FIGURE 6 | Comparison of the DNA methylation status at human *CNR1* **(A)** and *OPRM1* **(B)** promoters in the obese population stratified based on the years from obesity onset. The bars represent the mean of the % of methylation values of individual CpG sites under study as well as of the average (ave) of the CpG sites ± the SEM. Significant differences are indicated: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. obese > 5.

DISCUSSION

The first outcome of this study is the selective up-regulation of the expression of *Cnr1*, the gene coding for CB_1, and of *Oprm1*, the gene coding for MOP, in the hypothalamus of rats exposed to high fat diet for 5 and 21 weeks. These alterations were present at both time-points analyzed for CB_1, that appeared to be engaged in long-lasting effects, and only at the beginning of obesity development for MOP, that appeared to be engaged in obesity onset only. Of note, we failed to observe any alteration in any other component of the ECS and of the EOS, speaking in favor of a distinct role of the two receptors in obesity.

Our data are thus consistent with many studies that already showed the hyperphagic role of CB_1 (Williams and Kirkham, 1999; Jamshidi and Taylor, 2001; Verty et al., 2005; Koch et al., 2015) and MOP (Smith et al., 2002) in this brain region. Hypothalamic administration of the endocannabinoid anandamide (Jamshidi and Taylor, 2001) or of the cannabis extract tetrahydrocannabinol (THC) (Verty et al., 2005), both primarily acting though CB_1 in the brain, rapidly increases food intake in rats. Studies focused on the hypothalamus documented also that high-fat feeding increases MOP protein levels in Wistar rats (Smith et al., 2002), as well as in rats susceptible to obesity (Barnes et al., 2006).

The link between these two receptors in the promotion of feeding has been proposed by Kock and colleagues, who showed that this effect can be due to CB_1 activation on POMC neurons leading to the release of β-endorphin, an opioid neuropeptide acting on MOP (Koch et al., 2015).

Despite the great amount of research showing CB_1 levels dysregulation centrally in animal models of obesity (South and Huang, 2008; Massa et al., 2010), there is less information about altered hypothalamic *Cnr1* mRNA levels, which anyhow have been reported by some (Kempf et al., 2007; Gamelin et al., 2016; Ramirez-Lopez et al., 2016). Consistently with our present findings, *Cnr1* mRNA levels increased in female offspring hypothalami from rat dams fed a high caloric diet (Ramirez-Lopez et al., 2016). Moreover, receptor knockdown in

mice hypothalamus induced a reduction in body weight while increasing energy expenditure (Cardinal et al., 2012). On the other hand, receptor gene expression was down-regulated in the hypothalamus of rats fed with a palatable high-energy diet (Timofeeva et al., 2009). It seems noteworthy that in a recent analysis of the transcriptional regulation of ECS components in an animal model of binge eating behavior, we observed a selective epigenetic regulation of fatty acid amide hydrolase, the key enzyme for the degradation of anandamide, instead, *Cnr1* and all other ECS components were not affected (Pucci et al., 2018). This seems of particular relevance if one aims at finding specific biomarkers for different eating disorders and related disturbances (such as obesity).

The involvement of MOP gene regulation in obesity has also been investigated. MOP KO mice fed a high-fat diet were resistant to obesity (Tabarin et al., 2005), even though others also showed that MOP KO mice on a standard diet increased body weight in adulthood, when compared to wild-type littermates (Han et al., 2006). Analysis of gene expression showed increased MOP mRNA levels in hypohalami of the offspring of obese pregnant mice (Vucetic et al., 2010; Grissom et al., 2014), whereas no changes were observed in mice fed from weaning with high-fat diet for around 15 weeks (Vucetic et al., 2011).

In order to characterize MOP and CB_1 transcriptional regulation, we sought to compare hypothalamic genes DNA methylation. We observed a consistent reduction of the epigenetic hallmark at *Cnr1* promoter, in four of the eight CpG sites under study as well as in their methylation average, but only at the earliest time-point (5 weeks). However, no changes were observed in MOP DNA methylation. These findings suggest that the temporary hypomethylation at *Cnr1* gene at the beginning of obesity development might be responsible of the early changes observed in gene expression; yet, it does not seem necessary in the long term, when chromatin might be in a poised state with mRNA levels resulting still high. At any rate, this is the first report showing DNA methylation of *Cnr1* in obesity, whereas others have already addressed MOP epigenetic regulation in mice reward-related brain regions of the offspring of pregnant dams exposed to chronic high-fat diet (Vucetic et al., 2011). In partial agreement with our data, also the latter studies failed to show alterations of methyl CpG-binding protein, as well as of histone acetylation, at MOP gene promoter in the hypothalamus (Vucetic et al., 2011). Very recently the hypothalamic increase in histone acetylation was instead reported at *Cnr1* gene promoter, and was linked to increased hypothalamic receptor expression (Almeida et al., 2019).

With the aim to extend the study of CB_1 and MOP receptors to human obesity, we assessed whether PBMCs might mirror central nervous system defects, and we then evaluated DNA methylation at *CNR1* and *OPRM1* gene promoters in PBMCs of humans with obesity. We failed to observe any difference between controls and humans with obesity in the overall population, yet age-based stratification of the data clearly showed a significant reduction of the epigenetic hallmark at both *CNR1* and *OPRM1* promoters in younger (<30 years old) humans with obesity. As for *CNR1*, an age-dependent modulation was observed at one of the five CpG sites analyzed, as well as at their average, possibly driving an up-regulation of gene expression compared with normal weight subjects. Instead, in the same population DNA methylation was reduced in four of the five CpG sites analyzed. These differences for both receptors did not occur in subjects older than 30 years. Our findings appear of particular relevance if one attempts to identify early disease biomarkers. In agreement with this, a lower DNA methylation was also observed at genes promoters of humans with obesity with a shorter time length from disorder onset, and in those with lower BMI.

At the clinical level, it seems noteworthy that an increased *CNR1* expression in obesity has been shown at the peripheral level (Pagano et al., 2007; Sarzani et al., 2009), but never in blood samples from human subjects. Moreover, to the best of our knowledge, *OPRM1* expression in humans with obesity has not been studied yet, with the only available PET clinical study in different brain regions reporting lower MOP availability in obese subjects compared to controls (Karlsson et al., 2015).

Overall, our findings in human peripheral samples confirm the data observed centrally in the animal model, showing that regulation of *CNR1* and *OPRM1* genes is altered mainly at the early stage of phenotype development, at least in terms of the epigenetic hallmarks analyzed.

CONCLUSION

In conclusion, epigenetic switching appears dynamic and temporary in the preclinical and clinical models used here to investigate *CNR1* and *OPRM1*. Alterations of DNA methylation in these two genes, observed earlier in the obese phenotype as a result of early exposure (i.e., to high-fat diet or, for instance, to stressful conditions), disappear at a later stage in life. Thus, here we clearly show the relevant role of *CNR1* and *OPRM1* transcriptional regulation as a possible biomarker for obesity and, due to the reversible nature of the epigenetic hallmark, our data open new avenue for environmental strategies of intervention. As yet, conventional environmental strategies for the treatment of obesity include lifestyle modifications of diet and physical activity, and are often insufficient (Blevins, 2010). However, this might be also due to the lack of an early diagnosis. In line with this, our data support the relevance of a preventive therapeutic approach based on environmental factors.

Of relevance, a very recent study has reported altered DNA methylation on obesity-related genes in healthy adolescents, and these alterations were suggested as early life changes possibly associated with elevated disease risk in adulthood (He et al., 2019).

Study Limitations

We should point out that we analyzed PBMCs, composed of different cell types with different DNA methylation profiles (Bell et al., 2011). However, in order to extract homogeneous populations, cell manipulation is required, which is a laborious and difficult procedure to standardize, possibly affecting gene expression profiles (Whitney et al., 2003; Debey et al., 2004). Therefore, laser capture micro-dissected tissues might be used to address this issue in future studies. Moreover, for a better

understanding of the relationship between blood and brain in terms of epigenetic modulation, it would be of interest to study also in rats transcriptional regulation of genes in blood. In addition, it seems necessary to extend the present study to a larger cohort of human subjects, possibly by using innovative methods to achieve cell-sorting, and to add different preclinical experimental models of obesity to further characterize the epigenetic regulation of *CNR1* and *OPRM1* expression, as well as the relationship between their peripheral and central modulation.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of "name of guidelines, name of committee" with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethic Committee of IRCCS – Istituto Auxologico Italiano (RBFR12DELS_004/05C302_2013).

This study was carried out in accordance with the recommendations of Institutional Guidelines and complied with the Italian Ministry of Health and associated guidelines from European Communities Council Directive. The protocol was approved by Italian Ministry of Health no. 1610/2013.

AUTHOR CONTRIBUTIONS

MP, CD, CC, and MM conceived and designed the experiments. CD, MP, MM, VV, SM, EZ, and MMas performed the experiments. CD, MS, LP, MP, and CC analyzed the data. CD, CC, and AS contributed reagents, materials, and analysis tools. CD, MP, and MMac wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2019.00523/full#supplementary-material

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Transcriptome Analysis Reveals Differential Expression of Genes Regulating Hepatic Triglyceride Metabolism in Pekin Ducks During Dietary Threonine Deficiency

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Dietary threonine (Thr) deficiency increases hepatic triglyceride accumulation in Pekin ducks, which results in fatty liver disease and impairs hepatic function. However, the underlying molecular mechanisms altered by dietary Thr deficiency are still unknown. To identify the underlying molecular changes, 180 one-day-old ducklings were divided into three groups, including Thr deficiency group (Thr-D), Thr sufficiency group (Thr-S), and pair-fed group (Pair-F) that was fed with a Thr-sufficient diet but with reduced daily feed intake. The results showed that feed intake was similar between Thr-D and Pair-F groups, but weight gain rate and final body weight in the Thr-D group were lower than those in the Pair-F group. Feed intake, weight gain, and body weight in Thr-D and Pair-F groups were lower than those in the Thr-S group. The Thr-D diet reduced abdominal fat percentage but increased hepatic triglyceride content when compared with that of the Thr-S and Pair-F groups. The Pair-F reduced hepatic levels of C15:0, C17:0, C18:0, C20:0, C20:4n6, and C22:0 and also reduced total fatty acid, saturated fatty acid, and unsaturated fatty acid content when compared with those of the Thr-D and Thr-S groups. The Thr-D diet increased hepatic content of C6:0, C17:1, C18:3n6, C20:0, C20:1n9, and C22:2, as well as reduced the content of C18:2n6t and C23:0 when compared with those of the Thr-S group. Transcriptome analysis in the liver indicated that the Thr-D diet upregulated genes related to fatty acid and triglyceride synthesis and downregulated genes related to fatty acid oxidation and triglyceride transport. Gene ontology analysis showed that more genes related to lipid metabolism processes and molecular function were differentially expressed in the Thr-D group relative to Thr-S and Pair-F groups than in the Pair-F group relative to the Thr-S group. KEGG pathway analysis showed that differentially expressed genes were enriched in signal transduction, immune, hormone, lipid, and amino acid metabolism pathways. Our findings indicated that the Thr-D diet increased hepatic triglyceride and fatty acid accumulation *via* increasing fatty acid and triglyceride synthesis and reducing

fatty acid oxidation and triglyceride transport. These findings provide novel insights into our understanding of the molecular mechanisms underlying fat accumulation in the liver caused by dietary threonine deficiency.

Keywords: threonine deficiency, triglyceride accumulation, fatty acid oxidation, energy metabolism, fatty acid profile, Pekin duck

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is an increasingly prevalent disease worldwide that leads to chronic hepatic injury (Loomba and Sanyal, 2013). It is estimated that NAFLD affects approximately 25% of the world population (Lazo and Clark, 2008; Rector et al., 2008). Recently, the occurrence of NAFLD has been increasing in adolescents as the prevalence of childhood obesity rises (Janssen et al., 2005; Park et al., 2005). Lipid accumulation in the liver of people who drink little or no alcohol is the main characteristic of NAFLD. Such a fatty liver is more susceptible to inflammatory cytokines and oxidative stress (Browning and Horton, 2004; Cohen et al., 2011) and may gradually develop into steatohepatitis, liver fibrosis, and hepatocellular carcinoma (Chalasani et al., 2012).

Threonine (Thr) is an essential amino acid for humans and poultry (Kidd and Kerr, 1996) and plays important roles in various metabolic processes, such as hormone secretion and immune defense. Its importance is highlighted by the adverse effects of Thr deficiency in experimental animals, including anorexia, impaired immunity, excessive energy expenditure, and growth retardation (Ross-Inta et al., 2009; Xie et al., 2014; Jiang et al., 2016; Zhang et al., 2016). For example, dietary Thr deficiency reduces the food intake of poultry (Xie et al., 2014; Zhang et al., 2014; Jiang et al., 2016; Zhang et al., 2017), whereas such a reduced feeding leads to low fat deposition in young growing cattle (Basarab et al., 2003). Moreover, a threonine-deficient diet elicits lipid accumulation in the liver of rats (Methfessel et al., 1964). In broilers, dietary Thr deficiency increases fat content in breast muscle and whole body (Rangel-Lugo et al., 1994; Ciftci and Ceylan, 2004). More recently, we demonstrated that dietary Thr deficiency increases hepatic lipid deposition and reduces abdominal fat levels in ducks (Jiang et al., 2017).

Hepatic lipid accumulation is a complex process, and the role of Thr in it remains elusive. It is speculated that Thr deficiency increases hepatic lipid deposition by changing lipid catabolic and transport pathways, as well as promoting lipid synthesis (Methfessel et al., 1964). Indeed, we have shown that the expression of several hepatic genes related to lipid uptake, fatty acid synthesis, β-oxidation, ketogenesis, and triglyceride transport is affected by dietary Thr levels in Pekin ducks (Jiang et al., 2019). Nevertheless, the biochemical mechanisms whereby Thr deficiency causes hepatic lipid accumulation in ducks are still unclear.

Therefore, the present study was conducted to investigate the effects of dietary Thr deficiency and feed restriction on the growth performance, plasma parameters, hepatic lipid content, and hepatic fatty acid composition. Moreover, we also assessed the expression of hepatic genes by transcriptome analysis to explore potential pathways whereby Thr deficiency causes triglyceride accumulation in the liver of Pekin ducks. Noteworthy, because the reduced food intake associated with Thr deficiency (Jiang et al., 2017) may affect hepatic lipid levels, it is necessary to compensate for this effect by designing a paired fed group. We used such a group in this study, whose feed consumption was similar between animals fed with Thr-sufficient and Thr-deficient diets.

MATERIALS AND METHODS

Animals and Experimental Design

A completely randomized design with single factorial arrangement of treatments was used in this experiment. Dietary treatments included a Thr deficiency diet (Thr-D), a Thr sufficiency diet (Thr-S), supplemented with 0.21% (w/w) crystal Thr to basal diet as fed, and a paired fed group (Pair-F), which was fed with Thr sufficiency diet maintaining a similar daily feed intake to that of the Thr-D group by feed restriction. Thus, there were a total of three different treatments (Thr-D, Thr-S, and Pair-F).

One-day-old male Pekin ducks (Pekin Duck Breeding Centre of the Institute of Animal Science), 180 individuals, were randomly assigned to one of the three treatments composed of six replicate cages with 10 ducklings per cage according to average body weight (57.1 ± 3.01 g). All ducks were handled in accordance with the Pekin duck management guidelines. The birds were housed in raised wire floor pens (200 × 100 × 40 cm) with nipple drinkers and tubular feeders and maintained under constant light. The temperature was kept at 30°C from 1 to 3 days of age and then gradually reduced to 25°C until 21 days of age. Feed pellets and water were offered *ad libitum*.

The basal corn–wheat–peanut meal diet (**Supplementary Table S1**) was formulated to meet or exceed the current National Research Council recommendation (NRC, 1994), except for Thr concentration. First, a single batch of basal diet was mixed and then divided into two aliquots according to the experimental treatments. According to previous study (Zhang et al., 2014), each sublot was mixed with 0.21% crystalline Thr or cornstarch, which was used to replace crystalline Thr and maintain dietary characteristics. Dietary Thr concentrations in Thr-D and Thr-S diets were 0.53% (w/w) and 0.73% (w/w), respectively. Weight and feed intake per cage were measured weekly to calculate the daily body weight gain, daily feed intake, and gain:feed ratio from day 1 to day 21. All experimental procedures were approved by the Animal Management Committee (in charge of animal welfare issue) of the Institute of Animal Science, Chinese Academy

of Agricultural Sciences (IAS20160322, IAS-CAAS, Beijing, China) and performed in accordance with the guidelines. Ethical approval on animal survival was given by the animal ethics committee of IAS-CAAS.

Sample Collection and Preparation

At the 21st day of age, ducks were fasted for 12 h and weighed individually to calculate the average body weight of animals in each cage for each treatment. Then, three birds whose weights were close to the average body weight of animals in each cage were selected. Blood samples were collected by jugular vein puncture with heparinized syringes equipped with stainless steel needles. The plasma was separated by centrifugation and stored at -20°C until analysis. Then, the selected birds were killed by cervical dislocation, and left liver samples were collected. A liver sample from the same location was frozen in liquid nitrogen for analysis of gene expression, and the left sample was frozen at -20°C for analyses of total lipids, cholesterol, total triglycerides, and fatty acid compositions. The three duck samples were pooled into one sample with same volume or weight in individual cage, and then, pooled samples were used for liver lipid analysis, fatty acid analysis, gene expression analysis. Other three birds were selected from each cage to collect breast muscle, thigh muscle, abdominal fat, and liver; these organs were weighed to calculate their weight in relation to the whole body. Finally, other three birds from each group were killed by cervical dislocation, and liver samples were collected and frozen in liquid nitrogen for transcriptome analysis.

Dietary Protein and Amino Acids

Dietary protein levels were determined according to the Kjeldahl method (Thiex et al., 2002). The amino acid concentrations in diets were measured with an amino acid analyzer (L-800; Hitachi, Tokyo, Japan) after hydrolysis in 6-M HCl for 24 h at 110°C. Dietary tryptophan was determined according to the method of Official Journal of the European Communities (EU2000/45/EC).

Plasma Parameters

The levels of plasma parameters, alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), glucose, total triglycerides (TG), total cholesterol (CHO), high-density lipoprotein cholesterol (HDLC), and low-density lipoprotein cholesterol (LDLC), were measured using an automatic analyzer (Hitachi 7080, Tokyo, Japan) with commercial kit (Maccura, Sichuang, China).

Liver Lipids

Total lipids were extracted by homogenizing minced liver tissue samples in chloroform–methanol (2:1, v/v) as described previously (Folch et al., 1957). The extracts were evaporated under a stream of nitrogen, weighed, and resuspended in chloroform–methanol (2:1) containing 0.01% (w/v) butylated hydroxytoluene. The concentrations of TG and CHO were measured using commercial kits (BioSino Bio-technology and Science Inc, Beijing, China).

Fatty Acids Composition

Liver samples were prepared according to a previously described method (Yang et al., 2010a). Briefly, fatty acids were extracted from total lipids and methylated by adding 1-ml acetyl chloride/methanol (1:10, v/v) and 20 μl of 5 mg/ml nonadecanoic acid (used as internal standard) for 4 h at 80°C. Then 1 ml of hexane and 1.5 ml of 6% (w/v) K_2CO_3 were added into the tube. After shaking for 10 min, 400 μl of the hexane layer was transferred to a new injection vial after centrifugation for 10 min at 3,000×g for further gas chromatography–mass spectrometry (GC-MS) analysis. The fatty acid methyl esters were separated by GC (Agilent 6890, Agilent Technologies, Santa Clara, CA, USA) using a DB 23 capillary column (60 m × 0.25 mm × 0.25 μm, Agilent Technologies, Santa Clara, USA) with MS detection (5970C, Agilent Technologies, Santa Clara, CA, USA). Samples (1 μl) were injected using an autosampler. The oven was programmed as follows: 50°C for 1 min, ramp to 175°C at 25°C/min holding 3 min, ramp to 200°C at 3.5°C/min holding 3 min, and finally ramp to 230°C at 2°C/min holding 3 min with helium as the carrier gas at a split ratio of 1:50. The GC was operated at constant flow pressure of 33.357 kPa, and the injector temperature, transfer line, and ion source were 250, 250, and 230°C, respectively. Peaks were identified by comparing retention times with those of the corresponding standards (Sigma Aldrich, Saint Louis, MO, USA).

RNA Isolation

Total RNA was extracted from frozen liver samples with RNAiso Plus reagent (code no. 9109, Takara, Dalian, China) according to the manufacturer's instructions. For quantitative polymerase chain reaction (qPCR) analysis, RNA concentration was measured at 260 nm (NanoDrop™ 2000, Thermo Fisher Scientific, Waltham, MA, USA), and the RNA integrity was evaluated by agarose gel electrophoresis stained with GelRed® (Biotium, Fremont, CA, USA). The mRNA integrity and concentration of samples used in the transcriptome analyses were evaluated with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Transcriptomic Analysis

Whole transcriptomic expression profiles of hepatic RNA samples (n = 3) from each group were analyzed using a HiSeq X Ten system (Illumina, San Diego, CA, USA). STAR and edgeR were used to quantify transcriptomic data as previously described for differential gene expression within RNA samples (Robinson et al., 2010; Dobin et al., 2013). Changes in expression of genes affected by Thr deficiency were further confirmed by qPCR.

Pathway Analysis With Gene Ontology and Kyoto Encyclopedia Genes and Genomes (KEGG)

Differentially expressed genes ($|\log_2^{\text{Fold change}}| > 0.585$, $P < 0.05$) in liver were converted to their FASTA Protein Sequence, and gene enrichment was performed with the FASTA Protein Sequences by "Gene-list Enrichment" in Kobas 3.0 (http://kobas.cbi.pku.edu.cn/) (Wu et al., 2006; Xie et al., 2011). *Homo sapiens* was selected

as the reference species, and hypergeometric test/Fisher's exact test was applied as statistical method.

qPCR

RNA samples were reverse transcribed to cDNA with the use of PrimerScript™ RT Master Mix (Code No. RR036A, Takara, Dalian, China) following the manufacturer's instructions. qPCR analysis was performed using real-time PCR quantitative analysis in the fluorescence detection system (ABI Q7, Life Technologies, Shanghai, China) with Power Green Master Mix (Code No.4367659, Life Technologies, NY, USA). Primers used for amplification are listed in **Supplementary Table S2**. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a housekeeping reference gene to normalize the expression of the targeted genes (Vandesompele et al., 2002). Each specimen was measured independently in triplicate, and PCR amplification efficiency was close to 100%. Relative mRNA expression of target genes was calculated using the $2^{-\Delta\Delta ct}$ method as previously reported (Livak and Schmittgen, 2001).

Statistical Analyses

Data were subjected to one-way ANOVA using general linear model procedure of SAS (version 9.2; SAS Institute Inc). Differences among means were tested by Duncan's method. Data are expressed as mean and standard error of the mean (SEM). The level of statistical significance was set at $P < 0.05$.

RESULTS

Growth Performance

Although the feed intake of ducks in the Pair-F group was assured to be similar to that of the Thr-D group by feed restriction, Thr intake, body weight, and weight gain in the Thr-D group were lower (P < 0.0001) than those in the Pair-F group (**Table 1**). Both Thr-D and Pair-F diets reduced (P < 0.0001) body weight and weight gain of ducks compared with that of the Thr-S group. The gain/feed ratio was lower in the Thr-D group than that in the Pair-F group (P < 0.03).

Carcass Traits

Both Thr-D and Pair-F diets reduced the relative weights of breast muscle (P < 0.0001) and abdominal fat (P < 0.006) when compared to those of the Thr-S. These treatments had no effect (P > 0.05) on thigh muscle relative weight (**Table 2**). There were no differences in the relative weights of breast muscle and abdominal fat (P > 0.05) between Thr-D and Pair-F groups.

Hepatic Lipid Accumulation

The Pair-F diet increased the relative weight of liver tissues compared with those of Thr-D and Thr-S (P < 0.0001), but there was no difference in the relative weight of liver between Thr-D and Thr-S (**Table 3**, P > 0.05). The Thr-D diet increased hepatic triglyceride concentration in Pekin ducks (P < 0.05) compared with those of the Thr-S and Pair-F groups. However, Thr-D and Pair-F treatments did not affect (P > 0.05) total lipids and cholesterol concentrations in liver.

Plasma Parameters

The Pair-F treatment elevated (P < 0.02) the glucose and HDLC concentrations and decreased LDLC (P < 0.001) levels compared with Thr-D and Thr-S diets (**Table 4**). Thr-D increased (P < 0.001) plasma HDLC concentration compared with the Thr-S diet, but its level was still lower than (P < 0.05) that in the Pair-F group. The Thr-D diet did not affect (P > 0.05) glucose and LDLC concentration compared with Thr-S group diet. Moreover, there were no significant differences in the plasma activities of ALT, AST, and ALP, or the plasma concentrations of CHO and TG between any groups.

TABLE 1 | Effects of threonine deficiency and feed restriction on growth performance of ducks from 1 to 21 days of age.

Items	Thr-D	Pair-F	Thr-S	Pooled SEM	*P*-value
Body weight (g)	984^{c}	1029^{b}	1275^{a}	12.96	<0.0001
Weight gain (g/day)	44.3^{c}	46.3^{b}	58.0^{a}	0.06	<0.0001
Feed intake (g/day)	75.6^{b}	76.26^{b}	96.9^{a}	1.44	<0.0001
Gain : Feed (g/g)	0.59^{b}	0.61^{a}	0.60^{ab}	0.01	0.0219
Thr intake (g/day)	0.40 ^{c}	0.56 ^{b}	0.71^{a}	0.008	<0.0001

Thr-D, threonine deficiency; Thr-S, threonine sufficiency; Pair-F, pair-fed.
^{a,b,c}Mean values with unlike superscript letters were significantly different (P < 0.05).

TABLE 2 | Effects of threonine deficiency and feed restriction on carcass traits of ducks at 21 day of age.

Items	Thr-D	Pair-F	Thr-S	Pooled SEM	*P*-value
Breast muscle (% of live weight)	1.62^{b}	1.54b	2.5^{a}	0.063	<0.0001
Thigh muscle (% of live weight)	10.7	9.99	10.2	0.2741	0.2478
Abdominal fat (% of live weight)	0.76^{b}	0.81b	0.9^{a}	0.0248	0.0057

Thr-D, threonine deficiency; Thr-S, threonine sufficiency; Pair-F, pair-fed.
^{a,b}Mean values with unlike superscript letters were significantly different (P < 0.05).

TABLE 3 | Effects of threonine deficiency and feed restriction on liver lipids of ducks at 21 day of age.

Items	Thr-D	Pair-F	Thr-S	Pooled SEM	*P*-value
Relative liver weight (%)*	3.64^{b}	4.39^{a}	3.29^{b}	0.11	<0.0001
Total Lipid (% of fresh liver)	6.10	5.84	6.06	0.14	0.2502
Triglyceride (mg/g fresh liver)	6.66^{a}	5.37^{b}	5.48^{b}	0.37	0.0432
Cholesterol (mg/g fresh liver)	1.66	1.35	1.70	0.11	0.0668

Thr-D, threonine deficiency; Thr-S, threonine sufficiency; Pair-F, pair-fed.
**Relative liver weight values were calculated as liver weight/liver body weight × 100%.*
^{a,b}Mean values with unlike superscript letters were significantly different (P < 0.05).

TABLE 4 | Effects of threonine deficiency and feed restriction on plasma parameters of ducks at 21 day of age.

Items	Thr-D	Pair-F	Thr-S	Pooled SEM	P-value
ALT (U/L)	40.5	37.9	41.6	1.54	0.2538
AST (U/L)	9.06	8.44	8.86	1.70	0.5064
ALP (U/L);	679	634	704	20.9	0.0894
GLU (mmol/L)	9.21^{b}	9.69^{a}	9.35^{b}	0.11	0.0193
CHO (mg/dL)	7.13	7.14	6.91	0.15	0.4792
TG (mmol/L)	0.79	0.65	0.76	0.04	0.0586
HDLC (mmol/L)	3.90^{b}	4.22^{a}	3.64^{c}	0.08	0.0009
LDLC (mmol/L)	2.04^{a}	1.75^{b}	2.21^{a}	0.07	0.0008

Thr-D, threonine deficiency; Thr-S, threonine sufficiency; Pair-F, pair-fed; ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; GLU, glucose; TG, total triglyceride; CHO, cholesterol; HDLC, high-density lipoprotein cholesterol; LDLC, low-density lipoprotein cholesterol.

^{a,b,c}Mean values with unlike superscript letters were significantly different (P < 0.05).

Liver Fatty acid composition

The Thr-D diet increased the hepatic contents of C6:0, C17:1, C18:3n6, C20:0, C20:1n9, and C22:2 and reduced the contents of C18:2n6t and C23:0 compared with the Thr-S diet (**Table 5**). However, there were no differences in total contents of fatty acid (TFA), saturated fatty acid (SFA), unsaturated fatty acid (USFA), and polyunsaturated fatty acid (PUFA) between Thr-D and Thr-S groups. The Pair-F diet increased the hepatic content of C14:0 and reduced the contents of C15:0, C17:0, C18:0, C20:0, C20:4n6, and C22:0 compared with those of Thr-D and Thr-S groups ($P < 0.05$). Moreover, the Pair-F group had lower total contents of fatty acid (TFA), saturated fatty acid (SFA), unsaturated fatty acid (USFA), and polyunsaturated fatty acid (PUFA) than those of Thr-D and Thr-S groups.

Hepatic Transcriptome Analysis

To screen differentially expressed genes in liver changed by Thr-D and Pair-F diets, a transcriptome analysis was performed in three individual duck livers from the different groups (Thr-D, Thr-S, and Pair-F). The differential genes were identified using *t*-test in EdgeR software ($|\mathrm{Log_2}^{\text{Fold change}}| > 0.585$, P value < 0.05). Compared with the Thr-S group, Thr-D-fed animals had 1,125 differentially expressed genes: 488 were upregulated and 637 were downregulated (**Supplementary Table 3A**, **Figure 1**), and Pair-F-fed animals had 1,028 differentially expressed genes: 214 were upregulated and 814 were downregulated (**Supplementary Table 3B**, **Figure 1**). Compared with the Pair-F group, 948 genes were differentially expressed in the Thr-D group: 622 were upregulated and 326 were downregulated (**Supplementary Table 3C**, **Figure 1**).

Transcriptome Data Validation by qPCR

The hepatic gene expression patterns were verified using qPCR for 13 genes, which were selected to represent lipid metabolism pathways. The expression levels of 11 out of 13 tested genes were similar to those detected in the transcriptomic analysis (n = 6). The exceptions were *FADS1* and *ACADSB* (**Figure 2**).

TABLE 5 | Effects of threonine deficiency and feed restriction on hepatic fatty acid composition ducks at 21 day of age.

Fatty acid (mg/g)	Thr-D	Pair-F	Thr-S	Pooled SEM	*P*-value
C6:0	0.018^{a}	0.012^{b}	0.012^{b}	0.001	0.001
C10:0	0.036	0.036	0.035	0.0002	0.247
C12:0	0.065	0.065	0.063	0.001	0.331
C14:0	0.210^{b}	0.258^{a}	0.208^{b}	0.009	0.001
C14:1	0.039	0.039	0.035	0.001	0.073
C15:0	0.045^{a}	0.043^{b}	0.044^{a}	0.0004	0.003
C15:1	0.048	0.047	0.048	0.001	0.615
C16:0	16.6^{a}	14.4^{b}	15.5^{ab}	0.574	0.050
C16:1	0.556	0.690	0.624	0.041	0.101
C17:0	0.144^{a}	0.114^{b}	0.140^{a}	0.004	< 0.0001
C17:1	0.043^{a}	0.041^{b}	0.041^{b}	0.001	0.014
C18:0	16.0^{a}	12.2^{b}	15.0^{a}	0.385	< 0.0001
C18:1n9c	21.1	17.0	17.0	1.59	0.144
C18:2n6t	0.067^{b}	0.067^{b}	0.070^{a}	0.001	0.039
C18:2n6c	7.05	6.64	7.23	0.195	0.128
C18:3n6	0.089^{a}	0.084^{a}	0.067^{b}	0.005	0.011
C18:3n3	0.105	0.087	0.110	0.008	0.172
C20:0	0.238^{a}	0.211^{c}	0.225^{b}	0.004	0.0004
C20:1n9	0.297^{a}	0.250^{b}	0.260^{b}	0.012	0.032
C20:2	0.936	0.802	0.873	0.038	0.075
C21:0	0.081	0.080	0.083	0.088	0.391
C20:3n6	1.20	1.28	1.26	0.039	0.317
C20:4n6	24.9^{a}	21.0^{b}	26.1^{a}	0.792	0.001
C20:3n3	0.091	0.090	0.090	0.001	0.655
C20:5n3	0.142	0.179	0.186	0.014	0.079
C22:0	0.385^{a}	0.349^{b}	0.381^{a}	0.006	0.001
C22:1	0.070	0.070	0.072	0.001	0.217
C22:2	0.134^{a}	0.091^{b}	0.092^{b}	0.009	0.004
C23:0	0.213a^{b}	0.202^{b}	0.237^{a}	0.007	0.011
C24:0	0.420	0.413	0.410	0.005	0.332
C22:6n3	0.958	1.02	1.04	0.050	0.473
C24:1	0.290	0.243	0.247	0.020	0.207
TFA	92.6^{a}	78.0^{b}	87.8^{a}	2.80	0.007
SFA	34.6^{a}	28.4^{b}	32.4^{a}	1.02	0.003
UFA	58.0^{a}	49.7^{b}	55.2^{a}	1.78	0.015
MUFA	22.4	18.4	18.3	1.63	0.156
PUFA	35.7^{a}	31.2^{b}	37.1^{a}	0.952	0.001

Thr-D, threonine deficiency; Thr-S, threonine sufficiency; Pair-F, pair-fed; TFA, total fatty acid; SFA, saturated fatty acid; UFA, unsaturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

^{a,b,c}Mean values with unlike superscript letters were significantly different (P < 0.05).

Gene Ontology and KEGG Pathway Analysis of Differential Genes

To identify biological processes and KEGG pathways, the differentially expressed genes were decoded into their protein sequences. This resulted in 829, 682, and 733 protein sequences in Thr-D vs Thr-S, Thr-S vs Pair-F, and Pair-F vs Thr-S comparisons, respectively. An enrichment test was applied to search for significantly overrepresented GO terms (P value < 0.05) and KEGG pathways (P value < 0.05). The GO term enrichment showed that more genes related to lipid processes and molecular function were enriched in both Thr-D vs Thr-S and Thr-S vs Pair-F comparisons than in Pair-F vs Thr-S (**Figure 3**). Some KEGG pathways were also related to signal transduction, immunity, hormones, lipid, and amino acid metabolism (**Figure 4**).

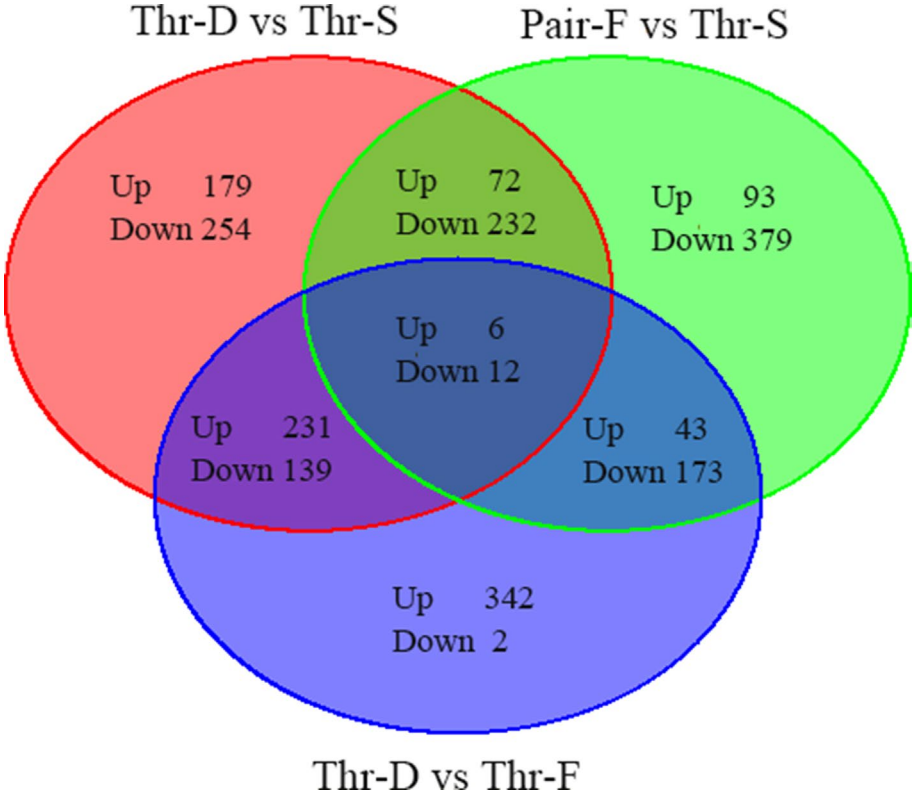

FIGURE 1 | The total differentially expressed genes between groups (Thr-D vs Thr-S, Thr-S vs Pair-F, Pair-F vs Thr-S).

DISCUSSION

Previous studies from our laboratory showed that dietary Thr deficiency increases hepatic triglyceride concentration and reduces the abdominal fat levels (Jiang et al., 2017; Jiang et al., 2019). In the present study, there were a reduction in growth and abdominal fat percentage and an increase in hepatic triglyceride concentration of ducks during dietary Thr deficiency (**Tables 1**, **2**, and **3**). Similar to those previous studies, we also observed a reduction in feed intake in the present study, which would affect physiological responses. Long-term (4–12 weeks) feed restriction reduces the concentration of ghrelin and increases the concentration of corticosterone in the plasma of turkeys (Vizcarra et al., 2018). In chickens, ghrelin activates the expression of corticotropin-releasing hormone and β-adrenergic receptor (Zendehdel and Hassanpour, 2014). In mammals, adrenaline can stimulate lipolysis by interacting with β-adrenergic receptor or inhibit lipolysis by interacting with α2-adrenergic receptor (Lafontan and Berlan, 1993; Robidoux et al., 2004). In addition, the reduction in feed intake reduces lipid deposition in cattle (Basarab et al., 2003). Therefore, the decreased feed intake itself observed in the present study might affect the hepatic lipid deposition in ducks.

Therefore, we assessed the role of feed restriction to distinguish its effects on hepatic triglyceride levels from those of Thr deficiency. By implementing feed restriction in the Pair-F group, we could assure that the feed intake of ducks in the Thr-D was similar to that of the Pair-F group. However, the growth performance of ducks in the Thr-D group was lower than that in the Pair-F group (**Table 1**), whereas ducks fed with the Thr-D diet increased their hepatic triglyceride concentration compared with those in the Pair-F group; Pair-F and Thr-S groups had similar hepatic triglyceride concentrations (**Table 4**). This indicates that low Thr intake directly increases hepatic triglyceride accumulation in ducks. These results differ from previous studies using broilers, in which feed restriction tends to reduce fat content in breast and thigh muscles (Wang et al., 2010) and reduce hepatic triglyceride content (Yang et al., 2010b). In lambs, feed restriction reduces total fatty acid, saturated fatty acid, and unsaturated fatty acid levels in the longissimus thoracis muscle (de Araújo et al., 2017). In the present study, the Pair-F group had increased hepatic content of C14:0 and reduced contents of C15:0, C17:0, C18:0, C20:0, C20:4n6, and C22:0, as well as reduced total fatty acid, saturated fatty acid, and unsaturated fatty acid contents, compared with Thr-D and Thr-S groups. The Thr-D group had increased hepatic fatty acid content (C6:0, C17:1, C18:3n6, C20:0, C20:1n9, and C22:2) compared with the Thr-S group. Ducks fed with the Pair-F diet had reduced hepatic fatty acid, whereas those fed with the Thr-D diet had increased hepatic triglyceride content. In addition, although there was a clear accumulation of triglycerides in the liver, its biological functions were not affected by Thr deficiency, as the plasmatic activities of AST, ALT, and ALP were unaffected by Thr-D and Pair-F diets. Nevertheless, Thr-D and Pair-F diets

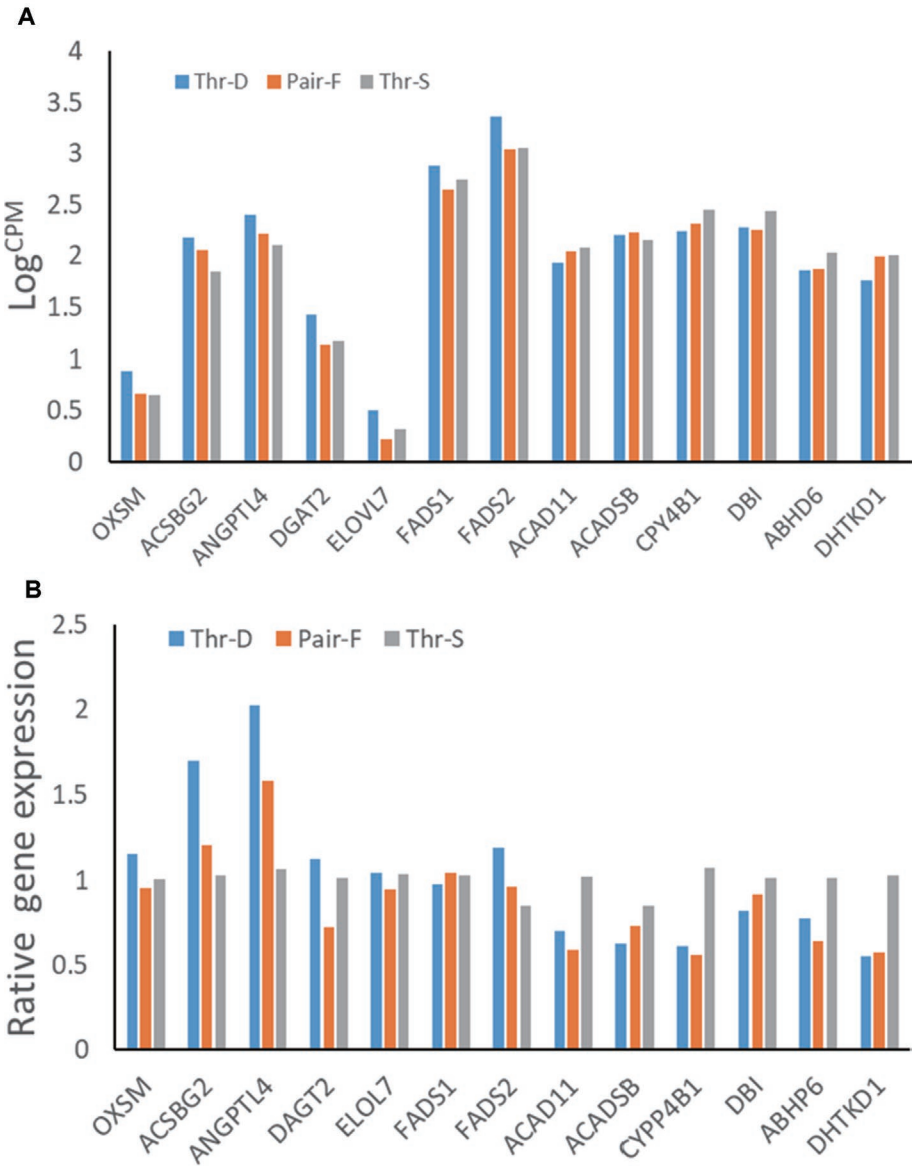

FIGURE 2 | Gene expressions from transcriptome data **(A)** and qRT-PCR **(B)** of hepatic gene expressions of 21-day-old ducks from threonine defciency (Thr-D), threonine suffciency (Thr-S), and pair-fed (Pair-F). *FADS2*, fatty acid desaturase 2; *ACSBG2*, acyl-CoA synthetase bubblegum family member 2; *OXSM*, 3-oxoacyl-ACP synthase; *ELOVL7*, ELOVL fatty acid elongase 7; *FADS1*, atty acid desaturase 1; *DBI*, diazepam binding inhibitor, acyl-CoA binding protein; *DGAT2*, diacylglycerol O-acyltransferase 2; *ABHD6*, abhydrolase domain containing 6; *ACADSB*, acyl-CoA dehydrogenase, short/branched chain; *ACAD11*, acyl-CoA dehydrogenase family member 11; *CYP4B1*, cytochrome P450 family 4 subfamily B member 1; *DHTK1*, dehydrogenase E1 and transketolase domain containing 1; *ANGPTL4*, angiopoietin like 4. Data are means ± SEM (n = 6). ^{a,b}Mean values with unlike superscript letters were significantly different ($P < 0.05$).

changed the concentration of GLU, HDLC, and LDLC in plasma, which indicates that Thr deficiency and feed restriction affect lipid metabolism.

In order to determine the underlying molecular changes caused by dietary Thr deficiency, we had previously assessed the expression of several genes involved in fatty acid synthesis and oxidation and triglyceride transport (Jiang et al., 2019). Unfortunately, the underlying mechanism of triglyceride accumulation caused by dietary Thr deficiency was still unclear after that study because of the large number of genes involved in the process. Therefore, here, we screened differentially expressed genes in response to dietary Thr deficiency using RNA sequencing techniques. It was observed that the lots of genes involved in amino acid, immune, energy, steroid, and lipid metabolism were differentially expressed.

Amino Acid Biosynthesis

In the present study, 29 differentially expressed genes were associated with amino acid metabolism in the comparison

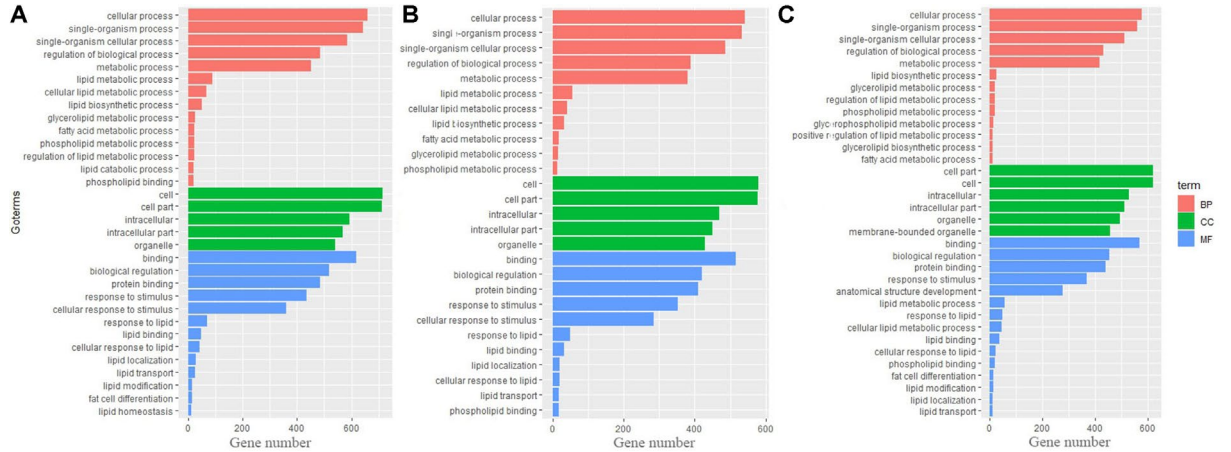

FIGURE 3 | The GO terms involved in lipid metabolism enriched by differentially expressed genes (**A**, Thr-D vs Thr-S; **B**, Thr-D vs Pair-F; **C**, Pair-F vs Thr-D)

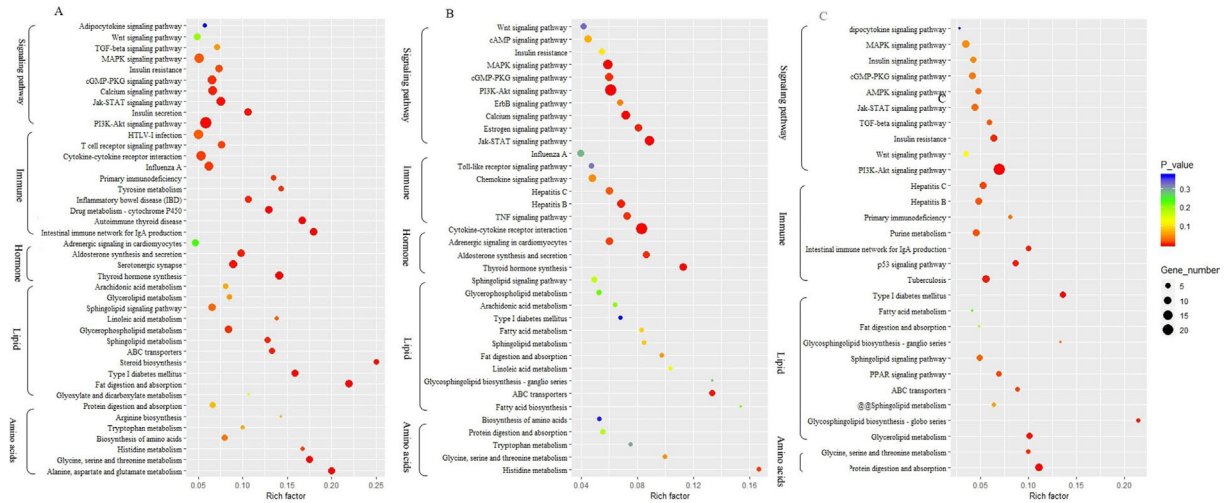

FIGURE 4 | The KEGG pathway involved in lipid metabolism enriched by differentially expressed genes (**A**, Thr-D vs Thr-S; **B**, Thr-D vs Pair-F; **A**, Pair-F vs Thr-D)

of the Thr-D group against the Thr-S group, of which nine genes were upregulated and 20 genes were downregulated (**Supplementary Table 4A**). When compared with the Pair-F group, the Thr-D group presented nine upregulated genes and nine downregulated gene expression involved in amino acid metabolism (**Supplementary Table 4B**). The Pair-F diet affected the expression of 14 genes compared with the Thr-S diet, of which four genes were upregulated and 11 genes were downregulated (**Supplementary Table 4C**). Dynamic research about amino acid networks showed that Thr have positive effects on methionine, valine, isoleucine, leucine, asparagine, glutamic acid, tyrosine, cysteine, and a negative effect on serine pathways (Shikata et al., 2007; Shikata et al., 2010). A previous study from our laboratory showed that dietary deficiency reduces the plasmatic concentration of methionine, cysteine, lysine, arginine, isoleucine, valine, histidine, and phenylalanine and increases that of serine and alanine (Jiang et al., 2017). In the present study, the differentially expressed genes included those associated with alanine, histidine, aspartate, glutamate, glycine, serine, threonine, and tryptophan metabolism, and most of these genes were downregulated by the Thr-D diet (**Supplementary Table 4A**). In addition, genes involved in amino acid biosynthesis were downregulated by threonine deprivation (**Supplementary Table 5A**). These results indicate that amino acid biosynthesis decreased, whereas amino acid degradation increased in the liver of ducks fed with the Thr-D diet.

Immunity

Threonine plays an important role in immune responses, as it affects the production of intestinal IgA in laying hens and pigs (Azzam et al., 2011; Zhang et al., 2017) and serum IgY in ducks (Zhang et al., 2014). In the present study, the enrichment analysis revealed that 57 genes were related to immune responses,

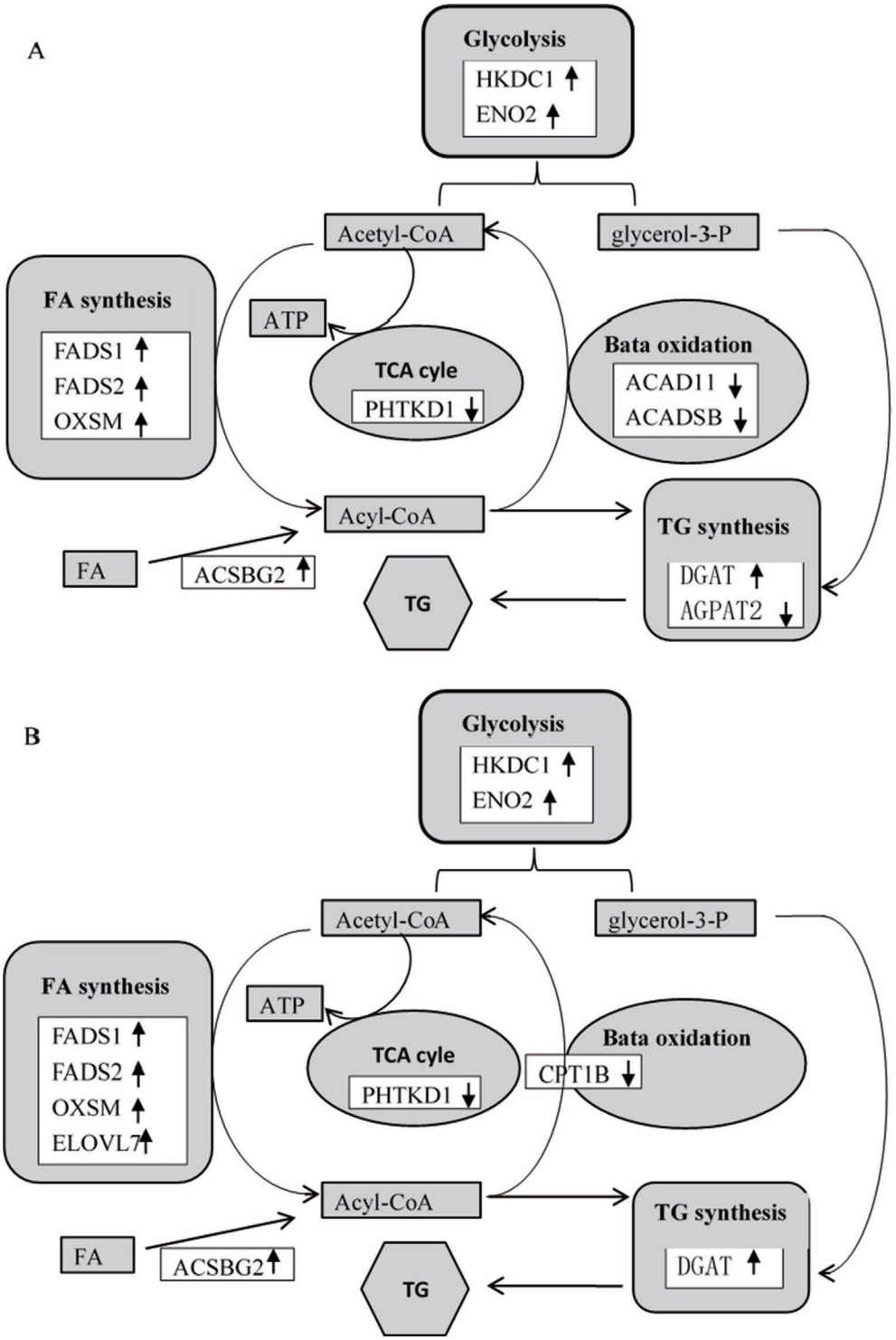

FIGURE 5 | Differentially expressed genes (**A**, Thr-D vs Thr-S; **B**, Thr-D vs Pair-F) involved in glycolysis, fatty acid synthesis and beta oxidation, triglyceride synthesis, and tricarboxylic acid (TCA) cycle. The black up arrows at right indicate increased gene levels in response to threonine deficiency, and the black down arrows at right indicate reduced gene levels in response to threonine deficiency. *HKDC1*, hexokinase domain containing 1; *ENO2*, enolase 2; *FADS1*, fatty acid desaturase 1; *FADS2*, fatty acid desaturase 2; *OXSM*, 3-oxoacyl-ACP synthase; *ACADSB*, acyl-CoA dehydrogenase, short/branched chain; *ACAD11*, acyl-CoA dehydrogenase family member 11; *ACSBG2*, acyl-CoA synthetase bubblegum family member 2; AGPAT2, 1-acylglycerol-3-phosphate O-acyltransferase 2; *DGAT2*, diacylglycerol O-acyltransferase 2; *DHTKD1*, dehydrogenase E1 and transketolase domain containing 1; *ELOVL7*, ELOVL fatty acid elongase 7; *CPT1B*, carnitine palmitoyltransferase 1B.

including T cell and Toll-like receptor signaling in the comparison of the Thr-D against the Thr-S group (**Supplementary Table 5A**). The comparison between the Thr-D and the Pair-F groups resulted in 45 differentially expressed genes associated with immune responses (**Supplementary Table 5B**). Only 32 genes involved in immune responses were differentially expressed between Pair-F and Thr-S groups (**Supplementary Table 5C**). T cells play a key role in cell-mediated immunity and T cell receptor activation, and they enhance a number of signaling cascades that determine cell fate by regulating cytokine production, cell survival, proliferation, and differentiation (Burbach et al., 2007). We found that seven genes related to the T cell receptor signaling were downregulated, whereas only one was upregulated in the liver of animals fed with the Thr-D diet. This indicates that T cell immunity was repressed by threonine deprivation.

Toll-like receptors (TLRs) are key components of innate and adaptive immunities. They control host immune responses against pathogens through recognition of molecular patterns that are specific to microorganisms. In the present study, five genes associated with Toll-like receptor signaling were upregulated, and one gene was downregulated by threonine restriction. The nucleotide-sensing TLR3 is activated by double-stranded viral RNA, which is a sign of viral infection. Its activation leads to NF-κB induction, IRF3 nuclear translocation, cytokine secretion, and the inflammatory response *via* TRIF/TICAM1 (Oshiumi et al., 2003; Johnsen et al., 2006). Toll-like receptor 5 (TLR5) participates in the innate immune response to microbial agents (e.g., bacterial flagellin) by activating NF-κB and promoting cytokine secretion and the inflammatory response (Hayashi et al., 2001). In the present study, TLR3 and TLR5 were upregulated by the Thr-D diet compared with the Thr-S diet. Moreover, their downstream gene, MAP3K8, was also upregulated. In addition, compared with the Pair-F group, the Thr-D group upregulated five gene expression related to Toll-like receptor signaling. Genes affected by the Pair-F diet were not enriched in genes associated with TLR signaling and T cell receptor signaling. Taken together, our results show that the Thr-D diet regulates the TLR signaling by affecting the expression of genes related to both the activation of TLR signaling and its downstream effectors.

Energy Metabolism

Previous studies reported that dietary Thr deficiency reduces hepatic cell respiration in rats (Ross-Inta et al., 2009). In the present study, the Thr-D diet had increased expressions of enolase 2 (*ENO2*) and hexokinase domain containing 1 (*HKDC1*) and reduced the expression of dehydrogenase E1 and transketolase domain containing 1 (*DHTKD1*) and 3-hydroxymethyl-3-methylglutaryl-CoA lyase (*HMGCL*) genes compared with the that of Thr-S group (**Table 6**). One of those genes, *HKDC1,* encodes hexokinase, which phosphorylates hexoses (Wolf et al., 2011; Guo et al., 2015). Enolase, which catalyses the production of phosphoenolpyruvate, is encoded by the *ENO2* gene (Marangos and Schmechel, 1987). Glycolysis supplies glycerol 3-phoshate for triglyceride synthesis and acyl-CoA for fatty acid synthesis or tricarboxylic acid (TCA). In that regard, *DHTKD1* is a key gene for the TCA cycle, which codes the enzyme that converts 2-oxoglutarate to succinyl-CoA, generating ATP (Danhauser et al., 2012). A previous study showed that dietary Thr deficiency decreases liver mitochondrial coupling, leading to a reduction in ATP production (Ross-Inta et al., 2009). Another key metabolic pathway, ketogenesis, depends on the activity of HMGCL enzyme, which catalyses acetoacetate synthesis in the liver from 3-hydroxy-3-methylglutaryl-CoA (Tuinstra and Miziorko, 2003). Acetoacetate is transported to extrahepatic tissues (e.g., the brain) as energy source. In addition, the Thr-D diet induced the expressions of acyl-CoA synthetase short-chain family member 1 (*ACSS1*) and 3-hydroxybutyrate dehydrogenase 1 (*BDH1*) genes compared with that of the Pair-F group (**Table 6**). The protein encoded by *ACSS1* converts acetate to acetyl-CoA, used in the TCA cycle to produce energy and electron carriers, whereas BDH1 enzyme catalyses the interconversion of acetoacetate to 3-hydroxybutyrate (Churchill et al., 1992). 3-Hydroxybutyrate is absorbed by target tissues and converted back to acetoacetate by the same enzyme (Newman and Verdin, 2014). Acetoacetyl-CoA can be converted to two acetyl-CoA molecules, and then, they can be oxidized and produced ATP in TCA (Fukao et al., 2004). The Thr-D group had increased expressions of *ENO2* and *HKDC1* and reduced expression of *DHTKD1* compared with the Pair-P group (**Table 6**). However, there were no differences in the expression of these genes between Pair-F and Thr-S groups. Therefore, threonine deprivation increases glycolysis and affects ATP production and ketogenesis (**Figure 5**).

Lipid Metabolism

In the present study, 64, 31, and 30 genes that were related to lipid metabolism were differentially expressed between Thr-D and Thr-S, Thr-D and Pair-F, and Pair-F and Thr-S groups, respectively (**Supplementary Table 4A**, **B**, **C**). Among those, genes involved in fatty acid and triglyceride synthesis were upregulated, whereas genes related to fatty acid and triglyceride transport and degradation were downregulated by either Thr-D or Pair-F diets. The gene upregulated by the Thr-D diet included 3-oxoacyl-ACP synthase (*OXSM*), very long chain fatty acids protein 7 (*ELOVL7*), and long chain fatty acyl-CoA ligase (*ACSBG2*), which are involved in *de novo* synthesis of fatty acids (Zhang et al., 2005; Ohno et al., 2010; Naganuma et al., 2011). However, acyl-CoA dehydrogenase, short/branched chain (*ACADSB*), acyl-CoA dehydrogenase family member 11 (*ACAD11),* and cytochrome P450 (*CYP4B*) were downregulated by the Thr-D diet. These genes are involved in fatty acid β-oxidation and ω-oxidation (Vockley et al., 2000; He et al., 2011; Hsu et al., 2017; Roellecke et al., 2017; Scott, 2017). The Thr-D diet downregulated the expression of carnitine palmitoyltransferase 1B (*CPT1B*) compared with the Pair-F diet. The product of this gene is a protein that transports long chain acyl-CoA molecules from cytoplasm into mitochondria and thus controls a key regulatory step of β-oxidation (Kerner and Hoppel, 2000). In addition, Thr-D and Pair-F diets downregulated the expression of acyl-CoA binding protein (*DBI*), apolipoprotein D (*APOD)*, and microsomal triglyceride transfer protein (*MTTP*). These genes participate in fatty acid and triglyceride transport (Rasmussen et al., 1994;

TABLE 6 | Differentially expressed genes in liver involved in lipid metabolism on day 21 of ducks caused by threonine deficiency.

Genes	Annotation	Function	$Log_2^{(Fold\ change)*}$		
			Thr-D vs Thr-S	Thr-D vs Pair-F	Pair-F vs Thr-S
ACOT12	Acyl-coa thioesterase 12	Fatty acid synthesis	1.81	1.74	–
FADS2	Fatty acid desaturase 2	Fatty acid synthesis	0.90	1.05	–
ACSBG2	Acyl-coa synthetase bubblegum family member 2	Fatty acid synthesis	0.98	–	–
OXSM	3-oxoacyl-ACP synthase	Fatty acid synthesis	0.65	0.71	–
ELOVL7	ELOVL fatty acid elongase 7	Fatty acid synthesis	–	0.89	–
FADS1	Fatty acid desaturase 1	Fatty acid synthesis	–	0.73	–
CPT1B	Carnitine palmitoyltransferase 1B	Fatty acid degradation	–	-0.77	–
ACADSB	Acyl-coa dehydrogenase, short/branched chain	Fatty acid degradation	-0.79	–	–
ACAD11	Acyl-coa dehydrogenase family member 11	Fatty acid degradation	-0.59	–	–
CYP4B1	Cytochrome P450 family 4 subfamily B member 1	Fatty acid degradation	-0.82	–	–
DBI	Diazepam binding inhibitor, acyl-coa binding protein	Fatty acid transporter	-0.65	–	-0.70
DGAT2	Diacylglycerol O-acyltransferase 2	Triglyceride synthesis	0.71	0.94	–
GPD1	Glycerol-3-phosphate dehydrogenase 1	Triglyceride synthesis	1.16	1.32	–
ABHD6	Abhydrolase domain containing 6	Triglyceride degradation	-0.67	–	-0.62
AGPAT2	1-acylglycerol-3-phosphate O-acyltransferase 2	Phospholipid synthesis	-0.87	–	-0.65
CHKA	Choline kinase alpha	Phospholipid synthesis	2.43	2.08	–
ETNK2	Ethanolamine kinase 2	Phospholipid synthesis	-1.71	-1.33	–
ENO2	Enolase 2	Glycolysis	2.04	1.91	–
HKDC1	Hexokinase domain containing 1	Glycolysis	1.03	1.12	–
DHTKD1	Dehydrogenase E1 and transketolase domain containing 1	ATP production	-0.94	-0.80	–
HMGCL	3-hydroxymethyl-3-methylglutaryl-coa lyase	Ketogenesis	-1.63	–	–
ACSS1	Acyl-coa synthetase short-chain family member 1	Ketogenesis	–	0.60	–
BDH1	3-hydroxybutyrate dehydrogenase 1	Ketogenesis	–	0.90	–
APOD	Apolipoprotein D	Lipid transport	-0.69	–	-0.63
ABCG1	Cholesterol transport	Lipid transport	-1.23	–	-1.28
SR-B1	Scavenger receptor class B member 1	Lipid transport	-0.61	–	–
IGFBP2	insulin-like growth factor-binding protein 2	adipokine	-1.24	-1.05	–
SOCS3	suppressor of cytokine signaling 3	adipokine	1.53	2.46	-0.93
ANGPTL4	angiopoietin like 4	adipokine	0.90	–	–

Thr-D, threonine deficiency; Thr-S, threonine sufficiency; Pair-F, pair-fed.

$Log2^{(Fold\ change)}$ is expressed as the logarithmic fold change between the two of three groups. The positive $Log2^{(Fold\ change)}$ indicates the upregulation genes, and the negative $Log2^{(Fold\ change)}$ indicates downregulation genes.

Faergeman and Knudsen, 1997; Xie et al., 2006; Walsh et al., 2015). Based on the aforementioned mRNA results, it would be expected that the fatty acid content in Thr-D and Pair-F groups should be higher than that in the Thr-S group. However, we observed similar total fatty acid levels between Thr-D and Thr-S groups. In Thr-D and Pair-F groups, only the levels of C22:2, C20:1n9, C18:3n6, C18:2n6t, and C17:1 were higher than those in the liver of animals fed with the Thr-S diet. Moreover, hepatic triglyceride content in the Thr-D group was higher than that in the Thr-S group, but both groups had similar total lipids contents, which indicated that dietary Thr deficiency affected the hepatic lipid compositions. However, it was still unclear and need further experiments to prove it. Therefore, it was speculated that the synthesis of triglycerides is favored during dietary Thr deficiency. The expression of diacylglycerol O-acyltransferase 2 (*DGAT2*) was upregulated by the Thr-D diet compared with those by the other two dietary treatments. The protein coded by this gene binds diacylglycerol to long-chain fatty acid acyl-CoA in the final reaction of triglyceride biosynthesis (Agarwal et al., 2011; McFie et al., 2011; Qi et al., 2012). On the other hand, the Pair-F diet did not change the expression of *DGAT2* compared with the Thr-S group diet. This observation is consistent with that of similar levels of total lipids between the Thr-S and Pair-F groups.

Steroid Biosynthesis

In the present study, the Thr-D diet upregulated the expression of 7-dehydrocholesterol r eductase (*DHCR7*), methylsterol monooxygenase 1 (*MSMO1*), NAD(P) dependent steroid dehydrogenase-like, lanosterol 14-alpha demethylase, squalene epoxidase (*SQLE*), and class I histocompatibility antigen compared with the Thr-S group diet. These genes are involved in steroid biosynthesis. To the best of our knowledge, there are no reports on the effects of dietary amino acid levels on the expression of genes related to steroid biosynthesis. However, feed restriction downregulates the expression of such genes in pigs and rats (Selman et al., 2006; Lkhagvadorj et al., 2009; Lkhagvadorj et al., 2010). In contrast, we found that the Pair-F diet did not change the expression of genes related to steroid biosynthesis compared with the Thr-S group diet. The discrepancy between the present study and previous studies might be due to species-specific characteristics.

Effects of Thr on Adipocytokines

Adipocytokines play key roles in the regulation of lipid metabolism. In the present study, several adipocytokine factors involved in lipid metabolism were differentially expressed, including *SOCS3*, *IGFBP2*, and *ANGPTL4*. These genes have

diverse functions, for example, *SOCS3* is a negative regulator of insulin signaling in skeletal muscle, adipose tissue, and the liver (Shi et al., 2004; Ueki et al., 2004; Yang et al., 2012), whereas it is also a negative regulator of leptin in the hypothalamus (Bjorbaek et al., 1999). The overexpression of *SOCS3* increases insulin and leptin resistance (Yang et al., 2012), which in turn increases hepatic lipid accumulation (Liu et al., 2014). In addition, *ANGPTL4* is expressed in the adipose tissue, liver, and placenta (Kersten et al., 2000; Kim et al., 2000; Yoon et al., 2000), where it regulates lipid metabolism primarily by inhibiting lipoprotein lipase activity (Ge et al., 2004; Köster et al., 2005) and promotes triglyceride uptake. The upregulation of *ANGPTL4* occurs in response to palmitic, oleic, arachidonic, and eicosapentaenoic acids (Gonzalez-Muniesa et al., 2011). Overexpression of *ANGPTL4* in mice increases the concentrations of triglycerides and cholesterol in plasma but does not increase body fat mass (Köster et al., 2005). Moreover, the overexpression of *ANGPL4* increases hepatic fat accumulation in mice (Xu et al., 2005). IGF binding protein 2 (*IGFBP2*) is expressed by several tissues, including the liver and white adipocytes (Gosteli-Peter et al., 1994), where it contributes to the prevention of diet-induced fat accumulation (Wheatcroft et al., 2007). Overexpression of *IGFBP2* reduces body fat accumulation in mice fed with a high-fat diet (Kammel et al., 2016) and decreases liver triglyceride deposition in ob/ob mice (Hedbacker et al., 2010). In the present study, the Thr-D diet downregulated *IGFBP2* and upregulated *ANGPTL4* and *SOCS3* in the liver of Pekin ducks, which agrees with the increased triglyceride deposition in the liver of these animals.

Effects of Thr on Pathway Related to Lipid Metabolism

In addition, we found that several cellular regulatory pathways involved in lipid metabolism were differentially expressed in Thr-D and Pair-F groups, such as PI3K-Akt, Jak-STAT, cGMP-PKG, and Wnt signaling pathway. Apoptosis-related pathways, such as PI3K/AKT-TOR, cAMP/PKA/CREB, and LKB1/AMPK-FOXO, regulate methionine-induced changes in hepatic lipid metabolism in fish and mammals (Gao et al., 2018). Signal transduction in the Jak-STAT pathway has been demonstrated to regulate lipid metabolism in mice (Xu et al., 2013; Shi et al., 2014). For instance, di-(2-ethylhexyl) phthalate reduces lipid hydrolysis and promotes triglyceride accumulation by regulating the activation state of the Jak-STAT pathway in the liver and adipose tissue in rats (Jia et al., 2016) and magnesium-induced reduction in hepatic lipid deposition in yellow catfish (Wei et al., 2017). In the present study, the Thr-D diet downregulated the expression of signal transducer and activator of transcription 4 (*STAT4*) and upregulated the expression of suppressor of cytokine signaling 3 (*SOCS3*), whereas the Pair-F diet did not change the expression of *STAT4* but downregulated the expression of *SOCS3* compared with the Thr-S group diet. Cytokines activate the JAK-STAT signaling pathway by binding to its receptor, leading to the expression of *SOCS* (Krebs and Hilton, 2000). The upregulated SOCS protein acts through a feedback mechanism to inhibit the JAK-STAT signaling pathway in the cytosol (Miao et al., 2006). Therefore, in Pekin ducks, the high expression of *SOCS3* might inhibit that of *STAT4* in the liver of animals fed with the Thr-D diet.

Activation of PI3K/AKT might lead to the expression of Fas cell surface death receptor and Sterol regulatory element-binding proteins (Jeon and Osborne, 2012; Liu et al., 2016). On the other hand, inhibition of PI3K/AKT impairs the suppression of very-low-density lipoprotein assembly and insulin secretion by regulating the secretion and degradation of apolipoprotein B and the gene expression of hepatic *MTTP* (Sidiropoulos et al., 2007). Moreover, inhibition of PI3K/AKT/mTOR pathway reduces hepatic lipid accumulation by increasing fatty acid oxidation and VLDL assembly and secretion in geese (Liu et al., 2016). In the present study, the Thr-D diet upregulated eight genes and downregulated 12 genes involved in the PI3K-AKT signaling pathway, as well as downregulated the expression of *MTTP*, compared with the Thr-S group diet. Feed restriction in the Pair-F group upregulated 15 genes and downregulated 59 genes in PI3K-AKT signaling pathway, when compared with that in the Thr-S group. Thus, it is clear that both Thr-D and Pair-F diets changed the expression of genes that compose the PI3K-AKT signaling pathway, but there were differences between Thr-D and Pair-F groups.

In summary, both Thr-D and Pair-F diets affected hepatic lipid metabolism in Pekin ducks. However, the molecular mechanisms underlying lipid metabolism changes differed between Thr-D and Pair-F groups. Animals in the Pair-F group had reduced hepatic individual fatty acid contents, whereas ducks in the Thr-D group had a similar fatty acid profile when compared with those in the Thr-S group. However, the Thr-D diet increased the accumulation of hepatic triglycerides, and the Pair-F diet had no effect on hepatic triglyceride accumulation. Transcriptome analysis showed that the Thr-D diet upregulated the expression of genes related to fatty acid and triglyceride synthesis and downregulated genes related to fatty acid oxidation and triglyceride transport. This indicates that threonine deprivation promoted the entrance of fatty acids into the triglyceride pathway. Both Thr-D and Pair-F diets induced regulatory changes involved in lipid metabolism that might be controlled by PI3K-AKT, Jak-STAT, and adipocytokine signaling pathways. The role of these signaling pathways in the regulation of lipid metabolism during dietary threonine deprivation warrants further research.

DATA AVAILABILITY

The datasets generated for this study can be found in NCBI. The number of BioProject is PRJNA530027, and the data can be visited at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA530027.

ETHICS STATEMENT

The study was approved by the Animal Management Committee (in charge of animal welfare issue) of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (IAS20160322, IAS-CAAS, Beijing, China) and performed in accordance with

the guidelines. Ethical approval on animal survival was given by the animal ethics committee of IAS-CAAS.

AUTHOR CONTRIBUTIONS

SH conceived and coordinated the study. YJ performed the study, was involved in all aspects of analysis, and drafted the manuscript. MX and SH were involved in experimental design. MX performed data analysis. JT, WF, and JX performed the sample analysis. GC participated in editing the manuscript. SH had primary responsibility for the final content. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2019.00710/full#supplementary-material

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Epigenetic Modifications Induced by Nutrients in Early Life Phases: Gender Differences in Metabolic Alteration in Adulthood

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Metabolic chronic diseases, also named noncommunicable diseases (NCDs), are considered multifactorial pathologies, which are dramatically increased during the last decades. Noncommunicable diseases such as cardiovascular diseases, obesity, diabetes mellitus, cancers, and chronic respiratory diseases markedly increase morbidity, mortality, and socioeconomic costs. Moreover, NCDs induce several and complex clinical manifestations that lead to a gradual deterioration of health status and quality of life of affected individuals. Multiple factors are involved in the development and progression of these diseases such as sedentary behavior, smoking, pollution, and unhealthy diet. Indeed, nutrition has a pivotal role in maintaining health, and dietary imbalances represent major determinants favoring chronic diseases through metabolic homeostasis alterations. In particular, it appears that specific nutrients and adequate nutrition are important in all periods of life, but they are essential during specific times in early life such as prenatal and postnatal phases. Indeed, epidemiologic and experimental studies report the deleterious effects of an incorrect nutrition on health status several decades later in life. During the last decade, a growing interest on the possible role of epigenetic mechanisms as link between nutritional imbalances and NCDs development has been observed. Finally, because of the pivotal role of the hormones in fat, carbohydrate, and protein metabolism regulation throughout life, it is expected that any hormonal modification of these processes can imbalance metabolism and fat storage. Therefore, a particular interest to several chemicals able to act as endocrine disruptors has been recently developed. In this review, we will provide an overview and discuss the epigenetic role of some specific nutrients and chemicals in the modulation of physiological and pathological mechanisms.

Keywords: epigenetics, nutrition, endocrine-disrupting chemicals, development, gender

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INTRODUCTION

A significant increase in human longevity has been observed in the last two decades, and life expectancy exceeds the age of 80 years in several countries (World Health Organization (WHO)) with a proportional increase of chronic diseases (Figueira et al., 2016). Noncommunicable diseases (NCDs), such as diabetes, sarcopenia, osteoporosis, cardiovascular diseases, neurological disorders, and cancers, increase with

age and seriously affect both subject's life and healthcare systems (Troesch et al., 2015). In fact, NCDs induce several and complex clinical manifestations that lead to a gradual deterioration of health status and quality of life of affected individual, making the subject frail and at greater risk of disability and mortality. Then, supporting healthy aging by preventing NCDs is a major priority for agencies such as the World Health Organization (WHO) and the United Nations [World Health Organization (WHO), 2018]. In particular, WHO has identified unhealthy diets, sedentary behaviors, excessive alcohol consumption, tobacco use, and pollution among the main modifiable risk factors, with nutrition as an important determinant of human health throughout life (Eggersdorfer and Walter, 2011).

Nutrition has a pivotal role in maintaining health, and dietary imbalances represent major determinants favoring chronic diseases through metabolic homeostasis alterations. Adequate nutrition and specific nutrients are important in all periods of life, but they appear essential during specific times such as in utero life and early years of postnatal life. In this context, large amount of epidemiologic and experimental data show that imbalanced diet can induce health consequences several decades after exposure, and during the last decade, an increased interest has been observed on the possible role of epigenetic mechanisms as link between nutritional imbalances and NCD development (Block and El-Osta, 2017).

Interestingly, the "developmental origins of adult disease" hypothesis originated in 1989 from epidemiological studies by David Barker and colleagues (Barker et al., 1989a; Barker et al., 1989b) that showed newborns with small weight at birth were at a major risk of heart failure in later phases of life. Hales and Barker (Hales and Barker, 1992) used the term "programming" to describe the "permanent or long-term change in the structure or function of an organism resulting from a stimulus or insult acting at a critical period of early life." Afterward, the concept of epigenetics was introduced to support the programming theory. Epigenetics can be described as cell-specific reversible modifications in DNA chromatin structure that modulate gene expression without altering DNA sequence. Epigenetic factors are heritable from cell to daughter cell within the same organism, and there is growing evidence that this heritability can be transgenerational among organisms (Heard and Martienssen, 2014; van Otterdijk and Michels, 2016). Indeed, the genetic heritage of each living being contains both DNA sequence information and epigenetic information, and their interaction maintains the function of organs and cells. The most studied epigenetic modifications are DNA methylation, histone modification, chromatin remodeling, and noncoding RNA, which all require the involvement of transcription factors. Further, during the last decades, several studies have confirmed the existence of specific human genes able to confer different susceptibilities to diseases (Jirtle and Skinner, 2007).

EPIGENETICS ALTERATION UPON EARLY EXPOSURE TO ALTERED DIET AND METABOLIC CONDITIONS

Transgenerational effects on metabolism and metabolic diseases have been known and studied before the advent of the field of epigenetics. In fact, the evaluation and characterization of children born during the Dutch Winter Famine (Lumey et al., 1993) showed a link between maternal nutrition and risk of metabolic disorders later in life, such as a Swedish study, which found that paternal and grand-paternal nutrition during childhood increased mortality for cardiovascular diseases and diabetes in later decades of life (Kaati et al., 2002).

These studies and following epidemiological observations show that unhealthy nutrition, not only undernutrition but also overnutrition, during in utero and early postnatal life increases susceptibility to metabolic alterations later in life by acting during the critical period of growth and by probably causing a mismatch between early and adult nutritional environments.

Maternal obesity is increasing, and several human and animal studies have demonstrated that offspring of obese mothers or mothers exposed to a high-fat diet present increased weight and fat mass at birth and during growth have an increased risk of developing nonalcoholic fatty liver disease (NAFLD), insulin resistance, altered glucose tolerance, obesity, hyperphagia, hypertension, and cardiovascular damage (Laker et al., 2013). Also, obesity and high-fat diet are associated with elevated circulating lipids that cross the placenta, through specific fatty acid transporters amplified in obese pregnant women, and they modulate cell signaling pathways by acting as ligands for nuclear receptors and altering gene expression by DNA hypermethylation. It seems that maternal high lipid levels interfere with the hypothalamic expression of leptin receptor, pro-opiomelanocortin (POMC), and neuropeptide Y in offspring, such as with the expression of SIRT1, specific factor involved in fat and glucose metabolism (Chen et al., 2008; Kim and Um, 2008). In particular, SIRT1 is principally involved in obesity, liver lipid metabolism (NAFLD), and brain neuronal degeneration. SIRT1 is a Nicotinamide Adenine Dinucleotide (NAD)+-dependent protein deacetylase, and it is involved in the deacetylation of the nuclear receptors, playing a critical role in insulin resistance development (Martins, 2013). In fact, SIRT1 is involved in metabolic regulation and in the repair of DNA damage with epigenetic alterations and maintains the DNA to prevent gene modification of various genes including CYP 450 enzymes and allows rapid metabolism of xenobiotics that enter the organism. Diet changes, as observed in underdeveloped countries' urbanization and Western countries, involve SIRT1 dysregulation, causing several alterations in transcriptional regulators and modification of chromatin that contribute to endocrine abnormalities such as insulin resistance, NAFLD, and energy balance disorders (Martins, 2017a; Martins, 2017b). Moreover, SIRT1 has been recently identified as an antiaging gene, both in humans and other animal species. SIRT1 is involved in telomere maintenance and DNA repair with its critical involvement chromosome stability and cell proliferation and is important to the regulation of other antiaging genes such as klotho, p66shc, and Forkhead box protein O1 with relevance to age-related diseases (Martins, 2018).

On the other hand, maternal elevated circulating lipids determine the activation of the inflammatory signaling, which lead to an increase in proinflammatory cytokines [tumor necrosis factor α, interleukin 1 (IL-1), IL-6, IL-8, and IL-18] and oxidative stress within the placenta, which results in an altered intrauterine and postnatal development (Laker et al., 2013). Just a few studies have investigated the effects of maternal

inflammation on offspring's postnatal life and have shown that it impairs nervous system and musculoskeletal development, while it promotes adipogenesis (Jonakait, 2007; Bayol et al., 2008; Tong et al., 2009). Interestingly, the effect of interpregnancy weight loss was studied, and a reduced risk to develop obesity and cardiovascular diseases was observed in siblings born after maternal bariatric surgery compared with those born before (Guénard et al., 2013). Moreover, in a mouse model, after a long-term high-fat diet, the development of obesity and mild glucose intolerance through specific gene expression alterations has been demonstrated. In particular, the authors have identified a histone acetylation among the gene expression profile in pancreatic islets, causing a dysregulation in fatty acid metabolism through the suppression of specific genes (NRF1, GABPA, MEF2A) involved in fatty acid signaling (Nammo et al., 2018). In another study conducted on pregnant rats, maternal dyslipidemia induced by an unsaturated fatty acid diet determines DNA methylation and histone acetylation in placenta and fetal liver with a subsequent accumulation of lipids in the fetal liver (Ramaiyan and Talahalli, 2018). On the other hand, the use of a hypolipidemic agent, such as *Quercus acutissima* fruit ethanol extract, exhibits antiobesity effects through inhibition of acetylation in 3T3-L1 preadipocytes and high-fat diet–fed obese mice (Hawang et al., 2017) (**Figure1**). Furthermore, recent investigations have also demonstrated that either a maternal fat overload diet or high-calorie diet can induce mitochondrial dysfunction, inflammation, and senescence-like characteristics in brown adipose cells likely leading to metabolic imbalance and increased risk of developing obesity in later phases of life (Lettieri Barbato et al., 2015; Lettieri-Barbato et al., 2017).

Like maternal obesity, gestational diabetes also has detrimental effects on both mother and fetus. Offspring of mothers with gestational diabetes present increased birth weight, adiposity, neonatal hypoglycemia, and obesity and have an increased risk of developing metabolic syndrome and type 2 diabetes later in life (Catalano, 2010). To date, several genes have been associated to diabetes, which, however, explain only a small proportion of heritability, whereas environmental factors seem to influence its pathogenesis in a significant manner. Then, the gestational diabetes represents an interesting model to study the epigenetic modifications determined by environmental influence (Nolan et al., 2011). Indeed, gestational diabetes is more frequent in daughters of diabetic mothers than in those of diabetic fathers (Harder et al., 2001; McLean et al., 2006), pointing to intrauterine glucose exposure as a relevant issue in addition to genotype (Hocher, 2014; Hocher et al., 2016; Reichetzeder et al., 2016). In fact, intrauterine exposure to hyperglycemia determines an impairment in placental cholesterol uptake and alters placental methylation of leptin and adiponectin, hormones that regulate energy balance and insulin sensitivity, leading to the development of both leptin and insulin resistance. Moreover, animal and human studies show that leptin and insulin resistance, such as undernutrition, act on hypothalamic receptors and appetite circuits leading to postnatal hyperphagia, decreased satiety, and subsequent development of metabolic syndrome (Block and El-Osta, 2017) (**Figure1**).

Finally, maternal restriction of proteins, folate, methionine, and B vitamins during periconceptional period, gestation, and lactation increases the risk of lower weight at birth and increased central adiposity, fatty liver, blood pressure dysregulation, and myocardium hypertrophy in offspring as consequences of an altered DNA methylation (Wu, 2009; Gueant et al., 2013). In particular, in an experimental mouse model, it has been demonstrated that a low-protein diet in pregnant mothers during a precocious gestational period, such as the preimplantation period, determines cardiovascular and metabolic diseases in offspring adults, through histone modifications of the *Gata6* gene (Sun et al., 2015). And, in male rat offspring, maternal protein restriction, during gestation and lactation, determines impaired glucose tolerance in adulthood by histone acetylation of the liver X receptor α, which is involved in the regulation of hepatic gluconeogenesis (Vo et al., 2013), as well as leads to histone modifications in GLUT4 promoter region in the

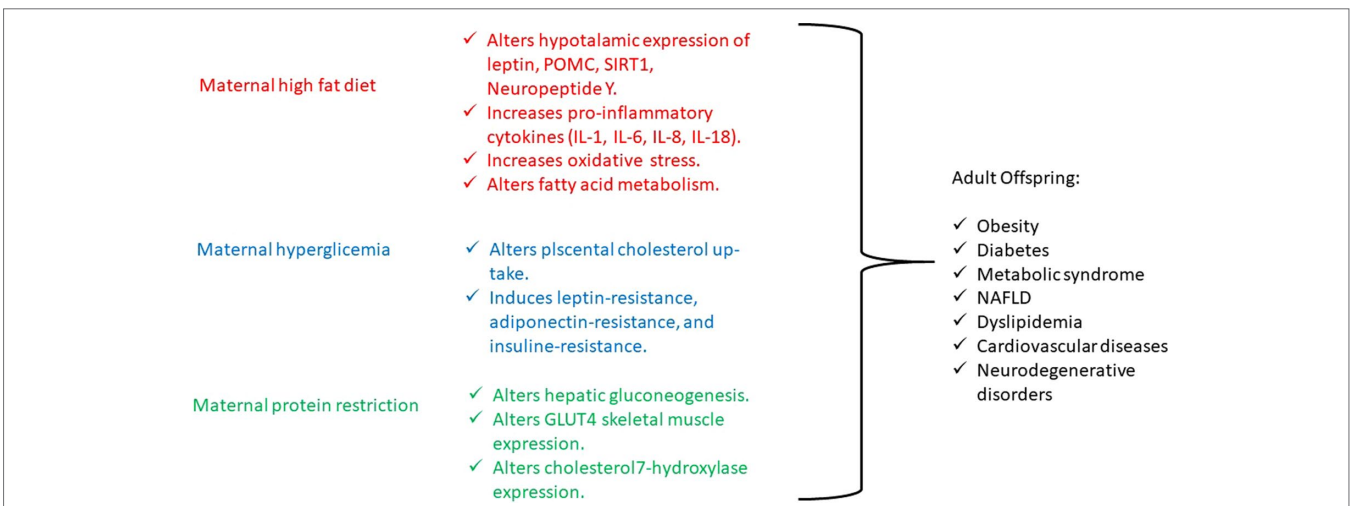

FIGURE 1 | Principal biological mechanisms of diet-induced epigenetic alterations.

skeletal muscle of female rat offspring (Zheng et al., 2012) and determines high cholesterol levels in adult rat offspring because of repressive changes in histone modifications at the cholesterol 7α-hydroxylase promoter (Sohi et al., 2011) (**Figure1**).

A last consideration must be made regarding alcohol consumption during pregnancy. In fact, recent animal studies show that prenatal ethanol exposure determines high fat mass at birth, altered β-cells structure, impaired glucose homeostasis, and insulin resistance (Yao and Nyomba, 2008; Dobson et al., 2012), by inducing anomaly in DNA methylation (Ungerer et al., 2013), likely due to reduced folate bioavailability and methionine levels (Halsted et al., 2002).

EPIGENETICS MODIFICATIONS UPON EARLY EXPOSURE TO CHEMICALS THROUGH FOOD CHAIN

Hormones play a pivotal role through life in the regulation of fat, carbohydrate, and protein metabolism, and hormonal alterations of these processes are likely to impair metabolism and fat storage. Many natural and synthetic chemicals, found in the environment, contaminating food through food chain, possess hormonal activity. These compounds, known as endocrine disrupters (EDCs), are exogenous substances endowed with the capacity to alter the function(s) of the endocrine system and thus represent a serious risk to health both in humans and animals (International Programme for Chemical Safety) (Li et al., 2013; Maradonna and Carnevali, 2018). Endocrine-disrupting chemicals belong to a heterogeneous class of chemicals dispersed in the environment. These compounds alter many aspects of the endocrine-metabolic homeostasis because of their ability to mimic and/or antagonize the biological activity of endogenous hormones (Pande et al., 2019), likely binding to specific receptors. Although the main EDC effect is on the reproductive system (McLachlan et al., 1984), growing evidence shows that some compounds can also impair body weight regulation by affecting metabolism (Migliaccio et al., 1996) and functional activity of adipocytes, often leading to obesity. These EDCs are defined as "obesogens" (Grun and Blumberg, 2006). Several chemicals have comprised obesogens with estrogen properties, such as tributyltin (TBT), generally used as biocide in antifouling paints applied to the hulls of ships; diethylstilbestrol, used to enhance fertility in farm animals; dichlorodiphenyltrichloroethane (DDT) and its breakdown product dichlorodiphenyl-dichloroethylene, used as insecticide; bisphenol A (BPA), used in the manufacture of plastics; polybrominated diphenyl ethers and 4-nonylphenol, used for industrial proceedings; parabens, generally used as antimicrobial agents for the preservation of personal care products, foods, pharmaceutical products, and paper products; phytoestrogens, naturally produced by plants and assumed by humans *via* ingestion of edible plants (Darbre, 2015). "Interestingly, several animal and human evidence shows that the exposure to obesogens, both prior to birth in utero and during neonatal period, leads to altered body weight at birth (both high weight and low weight) and increased body weight and obesity during growth with an increase in fat cell number permanently into adult life (Janesick and Blumberg, 2011). Moreover, many studies highlight that such effects can also be inherited through future generations even in the absence of additional exposure. Transgenerational studies have revealed that TBT exposure of pregnant mice generates offspring of both genders with larger fat deposits, and this phenotype is inherited up to the third generation, even without further TBT exposure (Chamorro-Garcia et al., 2013; Janesick and Shioda, 2014). Other heritable traits toward obesity in rodents have been observed after exposure to BPA, phthalates (Manikkam et al., 2013), and DDT (Skinner et al., 2013).

Obesogens induce weight gain by increasing both the number and size of adipocytes, by altering the endocrine pathways responsible for adipose tissue development, by changing lipid homeostasis, and by promoting adipogenesis and lipid accumulation. These events might occur through multiple mechanisms, such as interference with Peroxisome Proliferator-Activated Receptors (PPARs) and steroid receptors, alteration in fat cell recruitment, shifting of appetite, satiety, and food preferences (Darbre, 2017). In particular, it is thought that early life exposure to EDCs might influence epigenetic programming of obesity *via* the capacity of these compounds to bind nuclear receptors and other transcription factors and thus to influence consequent gene expression. For example, nuclear receptors, such as steroid receptors, can directly bind hormone-response elements present in the DNA upon activation by single or multiple ligands. Furthermore, they are able to recruit chromatin-modifying complexes including methyltransferases and acetyltransferases, which directly alter epigenetic marks involved in the regulation of target genes (Ozgyin et al., 2015). Therefore, EDCs can change the local chromatin state as well as modulate the expression of DNA or histone methyltransferases by activating or inhibiting nuclear receptors and other transcription factors (Rissman and Adli, 2014).

Among EDCs, phytoestrogens represent a diverse group of natural chemicals with structural and functional similarities to endogenously produced mammalian estrogens, able to bind the nuclear receptors and thus endowed of significant estrogen receptor (ER) modulatory activities. They are present in fruits, vegetables, and whole grains commonly consumed by humans, as well as in many dietary supplements, and are widely marketed as natural alternatives to estrogen replacement therapy. Recently, the nutritional changes leading to the inclusion of soy-derived products into human diets have consistently enhanced the exposure to these compounds. In fact, soy products are nowadays important components of food products consumed in both adult and infant human diets (McCarver G et al., 2011), with variable amounts assumed in different world regions (Jefferson et al., 2009). Phytoestrogens are polyphenolic structures classified as flavonoids (or isoflavones), coumestans, lignans, and stilbenes, with isoflavones representing major compounds in dietary sources (Rietjens et al., 2017). Various beneficial health effects have been ascribed to these compounds including cardiovascular diseases, obesity, metabolic syndrome, and type 2 diabetes, as well

as brain function disorders and some types of cancer (Guerrero-Bosagna and Skinner, 2014).

Phytoestrogens exert their potential health effects by different ways. Although the main mode of action relies on their binding to ER, several other pathways such as rapid nongenomic cellular response, antioxidant action, tyrosine kinase inhibition, PPAR-mediated action, and binding to nonclassic ER gp130 or aryl hydrocarbon receptor (AHR) have been largely described (Guerrero-Bosagna and Skinner, 2014). Compelling evidence suggests that epigenetic modifications link environmental insults occurring during development to disease susceptibility in the adult life. In this regard, the capacity of phytoestrogens to induce epigenetic effects has been described, in particular for the soy isoflavone genistein and to a lesser extent for daidzein and its microbial metabolite equol (Guerrero-Bosagna et al., 2008; Remely et al., 2015). A direct epigenetic effect of isoflavones was initially demonstrated upon exposure of newborn mice to coumestrol and equol that lead to increased methylation and subsequent inhibition of the proto-oncogene H-ras (Lyn-Cook et al., 1995) in both male and female mice. Furthermore, consumption of genistein was reported to alter DNA methylation pathways in mice (Day et al., 2002). In addition to direct effects, evidence has been achieved on the capacity of phytoestrogens to affect offspring methylation patterns as a result of maternal exposure. In this regard, dietary supplementation of pregnant mice with genistein altered coat color and protected A^{vy} mouse offspring from obesity development by modifying the epigenome of the fetus (Dolinoy et al., 2006).

Several studies have been carried out to assess the obesity-promoting or obesity-protective effect of maternal supplementation with phytoestrogens on the offspring. The results achieved are often difficult to compare because of several variables including the animal model, interspecies differences in isoflavone metabolism, diet composition, phytoestrogen concentration, and length of treatment, as well as confounding factors such as age and gender (Ørgaard and Jensen, 2008). In this regard, it is of interest that sex differences in human amniotic fluid levels of daidzein and genistein, with significantly higher concentrations among the female fetuses, have been reported (Jarrel et al., 2012). Likewise, genistein pharmacokinetics are faster in male rather than female rats (Sikker et al., 2001). Studies assessing the effects of in utero exposure to phytoestrogens in different preparations (genistein as supplement to standard diet, isoflavone-rich diet, soy protein–based diet) and for different periods after birth yielded contrasting results. Whereas some studies reported that in utero exposure results in a lower weight at birth (Cederroth et al., 2007; Guerrero-Bosagna and Skinner, 2009; Zhang et al., 2015), others reported obesity-promoting properties such as increased body weight and food intake (Vafeiadi et al., 2015; Jahan-Mihan et al., 2011; Jahan-Mihan et al., 2012; Cao et al., 2015; Walley and Roepke, 2018). Likewise, more than a decade ago, Ruhlen and colleagues (Ruhlen et al., 2008) reported either decreased or increased body weight of offspring upon in utero exposure depending on the period of life (adulthood vs. at birth).

Predisposition to diet-induced obesity as a consequence of prenatal following prenatal nutrient restriction has been reported to be gender related, with males more affected than females, as well as age at pubertal development (girls earlier than boys) (Rubin et al., 2017; Guerrero-Bosagna et al., 2008). Regardless of the effect induced in the offspring by in utero exposure to phytoestrogens, they appeared to be stronger in the male progeny with respect to females.

CONCLUSIONS

Obesity and NCDs are increasing burning health problems that greatly affect worldwide population. Several factors are claimed to play a role in the development and persistence of these metabolic chronic disorders through life, such as altered diet and sedentary life. Interestingly, in the last decades, several studies have pointed out the importance of perturbance during the early phases of life in the increased number of metabolic chronic disorders. In particular, altered diet and exposure to specific chemicals through food chain appear to play a pivotal role. Further studies, however, are needed to fully characterize and confirm this hypothesis in order to apply preventive actions to successfully approach this global health problem.

AUTHOR CONTRIBUTIONS

All authors have equally contributed in writing, revising, and finalizing the manuscript.

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REVIEW
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SIRT1 in the Development and Treatment of Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) is one of the most common causes of cancer-related death worldwide. Current treatment options for inoperable HCCs have decreased therapeutic efficacy and are associated with systemic toxicity and chemoresistance. Sirtuin 1 (SIRT1) is a nicotinamide adenine dinucleotide–dependent enzyme that is frequently overexpressed in HCC, where it promotes tumorigenicity, metastasis, and chemoresistance. SIRT1 also maintains the tumorigenic and self-renewal proprieties of liver cancer stem cells. Multiple tumor-suppressive microRNAs (miRNAs) are downregulated in HCC and, as a consequence, permit SIRT1-induced tumorigenicity. However, either directly targeting SIRT1, combining conventional chemotherapy with SIRT1 inhibitors, or upregulating tumor-suppressive miRNAs may improve therapeutic efficacy and patient outcomes. Here, we present the interaction between SIRT1, miRNAs, and liver cancer stem cells and discuss the consequences of their interplay for the development and treatment of HCC.

Keywords: miRNA, cancer, HCC, sirtuin 1, cancer stem cells

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most frequent primary liver malignancy and among the most common causes of cancer-related death worldwide (1, 2). The majority of cases occur due to HCV or alcoholic cirrhosis (3). However, HCC can also develop in obese individuals with non-alcoholic steatohepatitis (NASH) (4–7). The increasing incidence in diet-induced NASH is estimated to upsurge the number of patients with NASH-related HCC (3). The main therapeutic options for HCC include liver transplantation, surgical resection, and chemotherapy (8). However, most patients present with advanced-stage, unresectable HCC. Moreover, first-line treatment compounds such as sorafenib have low response rates (9) and are associated with systemic toxicity and chemoresistance (10, 11). Therefore, a better understanding of the underlying mechanisms that promote HCC development, chemoresistance, and metastases is vital for improving patient outcomes (12).

Mammalian sirtuins (SIRT1-7) are NAD^+-dependent deacetylases that are involved in a wide variety of biological processes including energy metabolism and lifespan and health span regulation (13). Mammalian sirtuins possess histone deacetylase, mono-ADP-ribosyltransferase, desuccinylase, demalonylase, demyristoylase, and depalmitoylase activity (14). SIRT1 is chiefly localized in the nucleus and plays a role in genomic stability, telomere maintenance, and cell survival (15, 16). SIRT1 regulates both histones and multiple downstream non-histone targets such as estrogen receptor-alpha (17), PPARγ (18), PGC-1α (19), androgen receptor (20), FOXO transcription factors (21), p53 (22), NF-κB (23), and Survivin (24). SIRT1 can also upregulate oncogenes: β-catenin (25), c-Myc (26), and HIF-1α (27) increasing their activity as a result.

SIRT1 is vital for the physiological function of healthy tissues. For instance, SIRT1 null mice have defects in hepatocyte metabolism and a shortened life span (28). SIRT1 deletion in mice hepatocytes results in hepatitis and hepatic steatosis (29). Oppositely, inducing SIRT1 activity in healthy tissues with synthetic activators or transgenic expression provided a plethora of benefits. SIRT1 overexpression reduced the release of pro-inflammatory cytokines and increased cell viability (23, 30, 31). SIRT1 also preserved the functions of hepatocytes and adipocytes against obesity (32). Overall, SIRT1 can be called a "Master Metabolic Regulator" (33), which is essential for normal hepatic function.

THE EXPRESSION AND FUNCTION OF SIRT1 IN HCC

SIRT1 has a multifaceted relationship with oncogenesis. SIRT1 is overexpressed in multiple malignancies, including human myeloid leukemia (34), colon cancer (35), prostate cancer (36), and squamous cell carcinomas (37). Conversely, SIRT1 expression is reduced in ovarian cancers and glioblastoma (38) when compared to corresponding normal tissues. Overall, SIRT1 may function as both an oncogene and tumor suppressor depending on subcellular localization, age, type of tissue, and concomitant mutations in related signaling pathways.

In HCC, SIRT1 was the only member of the Sirtuin family consistently overexpressed (39) and deemed vital for all stages of HCC tumorigenesis (39). Moreover, it was repeatedly demonstrated that SIRT1 was frequently overexpressed in HCC biopsies when compared to corresponding adjacent non-cancerous liver parenchyma (40–42) and its expression was necessary for the maintenance of HCC tumorigenesis (15, 43–45). Generally, SIRT1 mRNA levels are similar in HCC and non-malignant adjacent tissue, suggesting that SIRT1 is increased in HCC *via* a post-transcriptional mechanism (15). Hypermethylated in cancer 1 (HIC1) and p53 negatively regulate SIRT1 mRNA transcription and are often mutated or dysfunctional in HCC. Thus, SIRT1 overexpression may be partly accounted for by the decreased inhibition of its transcription. However, SIRT1 protein levels are also preserved post-translationally *via* reduced degradation and increased stability (15, 46).

Additionally, SIRT1 was overexpressed in a multitude of human HCC cell lines such as HKC1-4, SNU-423, HKC1-2, PLC5 SNU-449, SK-Hep-1, Huh-7, HepG2, and Hep3B (15, 45), when compared to normal liver cell lines (47).

However, there is still some controversy regarding SIRT1's role in HCC, as some reports showed that SIRT1 was downregulated in human HCC samples and hypothesized it had tumor-suppressive roles (38). The multifaceted role of SIRT1 in carcinogenesis suggests (48) that its function is dependent on cancer type and the state of downstream or upstream molecules that influence its oncogenicity (49). The role of SIRT1 in HCC may also depend on its subcellular localization. Although, in HCC cells, SIRT1 had a predominant nuclear localization where its expression promotes tumorigenesis, it was reported that cytoplasmatic SIRT1 may have tumor-suppressive roles (50).

Multiple lines of evidence suggest that SIRT1 expression has survival-promoting effects in both normal hepatocytes and in HCC cells. In healthy mice, SIRT1 overexpression protected against malignancies (51) and basal SIRT1 expression was vital for maintaining physiologic hepatic morphology and normal lifespan (44). However, basal SIRT1 levels were lower in mouse livers compared to other viscera, indicating that the hepatocytes may be more sensitive to the under- or overexpression of SIRT1 (44).

Similarly, SIRT1 expression is vital for the proliferation and survival of HCC cells (44). Malignant cells were shown to enhance their function by hijacking survival signaling pathways of non-malignant cells (52, 53). Therefore, SIRT1 activity may promote cellular function and survival and inhibit cancerous transformation in normal hepatocytes; after malignant transformation, SIRT1's functionality may be employed in promoting tumorigenesis and sustaining HCC survival (15). That is, SIRT1's activity may promote cellular survival independent of the cancerous or non-cancerous state of the hepatocytes. As of yet, there are no reports of experimentally induced oncogenesis *via* SIRT1 overexpression. Finally, SIRT1 overexpression does not appear to be a cancer-initiating event but rather a cancer-induced adaptive mechanism that promotes survival and proliferation (42). However, because SIRT1 simultaneously regulates a wide spectrum of biological processes, its role in HCC oncogenesis is incompletely understood and further research is warranted in order to clarify at which level and *via* what mechanisms do HCC cells increase and become dependent on SIRT1 expression. Additionally, the interplay between SIRT1 and the other six sirtuin family members and their role in HCC should be further explored.

Multiple studies evaluated the prognostic value of SIRT1 expression in HCC. SIRT1 overexpression correlated with the development of portal vein tumoral thrombosis, decreased overall survival rates, lower disease-free survival, and advanced TNM stages (54). Patients with SIRT1-positive HCC biopsies had a decreased 10-year survival compared to SIRT1-negative HCC patients. SIRT1 protein levels appear to be positively correlated with HCC grades; specifically, SIRT1 expression is higher in advanced HCC stages. One meta-analysis investigated the prognostic and clinical implications of SIRT1 expression in HCC. It showed that heightened SIRT1 expression was

associated with decreased patient overall survival and death-free survival. Moreover, increased SIRT1 expression correlated with larger tumor size, higher p53 expression, high alpha-fetoprotein (AFP) levels and advanced TNM stages (55). However, it was highlighted that, for the studies examined in the meta-analysis, there was no clear cutoff value or unified standard for the measurement of SIRT1 expression. Even though the statistical power was limited, it can be concluded that increased SIRT1 expression correlated with a poor HCC prognosis (26).

The deacetylation function of SIRT1 is vital for its oncogenic role in HCC. When the deacetylation domain of SIRT1 is mutated, the proliferation and colony formation ability of HCC cells are inhibited (40). Inhibition of SIRT1 in HCC cells, either through knockdown or administration of SIRT1 inhibitors, led to decreased tumor development *in vitro* and *in vivo* and exerted cytostatic as opposed to a cytotoxic effect (42, 44), while SIRT1 overexpression accelerated HCC growth (44). However, *in vivo* experiments indicate that other mutations in relevant cancer-related pathways might determine the function of SIRT1, thus, the role of SIRT1 should be viewed as context dependent (56). SIRT1 is also implicated in the malfunction of multiple HCC signaling pathways such as FOXO1, p53, and TGF (57–59). SIRT1 downstream targets involved in HCC progression include YAP (Yes-associated protein) (44, 60), PTEN/PI3K/Akt (61, 62), telomerase, and p53 (63). Overall, in HCC, SIRT1 acts as a potential oncogene (45). Further on, we will elaborate on the interplay between SIRT1 and the aforementioned pathways and molecules.

SIRT1 expression was also shown to prevent malignant development in a mouse model of metabolic-syndrome associated HCC. Communicable infectious diseases have been successfully dealt with in the past decades. However, in the early twenty-first century, non-communicable diseases have become a principal health hazard. The global spread of high calorie and low fiber, Western style foods, coupled with decreases in physical exercise led to a global epidemic of metabolic syndrome. The financial burden inflicted by metabolic syndrome is in the trillions (64). The epidemiology of obesity-associated HCC (65) and *in vitro* and *in vivo* experiments suggest that an obesogenic lifestyle, *via* pro-inflammatory cytokines, insulin resistance, steatosis, and lipotoxicity, may progress from metabolic syndrome to NASH (6) and HCC (65). Overall, diet-induced NASH is estimated to upsurge the number of patients with NASH-related HCC (3, 66–68).

SIRT1 expression promotes genomic stability in normal hepatocytes and appears to be protective against high-fat diet (HFD)-induced HCC. Moreover, the role of SIRT1 as a protector against metabolic syndrome is clear (69, 70). For instance, enhancing SIRT1 activity in a mouse model of type 2 diabetes leads to improved insulin resistance and controls hyperglycemia (7, 71, 72). Moreover, transgenic mice that systemically overexpress SIRT1 were protected from the hazards produced by a HFD (69, 73).

One model of metabolic syndrome-associated cancer examined the effects of a threefold systemic SIRT1 expression on diet-associated HCC.

Mice overexpressing SIRT1 systemically at approximately threefold that of the normal WT mice had measurably increased hepatic SIRT1 deacetylase activity. These mice had improved glucose tolerance, decreased adipose inflammation, and were protected from other negative effects of HFD such as hepatic steatosis. Moreover, compared to the control group, SIRT1-overexpressing mice displayed a lower incidence of HCC after the chronic administration of a HFD. Part of the protective effects of SIRT1 expression in HCC development was attributed to decreasing NF-κB-induced inflammation and malignant transformation (51).

Overall, systemic threefold SIRT1 overexpression protects hepatocytes but not fibroblasts from DNA damage and translates as safeguard against HFD-induced HCC (51).

SIRT 1 may promote protective effects against HCC *via* its effect on β-catenin (25) —an oncogene associated with epithelial cancer (74). This may account for the carcinoma-selective protection provided by SIRT1 overexpression.

Collectively, the current body of literature suggests that SIRT1 expression has a pro-tumorigenic role in HCC but is not a cancer-initiating event.

ROLE OF SIRT1 IN THE TUMORIGENICITY OF LIVER CANCER STEM CELLS

Multiple models have been proposed in order to explain the functional and histological heterogeneity of solid cancers. One of them proposes a hierarchical organization of tumoral cell populations where a minor cell population termed cancer stem cells (CSCs) with self-renewal and differentiation capacities repopulate tumors and establish the histological and functional heterogeneity characteristic of most cancers (75, 76). Intra-tumoral CSCs are capable of differentiation and self-renewal and give rise to tumors identical to the original one in primary and metastatic sites (76, 77). HCC tissue samples possess intra-tumoral heterogeneity (78, 79), and a subpopulation of cells with stem cell-like proprieties might give rise to HCC and accelerate cancerous proliferation (80–82). Therefore, due to their proliferation and differentiation abilities, liver CSC (LCSC) have been incriminated for HCC initiation (83), chemoresistance (84, 85), metastasis (83), recurrence, and overall dismal patient outcome (83, 86–89).

For instance, hepatoblasts are cell progenitors with the ability to differentiate into hepatocytes (90). During chronic liver inflammation, hepatoblasts and other hepatic progenitor cells accrue genetic and epigenetic modifications, leading to their conversion in LCSCs (91). Importantly, through a process called dedifferentiation, hepatocytes can also undergo malignant transformation by acquiring CSC phenotypes (92). HCC CSCs can be identified through multiple biological markers such as CD133, CD90, and CD13 and ubiquitin-specific protease 22 (USP22) (82, 93). It was shown that SIRT1 plays a vital role in the self-renewal and maintenance of embryonic stem cells (94) and hematopoietic stem cells (95) and is also implicated in the biology of LCSC.

SIRT1–MRPS5 Signaling Pathway

Liver cancer stem cells (LCSCs) use enhanced mitochondrial respiration to generate ATP. In contrast, cancer cells primarily rely on aerobic glycolysis (Warburg effect) to generate ATP. Metabolic reprogramming is a hallmark of cancer and plays a vital role in cancer progression (96); however, the regulator of metabolic reprogramming that drives the switch from oxidative phosphorylation to aerobic glycolysis in LCSC is not fully understood.

Mitochondrial ribosomal protein S5 (MRPS5) is required for the enhanced mitochondrial function of LCSCs and promotes cancer commencement and development (97, 98). Acetylation promotes the nuclear translocation of various proteins (99). SIRT1 is highly expressed in LCSCs (100, 100) where it deacetylates MRPS5, thus determining its subcellular localization. In LCSCs, deacetylated MRPS5 is located in the mitochondria where it promoted oxidative phosphorylation, stimulated NAD+ production, and improved mitochondrial function, thus preserving LCSC stemness. Increased NAD+ also maintains SIRT1 activity and promotes a SIRT1–MRPS5 positive feedback loop. In contrast, in HCC cells, acetylated MRPS5 translocates to the nucleus and consequently promotes metabolic flexibility and enhanced glycolysis (101).

Relevantly, multiple acetylated proteins act as metabolic enzymes in the extra nuclear environment and as transcription factors when located inside the nucleus (102). Nuclear translocation of MRPS5 led to enhanced expression of glycolytic proteins and a switch in metabolism, from oxidative phosphorylation to a Warburg-type metabolism. Thus, MRPS5 may function as a transcription factor when localized in the nucleus and consequently regulate the expression of glycolytic genes. However, the exact mechanism by which MRPS5 increases the expression of glycolytic proteins is not clear. Further research should establish whether acetylated MRPS5 acts as a glycolysis promoting transcription factor in HCC.

SIRT1 expression was higher in LCSC compared to HCC cells. The comparatively lower SIRT1 expression in HCC cells may account for the predominant nuclear localization of MRPS5. However, the tumor microenvironment is highly dynamic and SIRT1 expression may be heterogeneous in different cellular subpopulations at different time points. Thus, fluctuating SIRT1 expression may contribute to tumoral heterogeneity and self-renewal by facilitating the transition of LCSCs to HCC cells. This may explain why MRPS5 is found in higher concentration in HCC cell's nucleus even though SIRT1 is frequently overexpressed in HCC cells and it would be expected to deacetylate MRPS5 and thus promote its mitochondrial localization.

SIRT1 also induced the mitochondrial unfolded protein response (UPRmt) that preserves cell longevity and metabolic fitness (103, 104). LCSCs present accelerated oxidative phosphorylation, which is associated with increased ROS production. Therefore, the SIRT1–UPRmt axis maintained LCSC viability by reducing ROS.

Metformin was reported to be beneficial to HCC patients (58). Interestingly, metformin downregulates MRPS5, which inhibits the activity of mitochondrial complex 1, thus decreasing the function of LCSCs (see **Figure 1**).

Human HCC samples with increased SIRT1 expression and high cytoplasmic MRPS5 levels presented more CSCs and were associated with high metastases rates, cancerous embolization, increased tumor size, and decreased survival compared to patients whose HCC biopsies showcased low cellular SIRT1 expression and high nuclear MRPS5 levels. This further indicates that the metabolic reprogramming induced by SIRT1/MRPS5 axis is crucial for the stemness of CSCs.

In summary, the SIRT1/MRPS5 axis augmented the metabolic plasticity and reprogramming of LCSC by ameliorating and maintaining mitochondrial function, consequently promoting hepatocarcinogenesis. Further studies should explore the interaction between SIRT1 and other MRPs. Administration of LCSC-targeted oxidative phosphorylation inhibitors or MRPS5 inhibitors should be explored in future experiments.

SIRT1–SOX Signaling Pathway

In vivo mice models demonstrated that SIRT1 is necessary for maintaining the self-renewal and tumorigenicity of LCSCs. In those models, silencing SIRT1 expression reduced the incidence of HCC compared with the controls (100). SIRT1 is overexpressed in LCSCs where it is necessary for maintaining oncogenesis and self-renewal and is correlated with a poor prognosis of HCC patients.

The core embryonic transcription factor circuitry (SOX2, c-Myc, Oct4, Nanog) is implicated in the self-renewal of CSCs (105–107). SIRT1 induces tumorigenicity in a subpopulation of LCSC in a SOX2-dependent manner. SIRT1 knockdown in LCSCs decreased SOX2, Oct4, and Nanog expression levels. Treating LCSCs with SIRT1 inhibitors TV6 and EX-527 reduced SOX2 and Nanog (100).

SIRT1 regulates SOX2 gene expression thus primes LCSC for self-renewal. DNA (cytosine-5)-methyltransferase 3A (DNMT3A) catalyzes the transfer of methyl groups to CpG DNA structures. In LCSCs, SIRT1 expression inhibited DNMT3A, consequently promoting hypomethylation of the SOX2 promoter and activated SOX2 gene expression, consequently inducing self-renewal and oncogenicity (100). Notably, HCC stage and recurrence were correlated with SIRT1 and SOX2. Thus, in LCSCs, SOX2 is a prime downstream regulator of SIRT1-induced self-renewal and oncogenesis (100).

The Ubiquitin Proteasome Pathway (UPP) is the prime mechanism for protein catabolism in mammals. UPP is also responsible for degrading SIRT1 (108, 109). IGF1 was vital for the self-renewal and tumoral growth of LCSCs. Namely, IGF signaling inhibited the UPP pathway, thus increasing SIRT1 protein levels and function in LCSCs (100). Thus, IGF1 may enhance the self-renewal of LCSCs by mediating SIRT1 levels (100).

Inhibiting SIRT1 in LCSCs reduced SOX2 expression and strongly repressed tumor growth in both *in vivo* and *in vitro* models. Overall, SIRT1 deacetylase activity was vital for the oncogenicity and self-renewal of LCSCs. Selective SIRT1

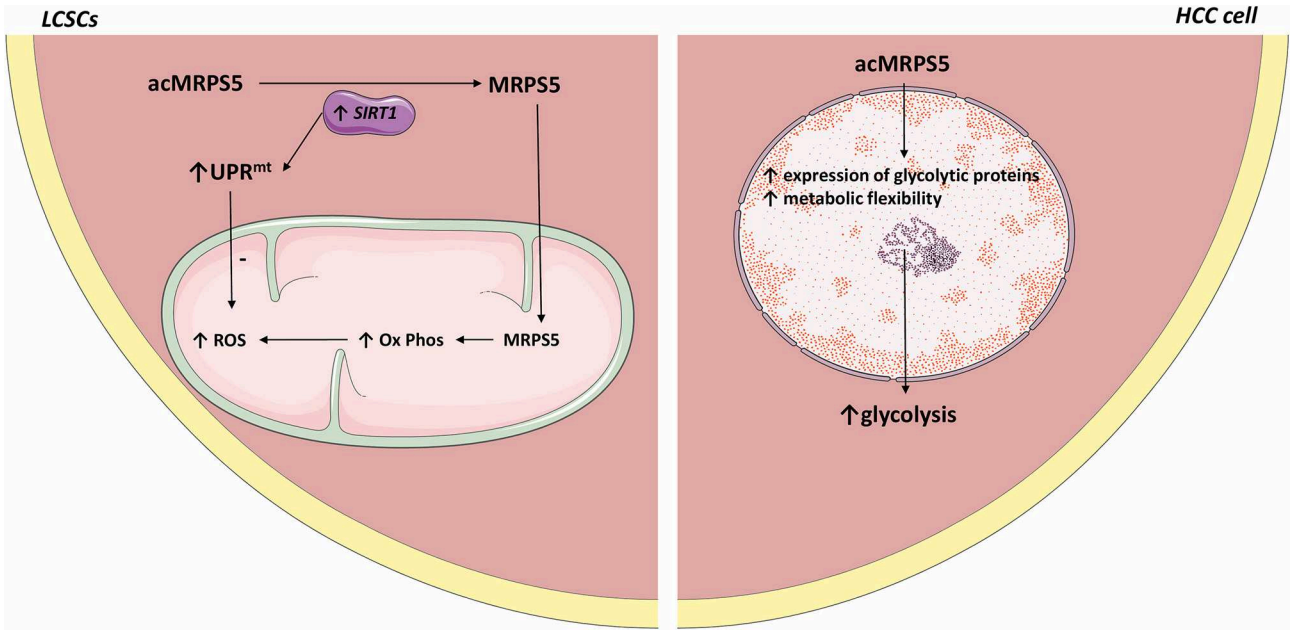

FIGURE 1 | Comparative representation of MRPS5 signaling in liver cancer stem cells and HCC cells. In LCSCs, SIRT1 deacetylates MRPS5, which promotes its mitochondrial localization. Mitochondrial MRPS5 increases oxidative phosphorylation and consequently ROS. However, SIRT1 also promotes UPRmt activity, which decreases ROS levels. Conversely, in HCC cells, MRPS5 is localized in the nucleus where its activity promotes increased cellular glycolysis. All the figures in this article were made using images from http://smart.servier.com.

inhibition in LCSCs is a potential therapeutic target hindering HCC development and progression.

SIRT1–MEK Signaling Pathway

Initially, HCC heterogeneity was attributed to hepatocytes since the liver was presumed to lack a distinct stem cell population (110). Nevertheless, accumulating evidence shows that HCCs display multiple cell subpopulations, some of which have stem cell characteristics (111, 112), and increased expression of CSC1 markers (Nanog, SOX2, Oct4) was identified in some HCC subpopulations (113, 114). These subpopulations were associated with increased HCC invasion and chemoresistance (115). The core embryonic transcription factor's circuitry (SOX2, c-Myc, Oct4, Nanog) is essential for LCSCs self-renewal (105–107).

Mitogen-activated protein kinase 1 (MAPK1/MEK1) is an oncogene implicated in cancer development and therapy resistance. Active MEK1 signaling is vital for the proliferation and oncogenic potential of LCSC. The interplay between MEK1 and SIRT1 was crucial for upholding the self-renewal and growth of LCSCs. Decreased MEK1 expression in LCSCs reduced the expression of Oct4, c-Myc, SOX2, and Nanog and significantly decreased LCSC self-renewal and proliferation (46). Mechanistically, MEK1 signaling activation increased SIRT1 expression and protein stability and inhibited the proteasomal degradation of SIRT1; this promoted self-renewal and oncogenicity in LCSCs, resulting in poor prognosis of HCC patients (46).

In a cohort of 148 HCC patients, the expression of the MEK1–SIRT1 pathway was strongly correlated with tumor size, vascular and capsular invasion, clinical tumor stage, and poor prognosis (46). MEK1 knockdown in LCSCs isolated from HCC samples lowered SIRT1's half-life, suppressed oncogenicity and self-renewal, and lessened the expression of stem cell markers. However, these results need to be further replicated and validated *in vivo*. Inhibiting SIRT1/MEK1 signaling impedes HCC oncogenesis and should be further explored as a possible therapeutic target (see **Figure 1**).

Notch3–SIRT1–LSD1–SOX2 Signaling Pathway

Lysine demethylase 1 (LSD1) is an epigenetic regulator responsible for demethylating various histones and controls the pluripotency of stem cells (116–118). LSD1 is overexpressed in HCC cells compared to normal hepatic parenchyma. Moreover, LSD1 is overtly expressed in LCSCs where it directly regulated the transcription of the SOX2 gen, promoted self-renewal and tumorigenesis, and was associated with a poor patient prognosis (119). Similar to the effect of SIRT1 *via* DNMT3A, LSD1 demethylated the SOX2 promoter and consequently increased its expression and improved LCSC stemness. Acetylation inhibits the enzymatic activity of LSD1 and stimulates its degradation *via* UPP. SIRT1 deacetylated LSD1 and thus increased its stability (see **Figure 2**).

Notch signaling is vital for cell proliferation and survival (120). In HCC, Notch receptors are mostly overexpressed and their ligand expression was associated with aggressive tumor phenotypes (121). Notch promoted HCC development and

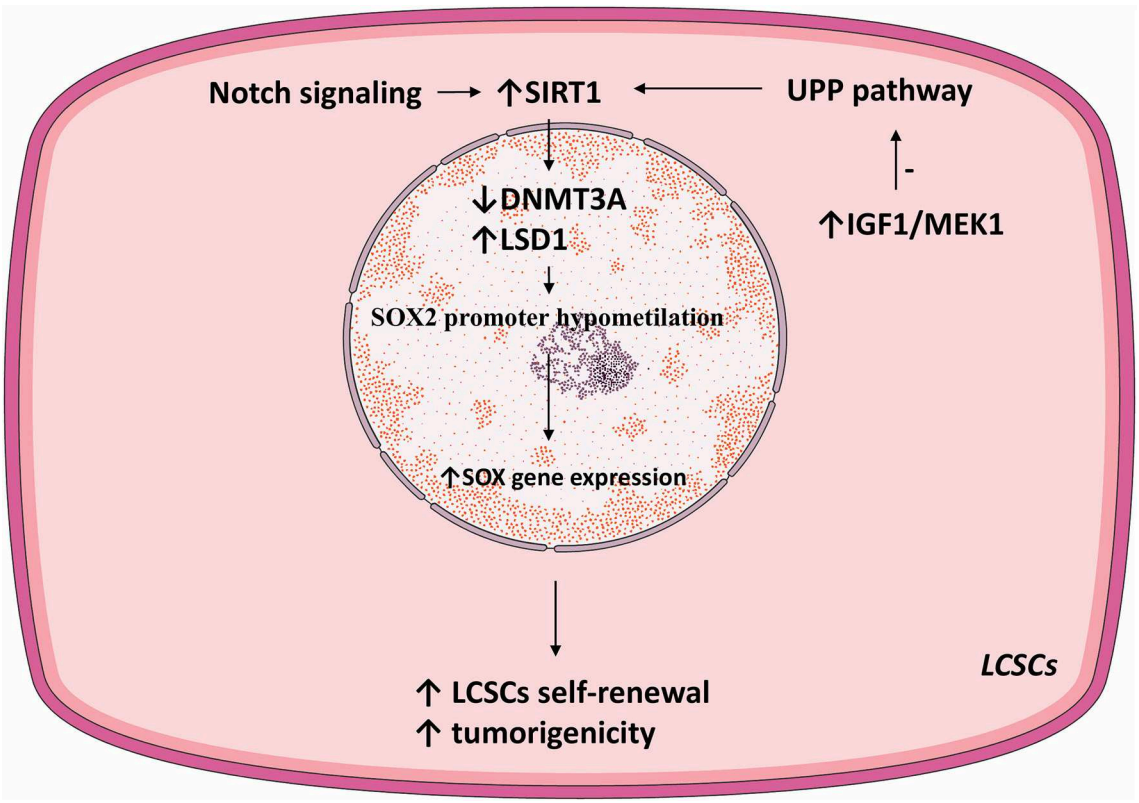

FIGURE 2 | SIRT1-related pathways involved in LCSC proliferation.

metastasis through activating the Wnt/β-catenin pathway (122, 123). Moreover, Notch signaling was shown to promote CSC self-renewal. Notch3 signaling induced SIRT1 expression and facilitated LDS1 deacetylation and activated LSD1, consequently promoting LCSC self-renewal. The Notch3-dependent pathway was crucial for LCSC self-renewal and *in vivo* tumor dissemination (see **Table 1**).

SIRT1–CPEB1 Signaling Pathway

Cytoplasmic polyadenylation element-binding protein 1 (CPEB1) mediates mRNA translation and negatively regulates HCC stemness and chemoresistance. Moreover, CPEB1 expression was low in HCC and LCSCs when compared to normal hepatocytes (124).

In HCC, CPEB1 upregulation decreased chemoresistance, accelerated doxorubicin-induced apoptosis, inhibited cell migration and self-renewal and decreased tumoral growth while CPEB1 knockdown had the opposite effect.

The 3′ untranslated region (3′UTR) of SIRT1 mRNA presents two cytoplasmic polyadenylation element (CPE) sequences. CPEB1 binds to SIRT1 mRNA and could curtail the poly(A) extremity of SIRT1 mRNA, thus decreasing SIRT1 protein levels. Thus, CPEB1 regulated SIRT1 expression at the post-transcriptional level. Hence, decreased CPEB1 expression may account for SIRT1 overexpression, which in turn promotes LCSCs self-renewal, chemoresistance, and HCC cell spheroid formation (124).

THE INTERPLAY BETWEEN miRNAs AND SIRT1 IN THE DEVELOPMENT AND TREATMENT OF HCC

MicroRNAs (miRNAs/miRs) are endogenous, single-stranded, non-coding regulatory RNAs (131) that exert their biological functions by integrating into the RNA-inducing silencing complex of their target mRNA where they attach to the 3′UTR and either inhibit mRNA translation or induce its degradation (47, 132). The function of multiple miRs was shown to be dysregulated in HCC and in a plethora of other cancers. Depending on the cellular environment and target genes, miRs can function as either oncogenes or tumor suppressors (47, 133, 134). miRs were demonstrated to play a role in HCC development, proliferation metastasis, and therapeutic resistance (121, 135, 136).

p53-miR-34a–SIRT1 Signaling Pathway

0404

0404, is a DNA-damaging compound with no cytotoxic effects on non-cancerous human hepatocytes. 0404 induced apoptosis and decreased growth in an *in vivo* HepG2 HCC model. However, P53

TABLE 1 | Molecules involved in the biology of LCSCs and their interaction with SIRT1.

	Role in HCC	Interaction with SIRT1	Comments	References
MRPS5	↑LCSCs mitochondrial function and NAD levels	SIRT1 deacetylates MRPS5 and promotes its nuclear localization	Metformin ↓MRPS5	(101)
SOX2	↑LCSCs self-renewal ↑Tumorigenicity	SIRT1 promotes SOX2 expression	SIRT1 inhibitors TV6 and EX-527 ↓SOX2	(88)
MEK1	↑Proliferation and oncogenesis of LCSC	MEK1 ↑ SIRT1 expression in LCSC	↑MEK-SIRT1 expression correlated with HCC metastasis	(46)
LSD1	↑LCSCs self-renewal and tumorigenesis	SIRT1 deacetylates LSD1 and prevents its degradation	↑SOX2 expression	(119)
CPEB1	↓Chemoresistance ↓Stemness ↑Apoptosis	↓SIRT1 expression	CPEB1 expression is ↓ in LCSCs and HCC	(124)

WT HepG2 cells were more responsive to 0404 compared to the p53 mutant Huh7 cell lines (137).

P53 modulates the transcription of multiple miRs. In turn, numerous miRs target the 3′UTR region of p53 mRNA. Hence, p53 and miRs may form a feedback loop (138).

The miR-34 family is typically silenced in multiple tumors and was identified as the most frequent p53-induced miRs (139, 140). miR-34a was shown to increase p53 transcription and acetylation and induced apoptosis in HCC cells. In HepG2 but not in Huh7 cell lines, 0404 upregulated p53 and miR-34a expression, increased acetylated p53, and downregulated SIRT1 protein expression, which consequently inhibited HCC growth (137). The anticancer mechanisms induced by 0404 and its toxicity and efficacy should be examined *in vivo* on multiple HCC cell lines.

Quercetin

Quercetin is a flavonoid with anti-cancer proprieties and low toxicity to non-cancerous cells. Quercetin activated apoptosis and cell cycle arrest in HepG2 and Huh7 cells. However, p53 status determined the sensitivity of HCC cells to quercetin. Namely, in p53 WT cell lines, proliferation was reduced by a significantly lower quercetin quantity, when compared to p53 mutants. HepG2 cells treated with quercetin showcased increased p53 expression, miR-34a activity, and SIRT1 inhibition (140, 141).

SIRT1 deacetylates p53, resulting in the cessation of its activity. miR-34a silences SIRT1 mRNA by binding to its 3′UTR region (142). Quercetin activated p53, which induced miR-34a and consequently silenced SIRT1 mRNA expression, leading to increased p53 acetylation, activity, and consequently apoptosis, thus forming a positive feedback loop. In summary, quercetin activates the p53-miR-34-SIRT1 axis and induces a positive feedback loop that suppresses tumor formation (143).

miR-34a-IL-24 Oncolytic Adenoviruses

miR-34a expression was shown to be downregulated in multiple cancers including HCC, where it exerts a tumor-suppressive role. In HCC, miR-34 levels were inversely correlated with vascular invasion, necrosis, and histological staging, and low miR-34a expression was associated with decreased overall survival (144).

miR-34a delivery *via* oncolytic adenovirus killed HCC cells *in vitro*, with low toxicity to normal hepatocytes. Importantly, miR-34a expressed in HCC *via* oncolytic adenoviruses, downregulated SIRT1 and Bcl-2 (144) expression, and induced cancerous cytotoxicity (144).

The cytokine IL-24 is known to inhibit tumoral angiogenesis and activates tumoral apoptosis. It was hypothesized that increasing the expression of both IL-24 and miR-34a would provide synergistic therapeutic benefits. Oncolytic adenovirus-mediated transfer of both miR-34a and IL-24 led to a more potent inhibition of HCC cell growth than administering either miR-34a or IL-24 separately. However, the antitumoral mechanisms of the oncolytic adenoviruses used in the abovementioned study are insufficiently understood and its effects on metastasis should also be explored.

Butyrate–miR-22–SIRT1 Signaling Pathway

Butyrate, a short-chain fatty acid, is produced by the intestinal microbiome *via* anaerobic fermentation and is subsequently absorbed by the hepatocytes (145). Butyrate was shown to induce apoptosis and decrease tumorigenesis in multiple malignancies (146, 147). Butyrate was reported to inhibit SIRT1 gene expression in some types of cancer, although this has not yet been demonstrated in HCC (148).

miR-22 was shown to be downregulated in HCC and its low levels contributed to tumorigenesis (149). miR-22 expression activated apoptosis and inhibited the *in vitro* proliferation of the Huh7 cells. Oppositely, SIRT1 expression was high in Huh7 cells and increased the expression of antioxidants such as superoxide dismutase (SOD), consequently maintaining cell proliferation (40).

In Huh7 cells, butyrate induced miR-22, which directly binds the 3′UTR region of SIRT1 and downregulates its expression; this reduced SOD activity and augmented ROS production, increasing caspase 3 and cytochrome c activity, thus promoting

apoptosis (150). Furthermore, by downregulating SIRT1, miR-22 increased PTEN and gsk-3 expression and downregulated β-catenin and p-akt expression and thus may promote apoptosis and decrease HCC proliferation (150).

However, multiple aforementioned experiments were only performed with 2D cultures or *in vitro* (150, 151). 3D cultures better mimic the *in vivo* environment (152) and it was reported that therapeutic approaches are less effective in 2D cultures when compared to 3D ones. Drug resistance was also reported to be higher in 3D cultures (153–155). Thus, replicating therapeutic interventions performed with monolayer cultures with spheroids, or *in vivo*, may offer a better understanding of their translational potential (see **Figure 3**).

miR-133b-Sirt1-GPC3-Wnt/β-catenin Signaling Pathway

The Wnt/β-catenin pathway is essential for the physiological functioning of the liver (156) and is also implicated in oncogenesis (157). In non-cancerous cells, SIRT1 deacetylates β-catenin, thus constricting it to the cytoplasm and limiting its ability to trigger transcription and induce cell proliferation (25).

The β-catenin gene is commonly mutated in HCC (158). SIRT1 is negatively associated with β-catenin mutation in HCCs, demonstrating that SIRT1 may promote oncogenesis in cancers independent of Wnt/β-catenin.

miR-133b mainly acts as a tumor suppressor and is markedly reduced in a multitude of cancers (159). miR-133b expression was decreased in a majority of HCC biopsies when compared to paired adjacent normal tissue (160). Moreover, miR-133b upregulation in HepG2 cells strongly repressed HCC cell proliferation and invasion and promoted apoptosis (47). Additionally, miR-133b upregulation decreased tumor growth, in nude mice with orthotopic HepG2 cell tumors. miR-133b directly targets and is inversely correlated with SIRT1 in human HCC cells. Enhanced miR-133b expression strongly decreased SIRT1 expression at both mRNA and protein levels. Overall, the anti-cancer effect of miR-133b in HCC cells appears to be achieved through inhibiting SIRT1 expression.

GPC3 stimulates HCC growth *via* Wnt signaling. It has been reported that SIRT1 inhibition decreases the expression of cancer markers AFP and GPC3. Decreased GPC3 and AFP expression indicate the development of an increasingly differentiated cell phenotype and may be beneficial (42). GPC3 is a membrane protein that is extremely overexpressed in HCC and is involved in hepatocarcinogenesis (161–163). GPC3 suppression in HCC cells inhibited proliferation and the expression of anti-apoptotic proteins (Mcl-1, Bcl-2, and Bcl-xL) and also increased TGF-β expression (47). GPC3 also promoted HCC cell EMT (164).

Malignant cells are characterized by inadequate intercellular adhesion and increased cellular motility. E-cadherin is a molecule vital for cell–cell adhesion in epithelial tissues (165). Dysregulation of E-cadherin-modulated intercellular adhesion is observed in human carcinomas and correlates with acquisition of metastatic potential (166). Downregulating SIRT1 decreased GPC3 mRNA and increased the mRNA expression of E-cadherin (42). miR-133b upregulation or GPC3 downregulation repressed GPC3, Mcl-1 Bcl-2, Bcl-xL, and SIRT1 expression and increased the expression of E-cadherin. Moreover, GPC3 downregulation canceled the SIRT1 overexpression-induced inhibition of apoptosis and accelerated invasion and proliferation (47). Additionally, in HCC cells, miR-133b overexpression inhibited GPC3 expression and cell proliferation.

GPC3 interacts with Wnt ligands, consequently stimulating cell migration and proliferation in HCC (167, 168). Activation of the Wnt signaling pathway induces cytoplasmic buildup and nuclear translocation of the transcription factor β-catenin. Intranuclear β-catenin induces the expression of genes that regulate cell differentiation, proliferation, migration, and apoptosis (168–170). SIRT1 upregulation increased the expression of GPC3, which consequently stimulated the Wnt/β-catenin pathway and induced cytosolic accumulation and nuclear translocation of β-catenin. Concluding, miR-133b suppresses cell proliferation and migration and activates cell apoptosis, by inhibiting the Sirt1-GPC3-Wnt/β-catenin signaling pathway (47).

However, SIRT1 was reported to suppress Wnt/β-catenin in multiple malignancies including mouse and human HCCs (171–175). Mechanistically, SIRT1 expression was shown to stimulate β-catenin phosphorylation, which promoted its degradation. SIRT1 regulation of β-catenin is contingent on protein kinase A (PKA). SIRT1 may stimulate PKAs auto-phosphorylation but may also influence PKA through transcriptional regulation of PGC1α–PKA's upstream regulator.

SIRT1 can activate the transcription of PGC1α in HCC cells. PGC1α was demonstrated to elevate cAMP, which, in turn, stimulates the activity of PKA. Thus, SIRT1 may collaterally promote the phosphorylation of PKA and β-catenin through a PGC1α-cAMP-dependent manner (175). SIRT1 also increases βTrCP gene expression. βTrCP is crucial for Sirt1-induced Wnt/β-catenin signaling inhibition. Namely, phosphorylated β-catenin is ubiquitinated by βTrCP and consequently degraded.

Overall, suppressing SIRT1 expression in HCC for therapeutic purposes may activate Wnt/β-catenin signaling and promote tumorigenesis. To short-circuit this potential side effect, the Wnt/β-catenin pathway should be simultaneously inhibited (175).

miR-449–SIRT1–SREBP-1c Signaling Pathway

Sterol regulatory element binding protein (SREBP)-1c is a transcription factor predominantly localized in adipocytes and hepatocytes, where it regulates lipid synthesis-related gene expression. Abnormal lipid metabolism has been connected to HCC development (65, 176) and SREBP-1c dysfunction is involved in oncogenesis (177–179). SIRT1 regulates lipid metabolism and its activation decreased the expression of hepatic SREBP-1c (180).

miR-449 is part of a miR family that regulates apoptosis and proliferation and may promote tumor suppression *via*

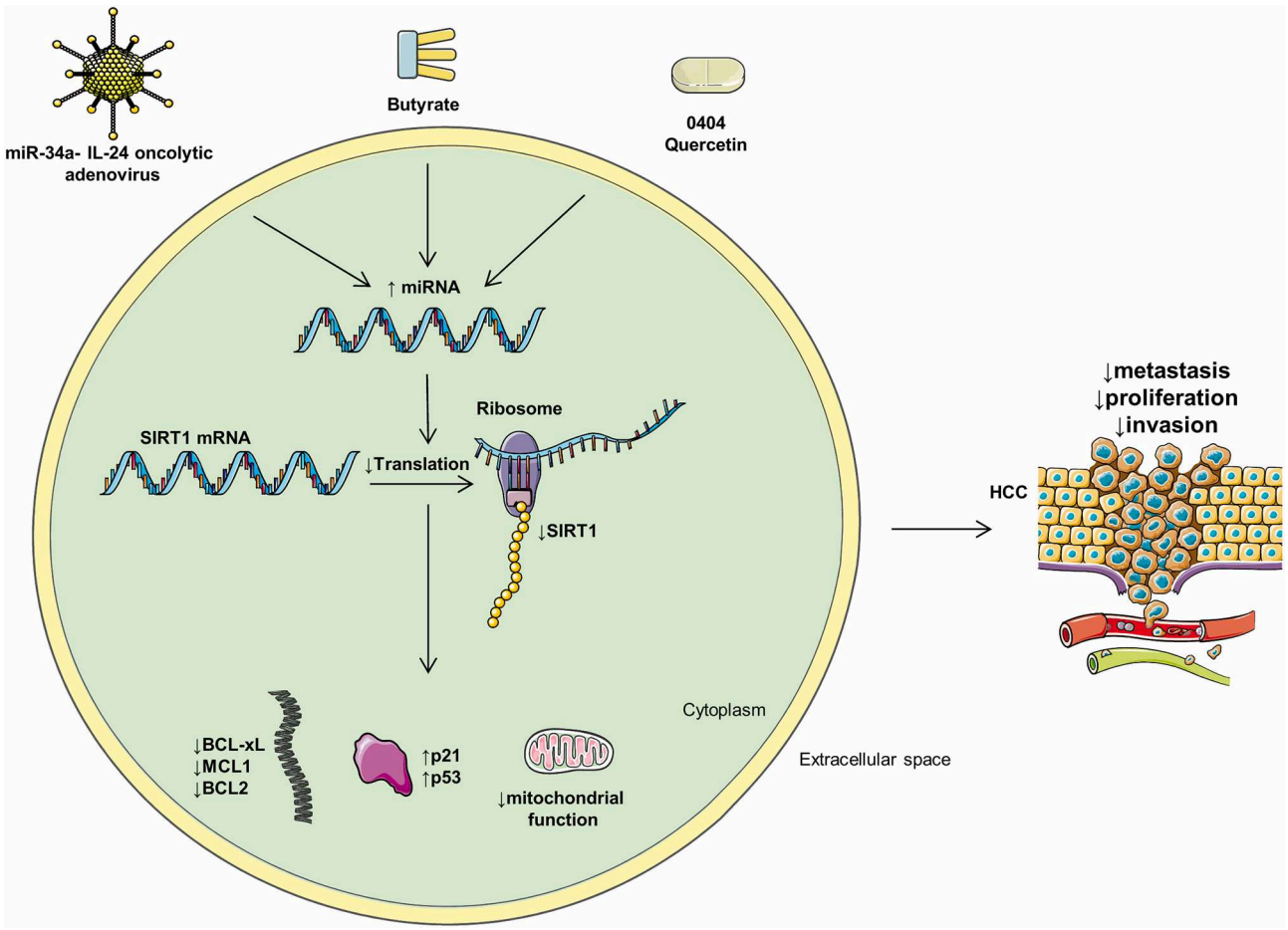

FIGURE 3 | Mediators of miRs and their intracellular effects. Multiple compounds may influence the levels of intracellular miRs and negatively regulate HCC progression. Oncolytic adenoviruses, butyrate, 0404, and quercetin positively regulate multiple HCC miRs and, as a consequence, decrease metastasis, proliferation, and invasion.

downregulating histone deacetylases (181, 182). In Huh7 and HepG2 cell lines, miR-449 directly targets and inhibits SIRT1 mRNA expression, which consequently inhibits SREBP-1c and thus constrains cholesterol and fatty acid biosynthesis (151). Additionally, the SIRT1-SREBP-1c downstream metabolic oncogenes 3-hydroxy-3-methylglutaryl CoA reductase (HMGCR) and fatty acid synthase (FASN) (177, 183) are also downregulated. Overall, miR-449 expression decreases mice HCC xenograft development (151, 184).

Thus, miR-449 inhibited the SIRT1–SREBP pathway which decreased proliferation and DNA synthesis, reduced lipid anabolism, and suppressed tumorigenesis in Huh7 and HepG2 cell lines (151).

MALAT1–miR-204-5p–SIRT1 Signaling Pathway

The lncRNA metastasis associated lung adenocarcinoma transcript 1 (MALAT1) is decidedly expressed in HCC where it stimulates growth and invasion. MALAT1 activates mechanistic target of rapamycin (mTOR) signaling (185) and enhances the development of HCC CSC (186).

Oppositely to MALAT1, miR-204 promotes apoptosis by activating p53 and inhibiting anti-apoptotic protein Bcl-2 (187). miR-204 also inhibited cancer stemness and EMT and increases chemosensitivity (187, 188). However, MALAT1 expression was negatively correlated with miR-204 levels. MALAT1 directly attaches to miR-204 and negatively regulates its expression (189). SIRT1 appears be a vital intermediary in the interplay between MALAT1 and miR-204. It is known that SIRT1 is vital for HCC EMT, migration, and invasion. SIRT1 is directly targeted and silenced by miR-204 (189). However, SIRT1 and MALAT1 attach to the same miR-204 site; thus, MALAT1 might be in competition with SIRT1 for binding miR-204; this decreases miR-204-induced SIRT1 inhibition. Overall, MALAT1 negatively regulated miR-204 activity and consequently increased SIRT1, which, in turn, induced HCC migration and invasion (189). Inhibiting MALAT1 decreased the aggressive behavior of HCC, which makes it a potential therapeutic target (189).

Moreover, it was shown that miR-204-5p expression was decreased in multiple human HCC cell lines (189). Decreased miR-204-5p levels were associated with HCC metastasis and poor patient outcome. In HCC cell lines, miR-204-5p binds to the 3′UTR region of SIRT1 and consequently decreases its expression at both mRNA and protein levels. Likewise, in these same cell lines, miR-204-5p was inversely associated with SIRT1 expression. miR-204-5p decreased the invasion and survival of HCC cells by reducing SIRT1 expression and protein levels both *in vivo* and *in vitro* (190). Overall, miR-204-5p induced post-transcriptional inhibition of SIRT1 in multiple human HCC cell lines. Importantly, miR-204-5p overexpression strongly downregulated both SIRT1 mRNA and protein levels in HCC cells and thus attenuated tumoral growth. As such, miR-204-5p is a potential diagnostic marker and increasing its expression and activity in HCC cells is a therapeutic option that needs to be further explored (190).

miR-486

miR-486 downregulation may be a hallmark of HCC development and contributed to the differentiation of LCSCs into HCC cells (43). miR486 was shown to be strongly downregulated in HCC samples and in LCSCs (43).

On the contrary, SIRT1 expression was increased in LCSCs and maintained the tumorigenic and self-renewal proprieties of LCSCs *in vivo*, and was inversely correlated with miR-486 levels in LCSCs (43). miR-486 directly targets and strongly suppresses SIRT1 expression and decreased the tumorigenic and chemo-resistant properties of LCSCs and suppressed HCC invasion and tumorigenicity (43).

Overall, this further validates the role of SIRT1 as a promoter of HCC development, invasion, and recurrence, in part through maintaining the stemness of CSCs (43).

miR-29c

Decreased levels of the miR-29 family were associated with poor HCC survival. The miR-29 family have tumor-suppressive roles by targeting Mcl-1 and Bcl-2. Relevantly, miR-29c exerts tumor-suppressing functions by inhibiting hepatocytic SIRT1. Ectopic miR-29c expression decreased SIRT1 expression and consequently repressed cell proliferation. miR-29c directly targets and suppresses SIRT1 mRNA translation in hepatocytes (191).

miR-29c was shown to be strongly downregulated in HCC biopsies and correlated with poor patient prognosis. The 5-year survival rate of HCC patients with low miR-29c expression was pointedly inferior to that of HCC patients with high miR-29c expression. Future interventions that upregulate miR-29c in HCC models may provide insights into a more efficient management of HCC (191).

miR-29a

Decreased miR-29a expression is common in HCC, where it promoted metastasis and, as a predictor of early post-surgical recurrence, diminished overall survival and disease-free survival (192). miR-29a suppressed HCC proliferation. Tellingly, miR-29a was strongly downregulated in HCC tissue biopsies when compared with adjacent normal tissue. miR-29a upregulation suppressed HCC cell proliferation and colony formation. Mechanistically, miR-29a increased p21 expression and decreased CDK4 and CyclinD1 expression, consequently suppressing cell cycle progression; it also targeted the 3′UTR region of SIRT1 mRNA and decreased SIRT1 mRNA and protein expression consequently overturning HCC cell proliferation (192). Oppositely, SIRT1 overexpression decreased the protective effects exerted by miR-29a. Overall, data suggests that miR-29a may serve both as a prognostic marker and as a therapeutic target for HCC suppression.

miR-138

It has been confirmed that miRs have suppressive or promotive effects on tumor metastases and invasion and impact HCC progression (193).

miR-138 functions as a tumor suppressor and it was found to be downregulated in multiple cancers (194). In HCC cells, miR-138 suppresses cell invasion and proliferation. Interestingly, miR-138 expression levels were inversely correlated with SIRT1 mRNA levels in HCC tissues (193). Upregulation of miR-138 expression downregulated SIRT1 at the level of mRNA and protein levels. miR-138 binds to the 3′UTR unique complementary site of the SIRT1 gene and directly inhibits SIRT1 expression, which leads to hindered HCC proliferation, migration, and invasion (193). miR-138 was inversely correlated with SIRT1 mRNA in HCC tissues.

Multiple studies showed that miR-138 expression was downregulated in a majority of examined HCC samples compared with peritumoral non-cancerous tissue (193). SIRT1 is overexpressed while miR-138 levels are decreased in HepG2, SMMC7721, Bel7404, and HCCM3 compared to the normal hepatic cell line L02 (193).

Tellingly, increasing miR-138 expression in HepG2 and SMMC7721 cell lines inhibited their proliferation (193). This suggests that decreased miR-138 expression was associated with increased SIRT1 mRNA expression in HCC (193). This validates the role of miR-138 in HCC proliferation and metastasis (195).

miR-34a

miR-34a is a tumor suppressor in breast, colon (196), and a plethora of cancers. miR-34a expression was correlated with HCC metastasis (197). miR-34a downregulated c-Met expression and consequently inhibited HCC invasion and migration (198), which have oncogenic or tumor suppressor functions (199).

miR34a expression was decreased in Hep3B and Huh7 cells when compared to normal hepatocyte cell lines. Inducing miR-34a overexpression in Hep3B and Huh7 significantly decreased

cellular invasion and migration (200). Moreover, miR-34a overexpression also decreased SIRT1 mRNA and protein levels and increased acetylated p53. Thus, miR-34a overexpression downregulated SIRT1 expression, which increased acetylated p53 levels and consequently suppressed HCC metastasis (200).

All in all, data suggest that the post-transcriptional overexpression of SIRT1 may be promoted by loss of suppressive mRNAs that normally target and inhibit its expression.

THE ROLE OF SIRT1 IN THE METASTASIS OF HCC

Metastases are a great contributor to HCC morbidity (12). SIRT1 overexpression in HCC samples correlated with advanced tumor stage and increased incidence of portal vein tumor thrombus. Moreover, SIRT1 overexpression in HCC facilitates invasion and proliferation and suppresses apoptosis (15, 126, 191). SIRT1 also increased the invasiveness and motility of human HCC cells and was necessary for HCC metastasis *in vitro*. Importantly, silencing SIRT1 reduced the aforementioned metastatic characteristics of human HCC cells (54). Accelerated cancer invasiveness is associated with increased mitochondrial activity, oxygen consumption, and ATP production (201).

One way in which SIRT1 promotes HCC metastasis is by its interaction with peroxisome proliferator–activated receptor γ coactivator 1α (PGC-1α). PGC-1α is a transcriptional co-activator that promotes mitochondrial biogenesis and respiration (19, 202). PGC-1α expression was demonstrated to enhance the invasion and migration of HCC cells (54). In HCC samples, SIRT1 overexpression was highly correlated with PGC-1α upregulation. Ectopic SIRT1 expression upregulated PGC-1α in HCC cells. Moreover, SIRT1 physically interacts with, deacetylates, and activates PGC-1α. SIRT1-induced PGC-1α increased mitochondrial copy numbers and mass, cellular ATP levels, DNA transcript levels, and mitochondrial biogenesis, which boosted the migration and invasion of HCC, thus promoting cancer dissemination (54, 203).

PGC-1α-induced mitochondrial biogenesis and oxidative phosphorylation were crucial for HCC metastasis (204)SIRT1 knockdown in HCC cells reduced the expression of mitochondria biogenesis-related genes, diminished mitochondrial mass and copy number, intracellular ATP, and mitochondrial DNA transcript levels. These changes led to decreased intracellular ATP production and impaired HCC metastasis (54).

The epithelial-to-mesenchymal transition (EMT) was shown to promote HCC metastasis (205). Through EMT, epithelial cells gain mesenchymal-like characteristics such as reduced intercellular junctions, enhanced invasiveness and motility, chemoresistance, and decreased polarization (206). SIRT1 expression promoted migration and invasion of HCC cell lines and metastasis in an *in vivo* xenograft mice model by activating EMT (45).

However, other reports suggest that SIRT1 expression was not occasionally activated in human HCC cell lines, suggesting that SIRT1-arbitrated metastasis did not implicate EMT (54). The difference may be attributed to the different HCC models used in the experiments.

Combined, these results suggest that SIRT1 expression promotes progression, metastasis, and invasion of HCC. SIRT1 knockdown decreased migration and invasion of HCC cells *in vitro*, decreased HCC invasion and metastasis *in vivo* (45, 54), and impaired mitochondrial function and biogenesis, suggesting that the SIRT1/PGC-1α axis may be a viable therapeutic target for decreasing metastasis (54).

IMPLICATIONS OF SIRT1 IN THE TREATMENT AND CHEMORESISTANCE OF HCC

HCC is a chemo-refractory cancer (207). Multidrug resistance (MDR) is partly incriminated for HCC metastasis and recurrence. The ATP binding cassette (ABC) transporters are involved in the cellular efflux of chemotherapeutics. ABC overexpression is partly incriminated for HCC MDR (208). The ABC transporters, P-glycoprotein, or multidrug resistance protein 1 (P-gp, MDR1) and multidrug resistance protein 3 (MRP3) are important for HCC chemotherapy (209–211).

SIRT1 expression was shown to stimulate oncogenesis and promote MDR in HCC (40, 212). SIRT1 overexpression upregulates MDR1 in HepG2 cells (213). Contrarywise, silencing FOXO1 or SIRT1 accentuates the cellular uptake of chemotherapeutics and reinstates HCC chemosensitivity. For instance, SIRT1 knockdown in human HCC cells enhanced doxorubicin-induced chemosensitivity and apoptosis (15).

Acetylated p53 promotes tumor suppression by interacting with its downstream targets. SIRT1 deacetylates both p53 and FOXO1, consequently suppressing their ability to induce apoptosis and cell growth arrest (214, 215).

FOXO1's activity is controlled by post-translational interventions, including acetylation, ubiquitination, and phosphorylation. SIRT1 deacetylates FOXO1 and thus promotes its nuclear localization and increases the FOXO-dependent transcription of stress-response genes (216, 217). Specifically, FOXO1 binds to the MDR1 gene promoter and increases MDR1 gene transcription.

Deleted in Liver Cancer-1 (DLC-1) is an established tumor suppressor that has important roles in cell motility and signal transduction pathways. Akt regulates DLC-1 activity by post-translational modifications. SIRT1, by inhibiting the PI3K/Akt pathway, could increase DLC-1 expression and thus promote HCC cell motility (218).

HULC/USP22/SIRT1/Autophagy Pathway

Autophagy is involved in the chemoresistance of cancer cells (219). Cisplatin, sorafenib, and 5-FU can induce autophagy in HCC cells, thus decreasing apoptosis and chemosensitivity (220). Long noncoding RNAs (lncRNAs) are transcripts involved in regulating gene expression. lncRNA HULC (highly upregulated

in liver cancer) is involved in HCC chemoresistance and autophagy. In HCC biopsies and cell lines, SIRT1 and HULC are both aberrantly upregulated.

Oxaliplatin and 5-FU upregulated HULC expression in HCC cells. In turn, HULC strongly increased USP22 protein levels and promoted SIRT1 deubiquitylation, consequently decreasing the UPP-mediated degradation of SIRT1 and increased its protein stability. miR-6825-5p, miR-6886-3p, and miR-6845-5p bind to the 3′UTR region of USP22 mRNA and thus decrease its levels. HULC downregulates the abovementioned miRs and strongly upregulates USP22. HULC-induced SIRT1 upregulation enhanced the deacetylation of key autophagy components such as Atg7 and Atg5 and triggered autophagy, which decreased HCC chemosensitivity. In summary, the HULC/USP22/SIRT1 pathway induces protective autophagy and decreases HCC chemosensitivity. *In vivo* knockdown of HULC or SIRT1 sensitizes HCC to oxaliplatin. Thus, combining chemotherapy with HULC or SIRT1 inhibitors in MDR HCC is a therapeutic option that needs further exploration (see **Table 2**).

Cambinol and EX-527

EX-527 and cambinol are cytotoxic to and trigger apoptosis in both 2D and 3D HCC cultures (42). Cambinol and EX-527 are SIRT1 inhibitors with antitumor effects that decrease MDR1 mRNA expression in a p53 status- and dose-dependent manner. This suggests that their administration and compatibility with other chemotherapeutics should be adjusted according to the particular genotype of the HCC treated (221).

Cambinol or EX-527 decreased SIRT1 activity and protein levels and thus increased apoptosis, reduced cell migration, and decreased the growth and viability of HCC cell spheroids (60, 126). EX-527 increased the acetyl-p53/p53 ratio in HCC cells and promoted apoptosis. Surprisingly, cambinol decreased the acetyl-p53/p53 radio in Huh7 cells, and increased p53 protein levels. The reason for this discrepancy and the consequences of cambinol on p53 acetylation should be further explored in multiple HCC models. However, both Ex-527 and cambinol decreased FOXO1 expression and increased FOXO1 acetylation, which consequently decreases P-gp in 2D HCC cultures. Whether this reduces chemoresistance should be further explored in HCC spheroids or *in vivo* models.

SIRT1 downregulation suppresses MRP1 and increases intracellular concentration of Adriamycin in MDRHCC. Downregulating SIRT1 *via* shRNA decreases MRP3 and MRP1 protein levels. However, the effects of cambinol and Ex-527 on those proteins in HCC have not yet been explored.

Sorafenib

Sorafenib is used in the treatment of advanced HCC. Notably, HCC cells resistant to sorafenib showcased increased MRP3 and P-gp expression. Thus, combining cambinol or EX-527, which downregulates P-gp and MRP3 in HepG2, with conventional chemotherapeutics may offer new treatment options against MDRHCC. Surprisingly, cambinol and EX-527 induced MRP3 and P-gp expression in Huh7 cells. Since HepG2 are p53 WT while Huh7 are p53 mutant, it was suggested that this may at least partially account for the discrepancy.

Therapy response may depend on p53 status, which appears to influence the expression of ABC transporters in MDRHCC. Therefore, the p53 status of each HCC has to be considered when evaluating for susceptibility to chemotherapy. This undermines the importance of characterizing the chemosensitivity of multiple HCC types and personalizing treatment accordingly.

USP22/SIRT1/AKT/MRP1 Signaling Pathway

Ubiquitin-specific protease 22 (USP22) is part of a subfamily of deubiquitinating enzymes and a CSC marker. USP22 is exceedingly expressed in some multidrug resistant human HCC cell lines (MDRHCC) where it decreases sensitivity to 5-fluorouracil (5-FU), doxorubicin, and methotrexate; specifically, it reduced the intracellular concentration of doxorubicin by promoting efflux pump activity and inhibited 5-FU-induced apoptosis. Similarly, SIRT1 expression in MDRHCC decreased intracellular doxorubicin concentrations and promotes resistance to 5-FU.

Inhibiting USP22 in MDRHCC strongly decreased ABCC1 expression [encodes MRP1 (resistance-associated protein 1)] and thus increased intracellular doxorubicin concentrations, but only dimly influenced ABCB1 expression (encodes P-gp). Thus, UPP22 may predominantly induce MDR *via* MRP1.

USP22 deubiquitinated SIRT1 (222, 223) and thus increased SIRT1 protein levels, which deacetylated and thus activated the PI3K/AKT pathway (224) and consequently increased MRP1s expression (225), which promoted MDR in HCC. However, this mechanistic chain needs to be further validated by other experiments. In 168 HCC biopsies, the protein expressions of MRP1 and USP22 were strongly correlated. Inhibiting the PI3K/AKT pathway in MDRHCC suppressed MRP1's expression and promoted 5-FU-induced apoptosis (226). SIRT1 inhibition increased the sensitivity of MDRHCC to 5-FU and increased intracellular concentration of doxorubicin. Simply put, USP22 may activate the SIRT1–AKT–MRP1 pathway and consequently promote MDR in human HCC cells (226). Future studies should explore the relationship between USP22 and other proteins involved in MDRHCC such as MRP1 and cytochrome P450, which is involved in the hepatic metabolism of xenobiotics.

SIRT1–YAP Signaling Pathway

SIRT1 stimulated the transcription of MKK3 and Yes-associated protein (YAP), which in turn promoted the nuclear localization of p38. SIRT1 activated p38 and consequently promoted HCC development (44). In HCC cells, SIRT1 expression activated YAP2 transcriptional activity and also accentuated the interaction between YAP2 and the transcriptional cofactor TEAD4, and thus promoted the transcription of their downstream targets, which consequently accelerated HCC cell growth and survival. SIRT1 deacetylates YAP2 both *in vitro* and *in vivo*, which upregulated the YAP2/TEAD4 axis and promoted HCC cell proliferation (60).

Treatment with cisplatin accentuated the interaction between SIRT1 and YAP2 and promoted the expression of the YAP2 downstream genes. As a response to cisplatin, YAP2 translocates to the nucleus where it is deacetylated by SIRT1,

TABLE 2 | Therapeutic substances that interfere with SIRT1-related pathways in HCC.

Medication	Targeted signaling pathways and biological processes	Effects on HCC	Type of study	References
Oxaliplatin and 5-FU	HULC-USP22-SIRT1	↑Autophagy ↑HCC chemosensitivity	*in vivo*	(125)
EX-527 and cambinol	SIRT1 inhibitors	↑Apoptosis ↓Cell migration ↓ Tumoral growth	*in vivo*	(126)
Metformin	↑AMPK activity ↑Acetylated p53 and p21	↑Senescence ↑Apoptosis ↓Proliferation	*in vitro* and *in vivo*	(127)
Gallotannin	↑AMPK phosphorylation ↓SIRT1 expression	↓Colony formation ↑Cytotoxicity ↑Senescence	*in vitro* and *in vivo*	(128)
Ku0063794 and Everolimus	↓SIRT1 expression	↓Proliferation ↓Autophagy ↑Apoptosis	*in vivo*, *ex vivo*, and *in vitro*	(129)
2-Unsubstituted 4,11-diaminoanthra[2,3-b]furan-5,10-dione derivatives	↓tNOX ↓Intracellular NAD+ ↓SIRT1 ↓Acetylated p53	↑ Apoptosis ↓Cell migration	*in vitro*	(130)

which consequently protects HCC cells from cisplatin-induced apoptosis. Overall, both YAP2 and SIRT1 confer protection against cisplatin (60). Silencing SIRT1 inhibited the nuclear translocation of YAP2 and promoted sensitivity to cisplatin.

Since SIRT1 inhibition induced cytostatic effects in HCC (42), combining it with cytotoxic chemotherapeutics should be considered. Moreover, SIRT1 inhibiting compounds used for the purpose of treating HCC should selectively target tumoral SIRT1 and spare normal hepatocytes in order to preserve liver function.

AMPK–SIRT1–p53 Signaling Pathway

By targeting transcription factors such as p53 and FOXO, SIRT1 suppressed cellular differentiation and senescence and may promote HCC growth (227). Mutual regulation ensues among p53 and SIRT1. SIRT1 deacetylates and inactivates p53 while acetylated p53 downregulates the translation of SIRT1 *via* miR-34a (142). Silencing SIRT1 in HCC cell cultures increases acetylated p53 and promotes growth arrest (41). This relationship is further validated by nicotinamide (NAM), a SIRT1 inhibitor. In mice treated with NAM, p53 presented increased acetylation, which led to decreased HCC oncogenesis. Importantly, the protective effects of NAM were attributed to the inhibition of SIRT1, not to NAM's antioxidant effect.

In HCC cells, SIRT1 deacetylates p53, thus repressing cellular senescence and apoptosis, and promotes tumorigenesis (63, 228). SIRT1 knockdown in HCC cells increased acetylated p53, decreased proliferative activity activated senescence, and induced a more differentiated cellular state (26, 42). However, the response to silencing SIRT1 may depend on the p53 status of HCC cells. Silencing SIRT1 in p53 WT HepG2 cells increased AMPK phosphorylation, reduced phospho-mTOR, and promoted G1 phase arrest (41, 63). However, silencing SIRT1 in p53 mutant HCC decreased phospho-AMPK and increased mTOR phosphorylation, which stimulated HCC tumorigenesis. Moreover, some reports indicate that inhibiting SIRT1 prevented cell proliferation irrespective of the p53 status of the HCC cells (42). The dynamic between the p53 status of HCC cells and how it affects SIRT1 inhibition is not yet clear.

5′ AMP-activated protein kinase (AMPK) is an enzyme implicated in glucose and fatty acid uptake; its activation stimulates hepatic fatty acid oxidation, ketogenesis, and lipogenesis (229). AMPK is a downstream target of tumor suppressor LKB1. The LKB1–AMPK pathway is vital for the suppression of mTOR signaling. mTOR signaling is overactive in multiple solid tumors and modulates cell proliferation (230). The AMPK-α2 subunit was shown to be strongly downregulated in HCC compared with the corresponding normal hepatic tissue and was correlated with poor patient prognosis.

In HCC cells, AMPK-α2 and SIRT1 are co-localized in the nucleus where they directly interact. AMPK phosphorylates SIRT1 at Thr344 and thus inhibits its deacetylase activity and substrate binding capacity and consequently maintains acetylated p53 levels, which promote apoptosis. Thus, AMPK promotes p53 acetylation and exerts antioncogenic functions in HCC (231).

Metformin

Inducing senescence may be a viable strategy for the chronic management of HCC, since it has fewer negative consequences than therapies that activate apoptosis (232, 233).

Metformin is a drug primarily used for the treatment of type 2 diabetes; it also has antitumoral effects in HCC (127, 234). A low dose of metformin strongly suppressed HCC growth *in vivo* and *in vitro* by inducing senescence, inhibiting proliferation, and activating apoptosis (127, 233). Metformin promoted phosphorylation and activated AMPK, which in turn phosphorylated SIRT1 and disabled its enzymatic activity. Consequently, levels of acetylated p53 and p21 were increased ensuing HCC senescence (127). Additionally, AMPK phosphorylation induced by metformin inactivated mTOR in p53 mutant HCC and negatively regulated oncogenesis (63). These

results need to be replicated with a larger variety of HCC cell lines (233).

However, both metformin and AMPK modulate NAD+ metabolism and can induce SIRT1 activity (127, 235, 236). Notably, SIRT1 can be phosphorylated at multiple sites that promote different phenotypes. Nevertheless, in HepG2 cells cultured in a high glucose medium, metformin activated both AMPK and SIRT1 and amplified p53 deacetylation contributing to p53 degradation (237). Specifically, metformin primarily targeted AMPK, which activated SIRT1. In this model, both AMPK and SIRT1 were required for metformin-induced p53 degradation (237).

Gallotannin

Gallotannin is a plant-derived compound with anticancer effects (125). Treating HCC cells with gallotannin *in vitro* resulted in reduced colony formation, amplified cytotoxicity, increased senescence, impaired autophagy, upregulated p21, and promoted cell death. In a mouse xenograft model, gallotannin decreased tumor growth (128). Mechanistically, gallotannin activated AMPK phosphorylation and decreased SIRT1 expression in both *in vitro* and *in vivo* HCC models (128).

Everolimus and Ku0063794

The PI3K/AKT/mTOR signaling pathway is involved in the development of multiple malignancies, including HCC (129).

Abnormal mTOR signaling is present in up to 48% of HCC patients and is associated with a meager prognosis. Everolimus is an mTOR complex 1 (mTORC1) inhibitor. However, targeting both mTORC1 and 2 is pivotal for evading drug resistance (129). Administering everolimus with Ku0063794, an mTORC2 inhibitor, produced a potent antioncogenic effect in HCC cells (130). mTOR and SIRT1 both regulate autophagy (130). SIRT1 may promote autophagy *via* deacetylating transcription factors such as E2F1, FOXO1, histone H4, and p53, which subsequently induce autophagy-related genes (238). Moreover, inhibiting mTOR can also activate autophagy (130, 239).

In HepG2 cells, combining Ku0063794 with everolimus decreased autophagy and inhibited SIRT1 expression, whereas individual monotherapy with either of the compounds did not inhibit SIRT1 and promoted autophagy (130, 240). Blocking autophagy stimulated apoptosis, decreased proliferation, and inhibited SIRT1 expression. This suggests that autophagy may promote survival in HCC cells (130).

Overall, combined use of Ku0063794 and everolimus downregulated autophagy by decreasing SIRT1 and consequently promoted antioncogenic effects in HepG2 cells (130). This experiment should be further validated with other HCC cell lines and by using spheroids or *in vivo* models. Moreover, testing whether mTORC1/2 inhibitor AZD8055 also inhibits autophagy would further validate the result obtained with Ku0063794 and everolimus in HepG2 cells (130).

In summary, *in vivo*, *ex vivo*, and *in vitro* results with HCC cells confirmed that combined Ku0063794 and everolimus therapy was superior to administering either compound alone as indicated by their increased reduction of cell invasion, migration, proliferation, and increased EMT inhibition (240). EMT inhibition was partly produced by decreased SIRT1 levels. Thus, combining everolimus with the mTORC1/2 inhibitor Ku0063794 provides potent anticancer effects (240).

tNOX-SIRT1-p53

First-line HCC treatment compounds such as doxorubicin are associated with systemic toxicity, inefficacy, and chemoresistance (10, 11). Recently, new anti-cancer compounds with high antiproliferative activity against chemoresistant cells have been developed (241, 242). The human tNOX gene encodes for a protein that is expressed in multiple solid malignancies where it is crucial for cellular migration and proliferation. tNOX converts reduced NADH to oxidized NAD. Importantly, tNOX inhibition reduces intracellular NAD concentration, which influences SIRT1 function (241, 243, 244). Suppression of tNOX by a multitude of agents activated apoptosis and diminished malignant cell growth (244, 245).

Two 2-unsubstituted 4,11-diaminoanthra[2,3-b]furan-5,10-dione derivatives promoted apoptosis in human HCC cells in a tNOX-dependent manner. In p53 WT HCC cells, these anti-cancer compounds bound and downregulated tNOX, which decreased intracellular NAD, and consequently suppressed SIRT1 activity. Decreased SIRT1 activity led to increased p53 acetylation and activation, which upregulated its downstream target, pro-apoptotic Bak and thus increased apoptosis (246). Augmented p53 acetylation promoted by SIRT1 inhibition was also associated with activation of PUMA, which upregulates Bak and prompts apoptosis (246, 247).

tNOX reduction reestablished non-cancer phenotypes, such as decreased migration, and amplified sensitivity to stress-induced apoptosis. Accumulating evidence suggests that suppressing tNOX may improve patient prognosis (246, 248–250).

c-Myc was demonstrated to be an HCC initiating oncogene (251). C-Myc expression is associated with HCC progression and poor patient prognosis (26, 252). In HCC cells, SIRT1 induced the expression of oncogenic c-Myc, which in turn increased β-catenin mRNA and protein expression and amplified the transcription and expression of the β-catenin target genes survivin and cyclin D1. Therefore, SIRT1 overexpression promoted oncogenesis *via* c-Myc activation (39, 246) and protected against p53 induced apoptosis (26).

c-Myc was shown to increase SIRT1 through transcriptional and post-transcriptional regulation. The SIRT1-c-Myc axis impacts cellular growth; however, the result of this interaction is still controversial (253–255).

CONCLUDING REMARKS

HCC accounts for immense mortality rates worldwide and poses difficult therapeutic problems. The therapies used today are inefficient at managing late-stage disease or metastasis. A better understanding of the underlying mechanisms that promote HCC development, metastasis, and chemoresistance may enable the development of more efficient therapeutic protocols. SIRT1 mediates LCSCs stemness, HCC metastasis, and chemoresistance. Targeting SIRT1 either to hinder the progression and metastasis of HCC or to decrease LCSCs stemness may be a viable therapeutic option. Directly inhibiting SIRT1 *via* miRs, exogenous compounds, or combining

conventional chemotherapeutics with tumor-selective SIRT1 inhibitors may improve treatment outcomes. However, a better understanding of the biology of SIRT1 in HCC is needed in order to efficiently inhibit related pathways and constrain HCC development.

AUTHOR CONTRIBUTIONS

IB-N and CC designed the study and approved the final version of the manuscript. MF, A-AG, and DG wrote the article, while CI and AI selected the most relevant articles to be included in the paper. The figures were created by MF and DG and tables were made by A-AG.

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Antidiabetic, Antihyperlipidemic, Antioxidant, Anti-inflammatory Activities of Ethanolic Seed Extract of *Annona reticulata* L. in Streptozotocin Induced Diabetic Rats

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Annona reticulata L. (Bullock's heart) is a pantropic tree commonly known as custard apple, which is used therapeutically for a variety of maladies. The present research was carried out to evaluate the possible protective effects of *Annona reticulata* L. (*A. reticulata*) ethanolic seed extract on an experimentally induced type 2 diabetes rat model. Male Albino Wistar rats were randomly divided into five groups with six animals in each group viz., control rats in group I, diabetic rats in group II, diabetic rats with 50 and 100 mg/kg/bw of ethanolic seed extract of *A. reticulata* in groups III and IV, respectively, and diabetic rats with metformin in group V. Treatment was given for 42 consecutive days through oral route by oro-gastric gavage. Administration of *A. reticulata* seed extract to diabetes rats significantly restored the alterations in the levels of body weight, food and water intake, fasting blood glucose (FBG), insulin levels, insulin sensitivity, HbA1c, HOMA-IR, islet area and insulin positive cells. Furthermore, *A. reticulata* significantly decreased the levels of triglycerides, cholesterol, LDL, and significantly increased the HDL in diabetic rats. *A. reticulata* effectively ameliorated the enzymatic (ALT, AST, ALP, GGT) and modification of histopathological changes in diabetic rats. The serum levels of the BUN, creatinine levels, uric acid, urine volume, and urinary protein were significantly declined with a significant elevation in CCr in diabetic rats treated with *A. reticulata*. MDA and NO levels were significantly reduced with an enhancement in SOD, CAT, and GPx antioxidant enzyme activities in the kidney, liver, and pancreas of diabetic rats treated with *A. reticulata*. Diabetic rats treated with *A. reticulata* have shown up-regulation in mRNA expression levels of nuclear factor erythroid 2-related factor 2 (Nrf2), NAD(P)H:quinone oxidoreductase 1 (NQO1), Heme oxygenase-1 (HO-1) and protein expression level of Nrf2 with diminution in Keap1 mRNA expression level in pancreas, kidney, and liver. From the outcome of the current results, it can be inferred that seed extract of *A. reticulata* exhibits a protective effect in diabetic rats through its anti-diabetic, anti-hyperlipidemic, antioxidant and anti-inflammatory effects and could be considered as a promising treatment therapy in the treatment of diabetes mellitus.

Keywords: ***Annona reticulata*, kidney, liver, pancreas, oxidative stress, inflammation**

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by the presence of high levels of glucose in blood that occurs either due to insulin's deficiency or malfunction (1, 2). Generally, people with diabetic condition encounter uncountable misery and devastating complications that may lead to morbidity and mortality (3). As per recent estimates by International Diabetes Federation, 5 million deaths were recorded due to diabetes (3). Earlier, it has also been reported that more than 2.5% of the world's total population suffer from DM (4). This increased prevalence rate, along with debilitating complications, warrants an urgent need to search for effective treatment strategies. Hyperglycemia-induced oxidative stress in terms of an increased generation of reactive oxygen/nitrogen species (ROS/RNS) and suppression of antioxidant defenses, such as SOD, CAT, GPx, play vital roles in the pathogenesis of DM (5, 6). This loss of balance in ROS/RNS and antioxidant defense mechanism may make tissues more vulnerable to oxidative stress that further aggravates the complications of DM. To negate the oxidative stress, cells are equipped with redox sensitive transcription factor Nrf2 to provide cellular protection (7). Earlier, it has also been reported that cellular redox homeostasis occurs primarily at the transcriptional level through the regulation of Nrf2/Keap1/ARE pathway (8, 9). It not only acts as a critical upstream regulator of global antioxidant response but also regulates the genes involved in inflammatory reaction (8, 10). Previously, it was reported that sustained oxidative stress through suppression of Nrf2 signaling pathway directs the overproduction of pro-inflammatory cytokines and chemokines, which activates the transcription factor NF-κβ that leads to chronic inflammation (11). Earlier, Pedruzzi et al. (12) reported that impairment of Nrf2 and its regulated genes results in systemic overload of oxidative stress and inflammation. Hence, the Nrf2/Keap1/ARE pathway represents an essential target to treat a broad spectrum of oxidative stress-mediated diabetic complications (8).

In addition to hyperglycemia-induced oxidative stress and inflammation, hyperlipidemia is another common feature that further progresses the diabetic complications (13). Further, the deterioration of liver and kidney functionality was evident from alterations in liver and kidney enzymatic and functional markers with deteriorated morphology in diabetic rats (14, 15).

Among the various treatment strategies, diet therapy, pharmacotherapy, and insulin therapy are the main treatment options available to control diabetes, in addition to wide range of glucose lowering drugs which exert their hypoglycemic effects through various mechanisms (16). However, these treatment options have not gained much significance as these treatment strategies are commonly associated with disadvantages, such as drug resistance, side effects, and toxicity. Hence, regardless of the presence of these hypoglycemic pharmacological drugs, supplementation of herbal based drugs to treat DM is now a promising and novel treatment strategy due to its safe and non-toxic nature (17).

Earlier, various herbal medicinal plants and herbs, such as *Averrhoa bilimbi* Linn. (18), *Potentilla discolor* Bunge (19), and *Semecarpus anacardium* Linn., which contain considerable amounts of antioxidant and anti-lipidemic components, have been found to be helpful in the management of DM and its associated complications. Considering the antioxidant and anti-lipidemic protective principles from herbal medicinal plants to fight DM, the present study is focused on *Annona reticulata* L.

The *Annona reticulata* belongs to the family Annonaceae and more than 100 different species of Annona genus have been identified (20). It is a traditional plant, commonly known as Bullock's heart and it has been used to treat various disorders such as epilepsy, cardiac problems, dysentery, worm infestations, bacterial infections, hemorrhage, dysuria, fever, and ulcers (21). The locals of Philippines, India and some other countries have claimed that this plant was traditionally used as anti-inflammatory, anti-stress, and anti-helminthic medications (22). Therefore, we chose to study the anti-inflammatory properties using a diabetic rat model to relate the pharmacological significance with the ethnobotanical claims by locals. Further, studies carried out using extracts of different parts of the plant have been reported to have anti-cancer (23), anti-inflammatory (24), anti-oxidant (25), hypoglycemic (26), analgesic (27), and anti-ulcerative effects (28), as well as wound healing activity (29).

Considering the diabetes-mediated complications and protective principles of *A. reticulata,* this study was performed to determine the anti-diabetic, anti-hyperlipidemic, antioxidant, and anti-inflammatory actions of *A. reticulata* seed in diabetic rats. The study also broadened its scope by studying the role of Nrf2/Keap1 molecular pathway in modifying the effects of *A. reticulata*.

MATERIALS AND METHODS

Chemicals

Streptozotocin was obtained from Sigma Chemicals, St. Louis, MO, USA. ELISA kits (NF-kβ, IL-1β, and IL-6) were procured from Abcam, UK. All other chemicals and solvents used were of analytical grade and procured from Sigma Chemicals, St. Louis, MO, USA. All primary antibodies used in this study were purchased from Santa Cruz Biotechnology, USA.

Preparation of Extract

The plant materials were bought from a local market and rinsed with tap water to clean from extraneous materials. The seeds of *A. reticulata* were dried under shade at room temperature, crushed by a mechanical grinder and were sieved through 40 mesh. The pounded materials were extracted with ethanol (95%) using Soxhlet extraction apparatus. The extract was concerted under reduced pressure. Thus, the ethanol free semi-solid mass gained was used for further studies.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

GC-MS analysis of the seeds of *A. reticulata* was performed using a GC-MS-QP2010 (Shimadzu, Japan), comprising of an AOC-20s headspace sampler and an AOC-20i autoinjector with a MS analysis capillary column (30 mm length × 0.25 mm diameter and 0.25 μm film thickness). Injector temperature was 250°C (Split injection mode). The temperature program was as follows:

80°C (3 min) and then with an increase of 10°C/min to 280°C. Carrier gas used was pure (99.999%) Helium (40.5 cm/s linear velocity) and constant column flow was 1.21 ml/min (total flow of 16.3 ml/min). Finally, the components of the seed extract were identified based on GC retention time and MS interpretation by matching spectra with NIST library.

Experimental Animals

Male healthy rats (150–250 g body weight) of Wistar strain were obtained from Central Animal House of our Institute and used for the study. The animals were housed for a week under standard conditions (12 h dark: 12 h light cycle, 50–60% relative humid and room temperature) before starting the actual experiments. This study was carried out as per recommendations from animal ethical committee of Changzhi Medical College by following the guidelines of National Institute of Health. The animal ethical committee of our institute approved the protocol. The rats were fed with normal rat feed and water *ad libitum*. All the experiments were done at regular time points.

Induction of Type 2 Diabetes Mellitus

For the induction of type 2 diabetes mellitus, overnight fasted rats were injected with nicotinamide (NA; 110 mg/kg; dissolved in saline) to minimize the streptozotocin (STZ) induced pancreatic β-cell damage. Fifteen minutes later, STZ (55 mg/kg) dissolved in 0.1 M citrate buffer (pH:4.5) injected intraperitoneally (i.p.) for the induction of type 2 diabetes (30). After 7 days of induction, the fasting blood sugar levels (FBG) were determined by glucometer and the rats with FBG level >250 mg/dL were considered to be diabetic and were used in the study.

Experimental Design

Male Wistar Albino rats were divided into five groups. Rats in group I served as normal controls, while rats in group II-V served as diabetic rats. Diabetic animals in group III and IV were administered orally with 50 and 100 mg/kg bw of *A. reticulata* seed ethanolic extract, respectively, and group V diabetic animals were treated orally with standard drug metformin at the dosage of 1 mg/kg bw for 42 consecutive days. The simplified scheme of treatment is given in **Figure 1A**.

Oral Glucose Tolerance Test (OGTT) and Insulin Tolerance Test (ITT)

The OGTT was carried out 1 week before the end of experiment. Overnight fasted rats were administered with glucose (2 g/kg) orally after administration of extract. Blood samples were collected from tail vein at 0, 30, 60, 90, and 120 min after glucose administration. ITT was performed 48 h before the last day of experimental period. Overnight fasted rats were injected intraperitoneally with 0.75 U/kg insulin. The blood glucose levels were estimated at 0, 30, 60, 90, and 120 min after insulin injection.

Blood Glucose Level and Biochemical Parameters Measurement

After 42 days of experimental period, the animals were sacrificed under chloroform anesthesia. The serum was separated from the collected blood and used for biochemical parameters. Liver, kidney, and pancreas were quickly removed and rinsed with saline. Glucose level was estimated by using Glucometer (One Touch Horizon, Lifescan, Johnson and Johnson Company). Homeostatic model assessment (HOMA)-IR, an indicator of beta cell function and insulin resistance, was done by the method of Matthews et al. (31). HOMA-IR was calculated from the following formula: HOMA-IR = [Fasting glucose (mmol/L) × fasting insulin (mU/L)]/22.5. Insulin was assayed by the solid phase enzyme-linked immunosorbent assay (ELISA). Lipoproteins were fractionated by a dual precipitation technique of Wilson and Spiger (32) and high-density lipoprotein (HDL), low-density lipoprotein. Total cholesterol (TC) and triglycerides (TG) were measured using commercially available kits (Abcam, UK) following manufacturer's protocol. Glycosylated hemoglobin (HbA1C) were estimated by the method of Rao and Pattabiraman (33).

Lipid peroxidation levels were determined in the liver, kidney, and pancreatic tissues spectrophotometrically by measuring the content of thiobarbituric acid reactive product, malondialdehyde (MDA) at 535 nm according to the method of Garcia et al. (34). The results were expressed as nanomole of MDA formed per milligram protein. Superoxide dismutase (SOD) activity was measured by rate of inhibition of pyrogallol auto-oxidation on a spectrophotometer at 470 nm according to the method of Marklund and Marklund (35). SOD activity was expressed as units per milligram protein. Glutathione peroxidase (GPx) activity was estimated by the rate of NADPH oxidization spectrophotometrically at 340 nm following the method of Rotruck et al. (36). GPx activity was expressed as micrograms of reduced glutathione (GSH) consumed/ min/ mg protein. Catalase (CAT) activity was estimated by monitoring the decomposition of H_2O_2 spectrophotometrically at 240 nm following the method of Sinha (37). CAT activity was expressed as micromoles of H_2O_2 consumed/min/mg protein. Nitric oxide (NO) concentrations were measured using Griess reaction following the manufacturer's protocol (Sigma Aldrich, St. Louis, USA). Sodium nitrite solution was used as a standard to measure the nitrite concentrations and the absorbance was read at 540 nm. Serum enzymes alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transferase (GGT) levels were estimated using commercial kits (Stanbio Reagent kit, TX, USA) by following the manufacturer's instructions. Tissue protein concentrations were estimated using a Randox kit with an automated Randox Daytona analyzer (Randox Laboratories Ltd., USA) following protocols from the manufacturer.

Urea was measured by the method of Natelson et al. (38), uric acid was measured by the method of Eichhorn and Rutenberg (39) and creatinine was measured by the method of Owen et al. (40) for the evaluation of kidney damage.

Quantitative Reverse Transcription Polymerase Chain Reaction (PCR) Analysis

The extraction of total RNA in the samples was performed using a TRIzol® Reagent method, quantified using Nanodrop 2000, and reverse transcription was done with the help of DNA synthesis kit (PE Applied Biosystems, Foster City, CA, USA).

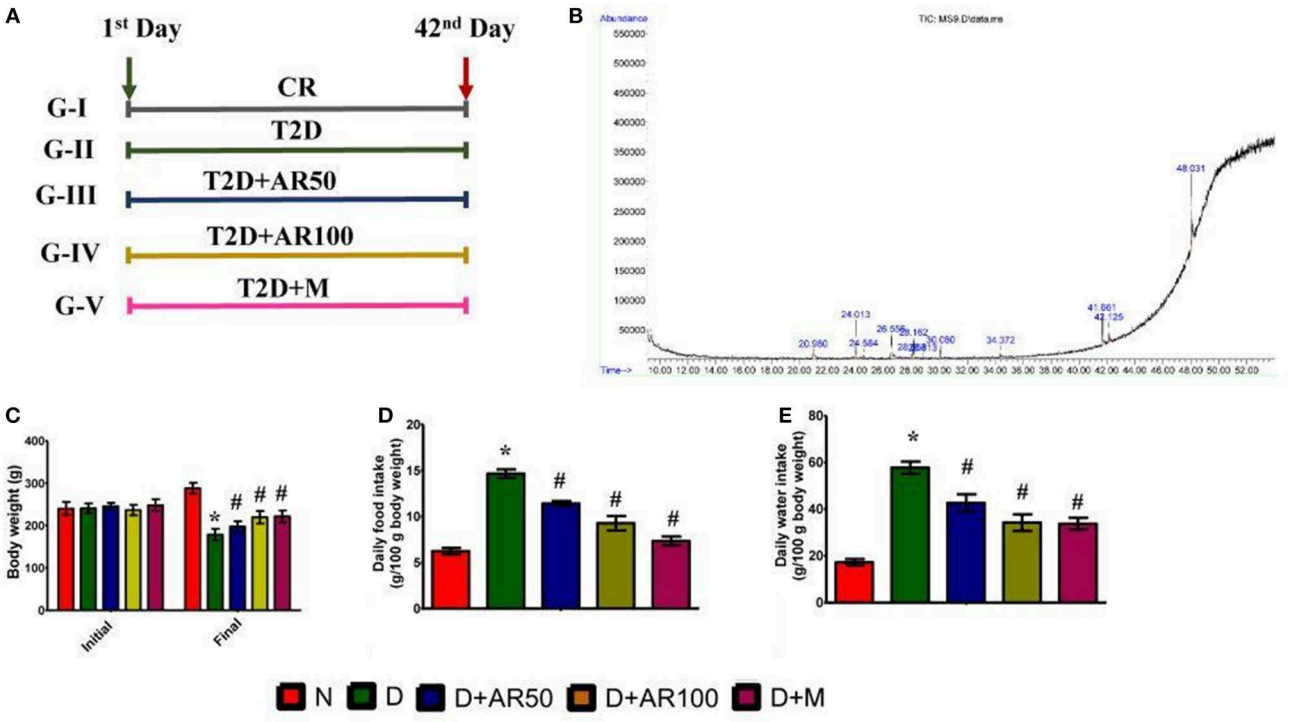

FIGURE 1 | (A) scheme of treatment, CR = Control rats; T2D = type 2 diabetic rats; T2D+AR50 = Type 2 diabetic rats + 50 mg/kg bw of *A. reticulata* seed ethanolic extract; T2D+AR100 = Type 2 diabetic rats + 100 mg/kg bw of *A. reticulata* seed ethanolic extract; T2D+M = Type 2 diabetic rats + Metformin; **(B)** GC-MS chromatogram of ethanolic seed extract of *Annona reticulata* L; Effect of *Annona reticulata* L on **(C)** Body weight **(D)** Daily food intake, and **(E)** Daily water intake. Each value is mean ± SD of six rats in each group. *significant compared to normal control group $p < 0.01$. #significant compared to diabetic group $p < 0.01$.

The following list of primers was used the analysis:
Nrf2: Forward - 5′AGCACATCCAGACAGACACCA3′,
Reverse - 5′TATCCAGGGCAAGCGACTC3′;
Keap1: Forward - 5′AGCAGGCTTTTGGCATCAT3′,
Reverse - 5′CCGTGTAGGCGAACTCAATTAG3′;
(NQO-1):Forward - 5′GAGAAGAGCCCTGATTGTACTG3′;
Reverse - 5′ACCTCCCATCCTCTCTTCTT3′;
HO-1):forward - 5′CTCCCTGTGTTTCCTTTCTCTC3′;
Reverse - 5′CTGCTGGTTTCAAAGTTCAG3′;
β-actin:Forward - 5′GGTATCCTGACCCTGAAGTA3′;
Reverse - 5′CACACGCAGCTCATTGTAGA3′.

RT-PCR was performed using the SYBR GREEN PCR master mix using Applied Biosystems Real-Time PCR System. The Relative quantification of mRNA expression was done using the $2^{-\Delta\Delta Ct}$ method. Ct values were normalized by β-actin to compare the expression among different groups.

Measurement of NF-κB p65, IL-1β, and IL-6

NF-κB p65, IL-1β, and IL-6 concentrations in various tissues of rat, such as pancreas, kidney, and liver, were measured using commercially available kits (as mentioned in Materials section) as per manufacturers' protocols.

Histological Analysis

The pancreas, liver, and kidney tissues from each group of animals were quickly removed after sacrificing the animals and washed immediately on ice cold saline. Each small portion of tissues was fixed in 10% neutral formalin fixative solution for carrying out histological studies. Tissues were embedded in paraffin after fixation and then solid sections were cut at 5 μm, which were then stained with haematoxylin and eosin. The photomicrographs of histological studies were captured.

Immunohistochemistry

Immunohistochemistry was carried out on formalin-fixed and paraffin-embedded sections of tissues. These sections were then deparaffinized, hydrated and washed in 0.1 M phosphate buffer saline (PBS). The presence of endogenous peroxidases was neutralized by treatment with H_2O_2 in the presence of methanol (Peroxidase acts as blocking solution), which was then washed in tris buffer saline (TBS). The imprecise binding of IgG was blocked with the help of normal goat serum, which was diluted (1:50) in 0.1% bovine serum albumin (BSA) with TBS for at least 30 min. The sections were incubated with the primary antibodies (Insulin, Nrf2, and NF-kβ), which were diluted and then left overnight. The sections were rinsed thrice each for at least 5 min in buffer and then incubated with biotinylated secondary antibodies diluted 1:1000 for a further 30 min, proceeding with washing. After this they were incubated further 30 min with Vectastain ABC kits (Avidin, Biotinylated horse radish peroxidase Complex) and washed for 10 min. Then the substrate, diaminobenzidine tetra hydrochloride (DAB) in distilled water,

was added and incubated for 5–10 min. The enzyme reaction was developed. The slides were lightly counterstained by haematoxylin to gain a good morphological identification of cells, and dehydrated by passing through ascending concentrations of alcohol then cleared by xylene. Permanent mounting media was used to put the cover slip. The substrate produces brown color at the immunoreactive sites.

Statistical Analysis

The results are presented as mean ± standard deviation (S.D). The differences between groups were analyzed using analysis of variance (ANOVA), followed by Bonferroni *posthoc* test. A value of $p < 0.05$ was considered statistically significant. Statistical software IBM SPSS (Version-18.0; Chicago, IL, USA) was used for analysis.

RESULTS

Effects of *A. reticulata* on Body Weight, Food, and Water Intake

The presence of bioactive compounds in the ethanolic extract of *A. reticulata* seeds was identified using GC-MS analysis. The GC-MS chromatogram (**Figure 1B**) expresses 11 peaks and has been identified after comparison of the mass spectra with NIST library (**Table 1**). The retention time, molecular formula, weight, and the percentage content in the seed extract were given. On the basis of these data, it was determined that 2,3-Dihydrobenzofuran (7.910%), Deconoin acid ethyl ester (14.730%), 2,3-Dimethoxy-succinicacid dimethyl ester (4.021%), 3-Hexadecyne (13.035%), Allo-Aromadendrene (1.970%), Allo-Aromadendrene (6.739%), Megastigmatrienone (1.901%), Ar-turmerone (3.952%), Oleic acid (10.028%), Gentisic acid (8.496%), and 13-Docosenamide (23.190%) were present in the *A. reticulata* seed extract. Among all, 13-Docosenamide was present in abundant amounts and megastigmatrienone was present in low quantity.

Figure 1C is depicting the alterations in body weight of control and experimental rats. In comparison with control rats, there was a significant decrease in the body weights of diabetic rats. On the other hand, treatment of diabetic rats with *A. reticulata* seed extract (50 and 100 mg/kg bw) and metformin caused a significant increase in the body weights when compared to body weights of diabetic rats. Diabetic condition has resulted in a drastic increase in daily food (**Figure 1D**) and water intake (**Figure 1E**), which decreased significantly upon treatment with 50 and 100 mg/kg *A. reticulata* seed extract and metformin, when compared to diabetic rats.

Effects of *A. reticulata* on Insulin Resistance and Pancreatic Functions

There was a significant increase in weekly fasting blood glucose in diabetic rats which did not reduce till 42 days when compared to control rats. Whereas, 50 or 100 mg/kg *A. reticulata* seed extract and metformin treatment for 42 days significantly reduced the fasting glucose levels to near normal levels (**Figure 2A**).

The prime hallmark of DM i.e., levels of serum insulin were drastically declined in diabetic rats, while *A. reticulata* seed extract or metformin treatment to diabetic rats significantly improved the insulin levels, compared to that of same levels in diabetic controls (**Figure 2B**).

Figure 2C shows the glucose tolerance as measured by OGTT. Control rats showed an increase in blood glucose levels by 30 min, which reverted to normal levels upon physiological hormonal control. In contrast, diabetic rats showed impaired glucose tolerance and did not revert to normal level. While, *A. reticulata* ethanolic seed extract treatment to diabetic rats significantly improved the glucose tolerance similarly to the standard drug. This finding was further evident by insulin tolerance test as shown in **Figure 2D**.

Hemoglobin, the oxygen carrier of blood, is glycated by blood glucose and the measurement of glycated hemoglobin (HbA1c) portrays the average blood glucose levels. The value below 6.0 is considered to be normal and above 6.5 is considered to be diabetic. In the present study, diabetic rats showed high HbA1c i.e., above 6.5, while this was lowered on treatment with *A. reticulata* seed extract or metformin. This may be due to the fact that *A. reticulata* seed extract or metformin lowered the blood glucose levels and, thus, the process of glycation is suppressed (**Supplementary Figure 1**).

The results also showed a drastic increase in HOMA-IR in diabetic rats, indicating the insulin resistance, while this was decreased on treatment with 50 or 100 mg/kg *A. reticulata* seed extract or metformin (**Figure 2E**).

A significant increase in pancreas index of diabetic rats was observed when compared with normal rats, which resulted in a significant decline in pancreas index upon treatment with either *A. reticulata* ethanolic seed extracts or metformin, compared to diabetic rats (**Figure 2F**).

Islets of Langerhans are the endocrine patches of pancreas, wherein alpha cells produce glucagon, beta cells produce insulin and delta cells produce somatostatin. Glucagon and insulin play vital roles in glucose homeostasis. The H&E staining of pancreas depicted the morphology of the pancreas, which shows that diabetic rats presented with small Islets of Langerhans with loss of shape, while 50 or 100 mg/kg *A. reticulata* seed extract or metformin treatment restored the shape and size of islet cells (**Figure 2G**).

The measurement of islet area is important in assessing the anti-diabetic effect of any drug. In the present study, there was a significant decrease in the islet area in diabetic rats, showing the destruction of beta cells that produce insulin. Islet area was significantly increased upon treatment with 50 or 100 mg/kg *A. reticulata* seed extract or metformin (**Supplementary Figure 2**). IHC of pancreas revealed the presence of less insulin in diabetic rats, while the treatment of diabetic rats with 50 or 100 mg/kg *A. reticulata* seed extract or metformin caused an increase in insulin (**Supplementary Figure 3**). The results were further supported by the presence of fewer insulin immunopositive sites in diabetic pancreas, however the treatment of 50 or 100 mg/kg *A. reticulata* seed extract or metformin significantly improved the insulin immunopositive sites in diabetic rats (**Supplementary Figure 4**).

Effects of *A. reticulata* on Lipid Profiles

Diabetes is often associated with alterations in lipid metabolism and hence lipid profile was studied in the present study.

TABLE 1 | GC–MS data of the different compounds present in ethanolic extract of *Annona reticulata*.

S. No	RT	Name of compound	Molecular formula	MW	Peak area %
1	20.978	2,3-Dihydrobenzofuran	C_8H_8O	120	7.910
2	24.015	Deconoin acid, ethyl ester	$C_{12}H_{24}O_2$	200	14.730%
3	24.581	2,3-Dimethoxy-succinicacid dimethyl ester	$C_8H_{14}O_6$	206	4.021%
4	26.552	3-Hexadecyne	$C_{16}H_{30}$	222	13.035%
5	28.054	Allo-Aromadendrene	$C_{15}H_{24}$	204	1.970%
6	28.157	Allo-Aromadendrene	$C_{15}H_{24}$	204	6.739%
7	28.814	megastigmatrienone	$C_{13}H_{18}O$	190	1.901%
8	30.082	Ar-turmerone	$C_{15}H_{20}O$	216	3.952%
9	41.663	Oleic acid	$C_{18}H_{34}O_2$	281	10.028%
	42.123	Gentisic acid	$C_7H_6O_4$	370	8.496%
10	48.031	13-Docosenamide, (Z)-	$C_{22}H_{43}NO$	337	23.190%

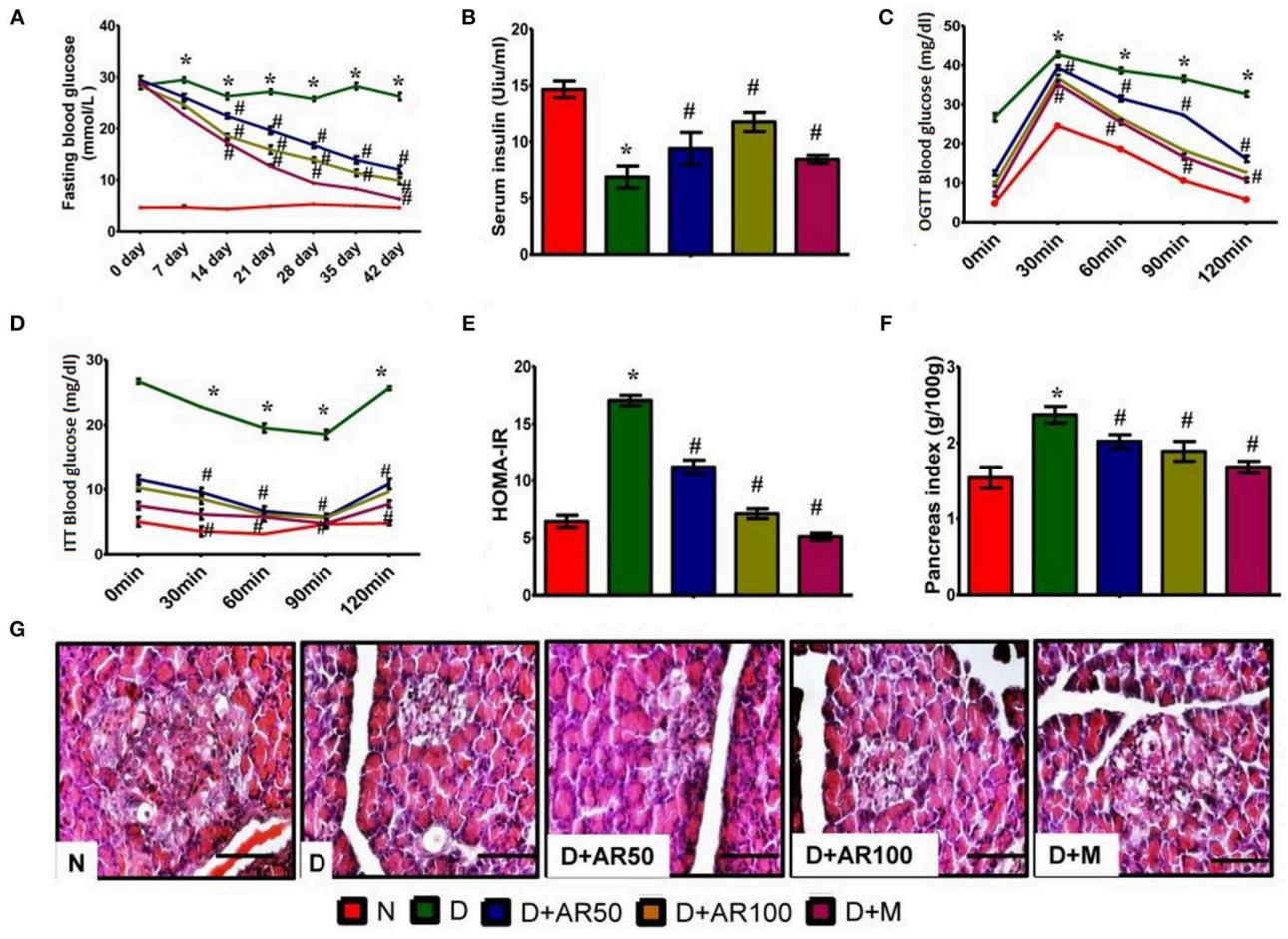

FIGURE 2 | Effect of *Annona reticulata* L on **(A)** fasting blood glucose levels **(B)** serum insulin levels **(C)** Oral Glucose Tolerance Test (OGTT) **(D)** insulin tolerance test (ITT) **(E)** HOMA-IR **(F)** pancreas index **(G)** pancreas histology. Each value is mean ± SD of six rats in each group.*significant compared to normal control group $p < 0.01$. #significant compared to diabetic group $p < 0.01$.

The results showed that significantly increased levels of total cholesterol (**Figure 3A**), triglycerides (**Figure 3B**), and LDL (**Figure 3C**) with significantly decreased levels of HDL (**Figure 3D**) were observed in diabetic rats compared to control rats. Moreover, 50 or 100 mg/kg *A. reticulata* seed extract or metformin treatment to diabetic rats for 42 days significantly improved the above mentioned lipid metabolism biomarkers.

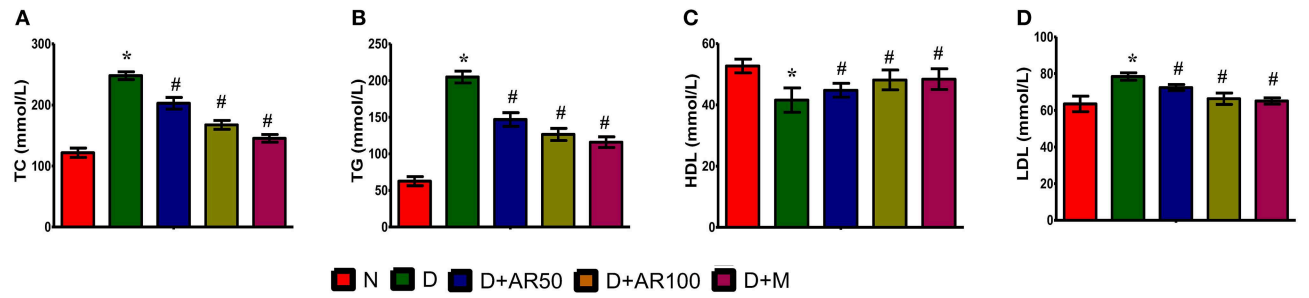

FIGURE 3 | Effect of *Annona reticulata* L on **(A)** Total cholesterol (TC) **(B)** Triglycerides (TG) **(C)** High-density lipoprotein (HDL) and **(D)** Low-density lipoprotein (LDL). Each value is mean ± SD of six rats in each group.*significant compared to normal control group $p < 0.01$. #significant compared to diabetic group $p < 0.01$.

Effect of *A. reticulata* on Kidney Histology and Function

Diabetic nephropathy is a common complication associated with kidney as this organ is highly susceptible to high glucose concentrations. As shown in **Figure 4A**, the histopathological sections of kidney in diabetic rats presented with distorted glomerular morphology, indicating kidney damage. On the other hand, 50 or 100 mg/kg *A. reticulata* seed extract or metformin treated diabetic rats showed an improvement in glomerular injury, in comparison with diabetic rats. The histopathological observations were supported by renal parameters, such as kidney index (**Figure 4B**), diameters of renal corpuscles (**Figure 4C**), glomerulus (**Figure 4D**), and Bowman's capsule (**Figure 4E**), in terms of deteriorated alterations in diabetic rats, which showed improvement in all these parameters after treatment with either *A. reticulata* seed extract or metformin. Also, urine volume (**Figure 4F**) and urinary protein excretion (**Figure 4G**), as a measure of polyuria and proteinuria, respectively, were significantly higher in diabetic rats compared to non-diabetic controls. Whereas, 50 or 100 mg/kg *A. reticulata* seed extract or metformin treatment to diabetic rats significantly decreased the urine volume and urinary protein, compared to diabetic controls. Further, serum creatinine (**Figure 4H**), uric acid (**Figure 4J**), and BUN (**Figure 4K**) were significantly increased with a significant decrease in CCr (**Figure 4I**) was observed, indicating a kidney incapable of eliminating waste products in diabetic rats compared to that of non-diabetic rats. Treatment of diabetic rats with 50 or 100 mg/kg *A. reticulata* ethanolic seed extract or metformin significantly reduced the serum creatinine, uric acid and BUN with significantly increased CCr, indicating *A. reticulata* seed extract or metformin ameliorated the renal injury.

Effect of *A. reticulata* on Liver Histology and Function

Liver is the major site of glucose metabolism where glycogenolysis takes place. The histopathological changes of liver in diabetic rats demonstrated distorted morphology with hepatocellular necrosis and vacuolization, while diabetic rats treated with 50 or 100 mg/kg *A. reticulata* seed extract or metformin displayed no major alterations and were near to normal liver structure (**Figure 5A**). The liver index (**Figure 5B**), steatosis (**Figure 5C**), hepatocyte ballooning (**Figure 5D**), and necroinflammation (**Figure 5E**) were significantly increased with significantly decreased glycogen content (**Figure 5F**) in diabetic rats. While, 50 or 100 mg/kg *A. reticulata* and metformin treatment to diabetic rats significantly improved all of these parameters when compared to that of diabetic rats.

The activities of ALT, AST, ALP, ACP, and GGT are considered to be reliable markers to assess liver function. In the present study, serum levels of ALT (**Figure 5G**), AST (**Figure 5H**), ALP (**Figure 5I**), ACP (**Figure 5J**), and GGT (**Figure 5K**) were significantly elevated in diabetic rats compared to non-diabetic rats, while diabetic rats treated with 50 or 100 mg/kg *A. reticulata* ethanolic seed extract and metformin for 42 days had much lower serum levels, which markedly alleviated liver damage.

Effect of *A. reticulata* on Nrf2/Keap-1 Pathway

Figure 6 illustrates the Nrf2 mRNA expression levels, Nrf2% immunopositive area, HO-1 mRNA expression levels, NQO-1 mRNA expression levels and Keap-1 mRNA expression levels in pancreas, kidney, and liver. The diabetic rats showed down-regulated expression in the mRNA expression levels of Nrf2 (**Figures 6A–C**), with increased Nrf2 immunopositive area (**Figures 6D–F**) in pancreas, kidney and liver. Furthermore, diabetic rats presented with down-regulated mRNA expression levels of HO-1, NQO-1 with an up-regulated expression of Kelch-like ECH associated protein 1 (Keap-1) mRNA expression levels in pancreas (**Figure 6G**), kidney (**Figure 6H**), and liver (**Figure 6I**). Treatment of diabetic rats with 50 or 100 mg/kg *A. reticulata* ethanolic seed extract and metformin for 42 days significantly elevated the mRNA expression levels of Nrf2, HO-1, NQO-1 with reduced Keap1 mRNA expression and Nrf2 immunopositive area in the pancreas, kidney, and liver.

Effect of *A. reticulata* on Anti-oxidant Enzymes

Figure 7 depicts activities of enzymatic antioxidants, such as SOD (**Figures 7A–C**), CAT (**Figures 7D–F**), and GPx (**Figures 7G–I**), along with the levels of MDA (**Figures 7J–L**) and NO (**Figures 7M–O**) in pancreas, kidney, and liver of control and experimental rats. The results revealed significantly increased levels of MDA and NO and significantly decreased activities of

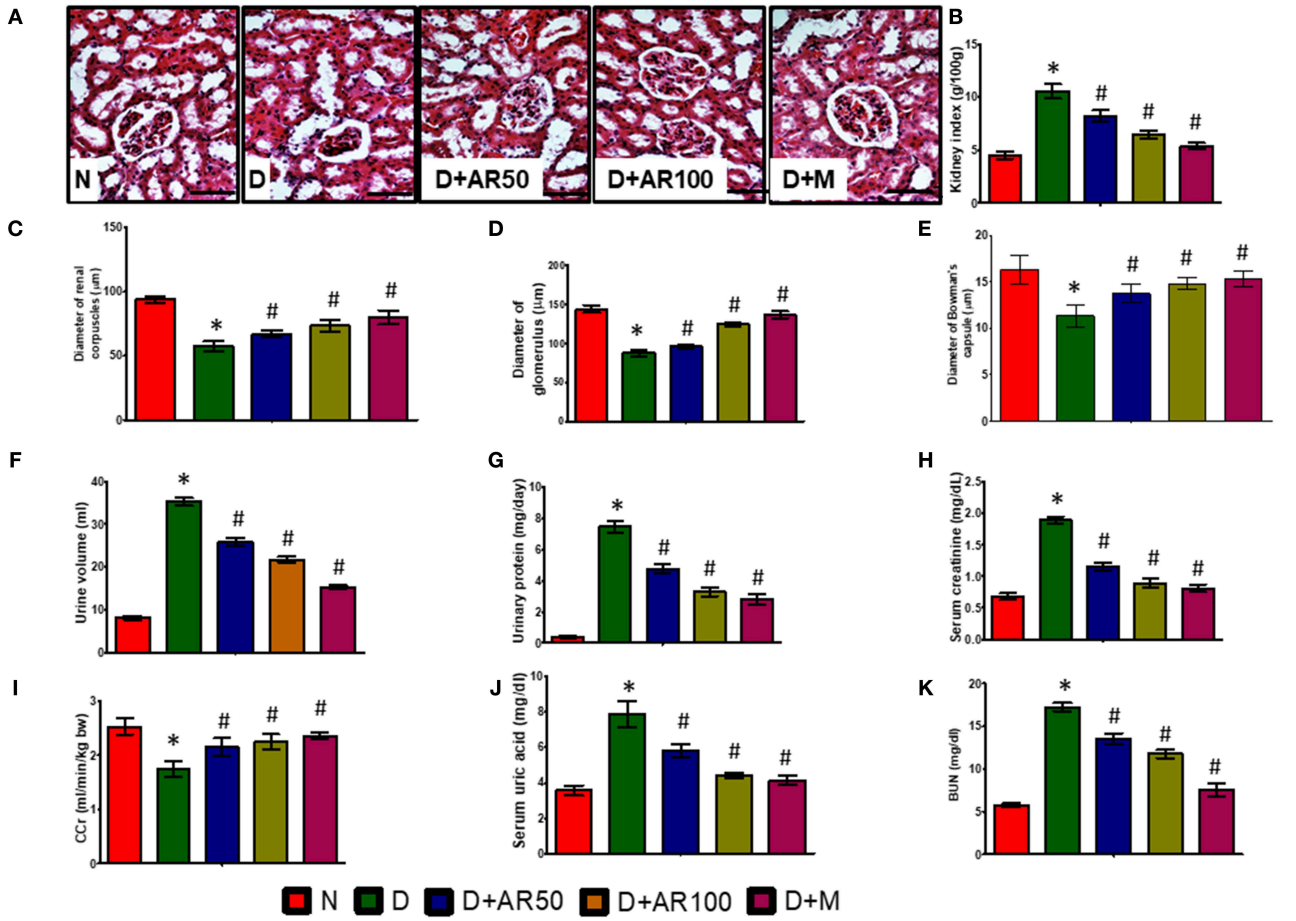

FIGURE 4 | Effect of *Annona reticulata* L on **(A)** Histology of kidney **(B)** kidney tissue somatic index **(C)** Diameter of renal corpuscles **(D)** Diameter of glomerulus **(E)** Diameter of Bowman's capsule **(F)** Urine volume **(G)** Urinary protein **(H)** Serum creatinine **(I)** Creatinine Clearance (CCr) **(J)** Serum uric acid **(K)** Blood urine nitrogen (BUN). Each value is mean ± SD of six rats in each group.*significant compared to normal control group $p < 0.01$. #significant compared to diabetic group $p < 0.01$.

SOD, CAT, and GPx in pancreas, kidney, and liver of diabetic rats. However, treatment of diabetic rats with 50 or 100 mg/kg *A. reticulata* ethanolic seed extract or metformin for 42 days significantly improved the oxidative status by means of inducing a significant decrease in levels of MDA and NO and a significant increase in activities of SOD, CAT, and GPx in pancreas, kidney, and liver.

Effect of *A. reticulata* on Pro-Inflammatory Parameters

ELISA results demonstrated the inflammatory reaction in pancreas (**Figures 8A,B**), kidney (**Figures 8C,D**), and liver (**Figures 8E,F**) of diabetic rats, as evidenced by increased levels of inflammatory cytokines IL-6 and IL-1β. Further, the immunohistochemistry results depicted an increase in expression of pancreatic, kidney, and liver NF-κB p65 in diabetic rats, when compared with that of the same expression in control rats (**Figures 8G–I**). On the other hand, diabetic rats administered with either 50 or 100 mg/kg *A. reticulata* ethanolic seed extract and metformin for 42 days resulted in reduced IL-6, IL-1β, and NF-κB p65 expression in pancreas, kidney, and liver, in comparison with IL-6, IL-1β, and NF-κB p65 expression in diabetic rats.

DISCUSSION

Diabetes mellitus is a metabolic disorder characterized by the presence of high levels of glucose in blood that occurs either due to insulin's deficiency or malfunction (41). In recent years, the prevalence of diabetes has been increasing at an alarming rate and is the reason for uncountable human misery and millions of deaths worldwide. This dramatic increase in prevalence of diabetes warrants an urgent need to search for effective treatment strategies. Although a wide range of glucose lowering drugs are currently available in the market, they have not gained much significance due to drug resistance, side effects and toxicity. Now-a-days, usage of plant-based drugs is gaining importance because of its safe to use and non-toxic nature. At this juncture, the present study was carried out to assess the possible protective effect of 50 or 100 mg/kg body weight ethanolic seed extract of

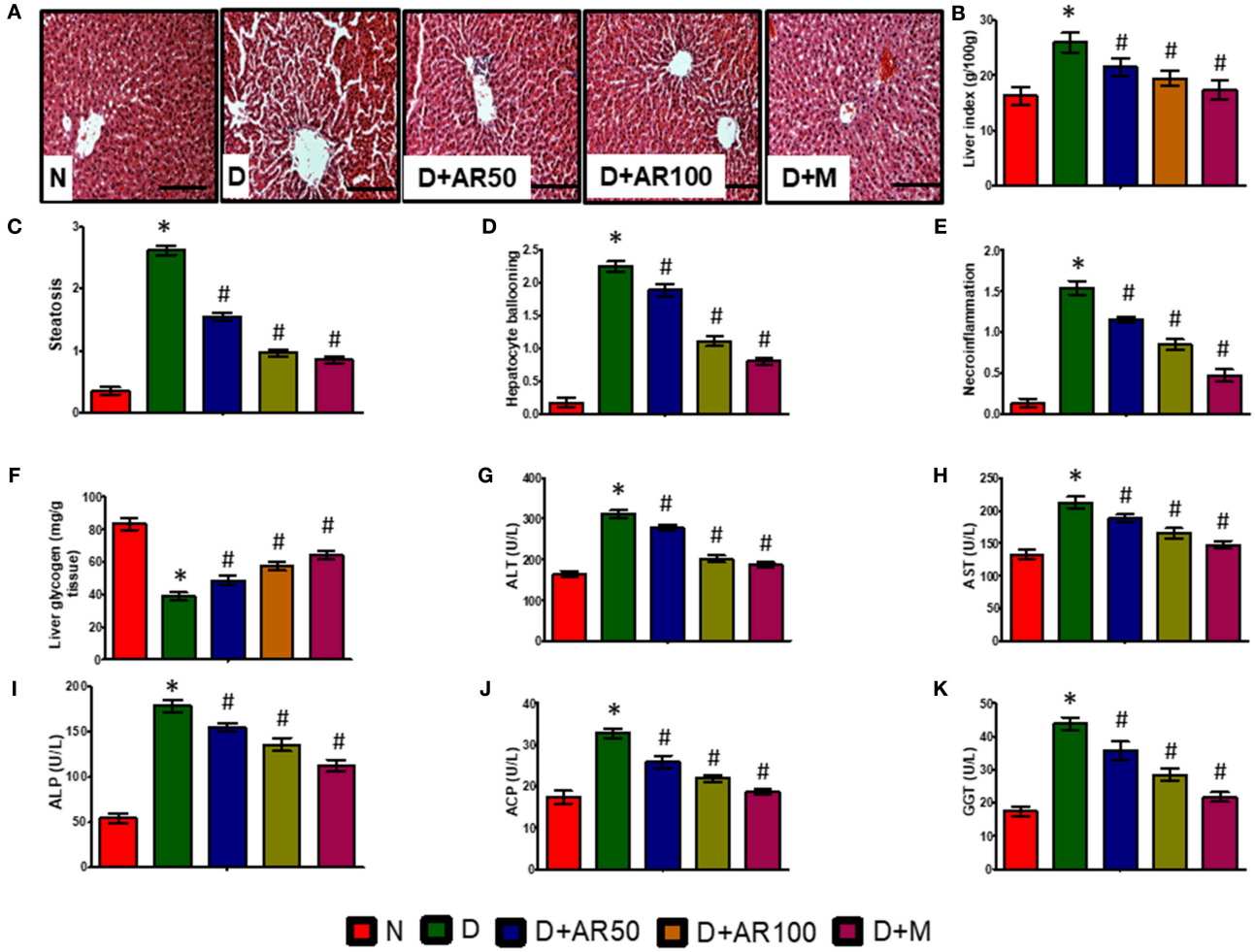

FIGURE 5 | Effect of *Annona reticulata* L on **(A)** Histology of liver **(B)** Liver tissue somatic index **(C)** Steatosis **(D)** Hepatocyte ballooning **(E)** Necroinflammation **(F)** Liver glycogen **(G)** Alanine aminotransferase (ALT) **(H)** Aspartate aminotransferase (AST) **(I)** Alkaline phosphatase (ALP) **(J)** Acid phosphatase (ACP) **(K)** Gamma-glutamyl transferase (GGT). Each value is mean ± SD of six rats in each group. *significant compared to normal control group $p < 0.01$. #significant compared to diabetic group $p < 0.01$.

A. reticulata on type 2 diabetes rat model. Type 2 diabetes was induced by intraperitoneal injections of STZ and nicotinamide. In this study, one group of rats was also treated with metformin, the first line treatment option to manage type 2 diabetes. The results revealed that administration of either 50 or 100 mg/kg ethanolic seed extract of *A. reticulata* is effective in reducing DM complications through its anti-diabetic, antioxidant, anti-hyperlipidemic, and anti-inflammatory properties.

The results showed a significant weight loss with a significant increase in food and water consumption in STZ-induced diabetic rats, which are mainly owing to high wasting of muscles, rather than utilizing glucose as an energy source (42). The results are in consonance with the earlier findings of Guo et al. (43). Treatment of diabetic rats with *A. reticulata* extract or metformin significantly increased the body weight with a concomitant decrease in food intake and water consumption. This may be because of the defending nature of the *A. reticulata* extract or metformin on glucose metabolism by improving glucose utilization in insulin target tissues and by decreasing the activities of the gluconeogenic enzymes, thereby preventing muscle wasting.

Persistent hyperglycaemia, along with impaired glucose tolerance, higher hemoglobin glycation, reduced glycolysis and increased gluconeogenesis are some of the characteristic features of diabetes mellitus. In this study, constant hyperglycemia, impaired glucose tolerance, declined serum insulin levels and a significant elevation in HbA1c and HOMA-IR were observed in diabetic rats. A large body of literature also supported the present findings of this study (44–46). Treatment of diabetic rats with the 50 or 100 mg/kg *A. reticulata* ethanolic seed extract or metformin significantly reduced the blood sugar concentrations, with a rise in insulin levels and improvement in glucose tolerance, HbA1c, HOMA-IR, and insulin sensitivity. The data was also supported by the findings of histopathological studies where *A.*

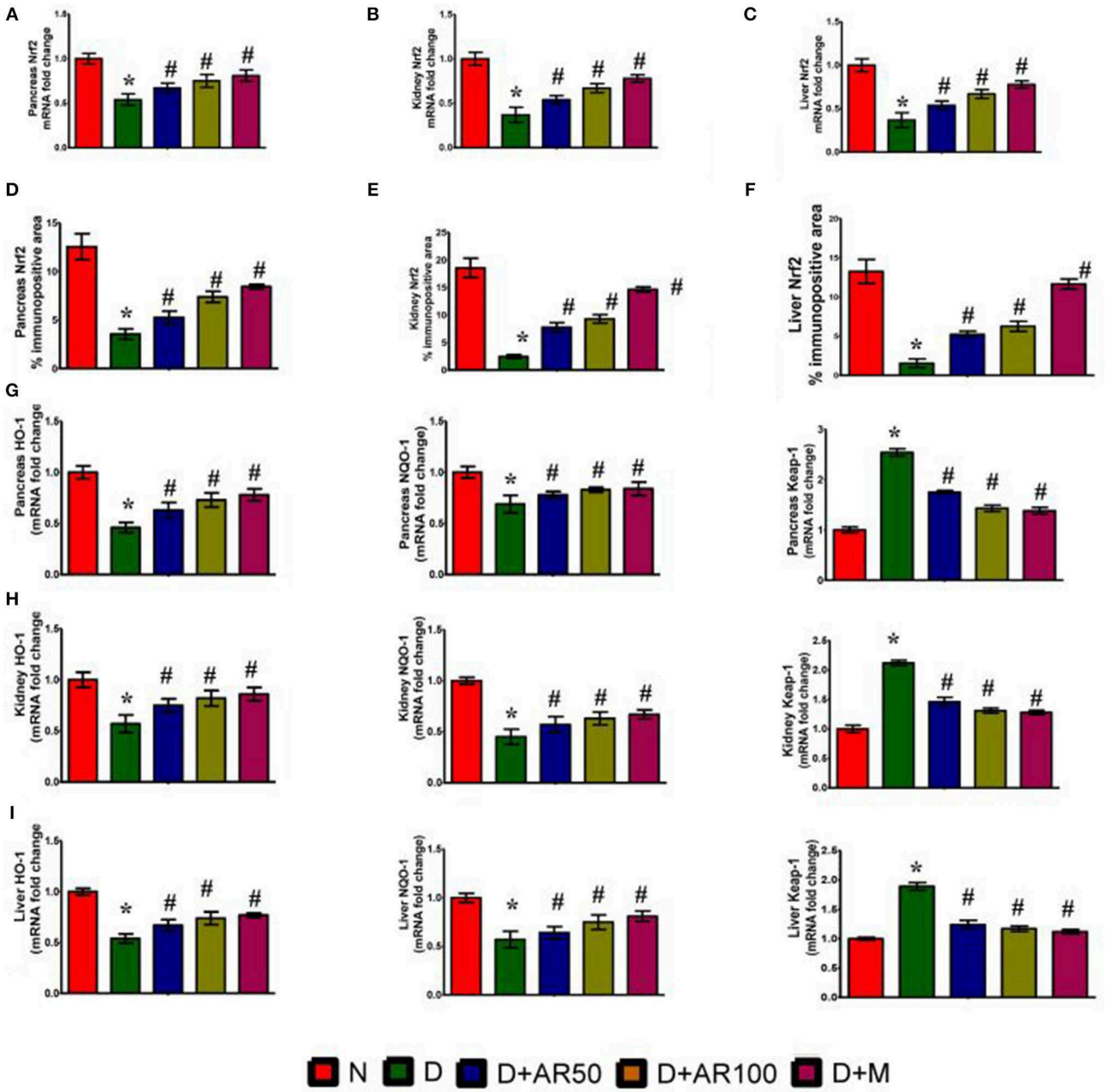

FIGURE 6 | Effect of *Annona reticulata* L on **(A)** Pancreas Nrf2 mRNA fold changes **(B)** Kidney Nrf2 mRNA fold changes **(C)** Liver Nrf2 mRNA fold changes **(D)** Pancreas Nrf-2% immunopositive area **(E)** Kidney Nrf-2% immunopositive area **(F)** Liver Nrf-2% immunopositive area **(G)** Pancreas HO-1, NQO-1, and Keap-1 mRNA fold changes **(H)** Kidney HO-1, NQO-1, and Keap-1 mRNA fold changes and **(I)** Liver HO-1, NQO-1, and Keap-1 mRNA fold changes. Each value is mean ± SD of six rats in each group.*significant compared to normal control group $p < 0.01$. #significant compared to diabetic group $p < 0.01$.

reticulata seed extract treatment to diabetic rats resulted in a significant increase in islet area and insulin positive cells. These results indicate the shielding effect of *A. reticulata* extract on the β-cells of the pancreas through its anti-diabetic potential and thereby restores the normal functioning of cells (47). The effects of 100 mg/kg *A. reticulata* seed extract and metformin are almost similar with insulin levels being higher in *A. reticulata* seed extract treated rats compared to metformin treated rats. The improvement in these metabolic alterations proved the effectiveness of *A. reticulata* seed extract in combating diabetic complications through its anti-diabetic nature.

Hyperlipidemia, a potential risk factor for cardiovascular disease, is a common complication associated with type 2 diabetes condition (48). Under normal physiological conditions, insulin activates lipoprotein lipase, which acts on triglycerides to separate fatty acids and glycerol. These fatty acids then undergo either

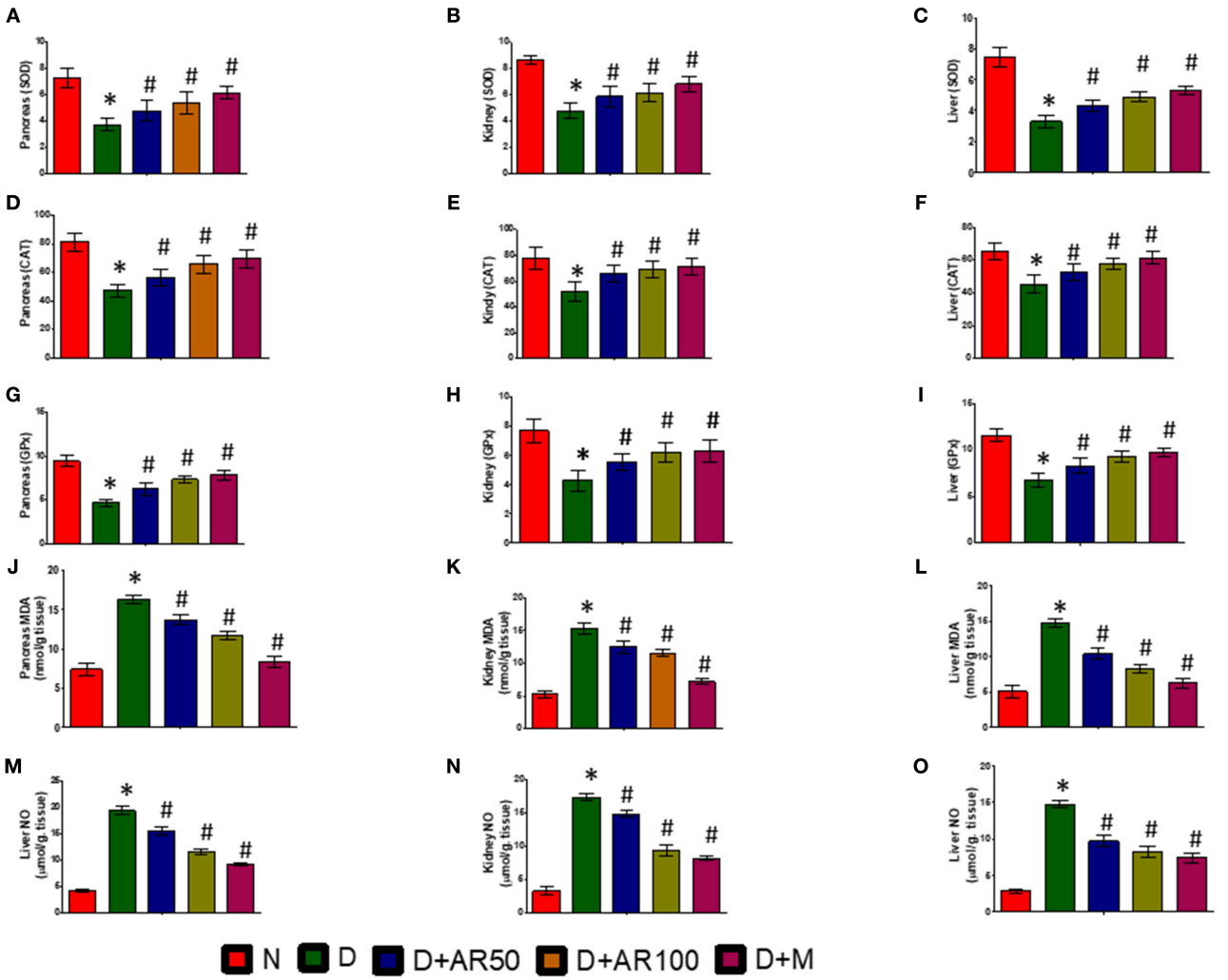

FIGURE 7 | Effect of *Annona reticulata* L on **(A)** Pancreas SOD **(B)** Kidney SOD **(C)** Liver SOD **(D)** Pancreas CAT **(E)** Kidney CAT **(F)** Liver CAT **(G)** Pancreas GPx **(H)** Kidney GPx **(I)** Liver GPx **(J)** Pancreas MDA **(K)** Kidney MDA **(L)** Liver MDA **(M)** Pancreas NO **(N)** Kidney NO **(O)** Liver NO. Each value is mean ± SD of six rats in each group.*significant compared to normal control group $p < 0.01$. #significant compared to diabetic group $p < 0.01$.

oxidation to generate energy or re-esterification to store it in body tissues (49). Under diabetic condition, insulin resistance and/or deficiency inactivate lipoprotein lipase, which leads to a condition of a hyperglyceridemia. A normal biological level of LDL plays a role in the transportation of cholesterol from liver to other tissues of the body, whereas in contrast, HDL transports endogenous cholesterol from body tissues to liver where they get metabolized and excreted. Increased levels of LDL, which deposits cholesterol in arteries, leads to coronary heart disease (50), while high levels of HDL avoids atherosclerosis by preventing cholesterol deposition (51). In this study, the induced type 2 diabetic condition in rats resulted in an increased level of total cholesterol, triglycerides, LDL, in association with reduced levels of HDL. The observed findings are consistent with other published reports (52, 53). The administration of 50 or 100 *A. reticulata* seed extract or metformin to diabetic rats markedly improved hyperlipidemic condition in rats by either improved secretion of insulin and/or enhanced insulin sensitivity.

The assessment of kidney functional markers gives valuable information regarding the functionality of kidneys. In this present investigation, the serum levels of the BUN, creatinine levels, uric acid, urine volume, and urinary protein were significantly elevated with a significant decline in CCr, indicating the deviation from normal kidney functioning to eliminate waste from kidneys in diabetic rats. The deteriorated alterations in kidney parameters, such as kidney index, diameters of renal corpuscles, glomerulus and Bowman's capsule, in association with distorted glomerular morphology, are an indication of kidney damage in diabetic rats. The results supported the findings of Giribabu et al. (15) who reported deteriorated alterations in kidney functional markers and histopathological observations in diabetic rats. However, 50 or 100 mg/kg *A.*

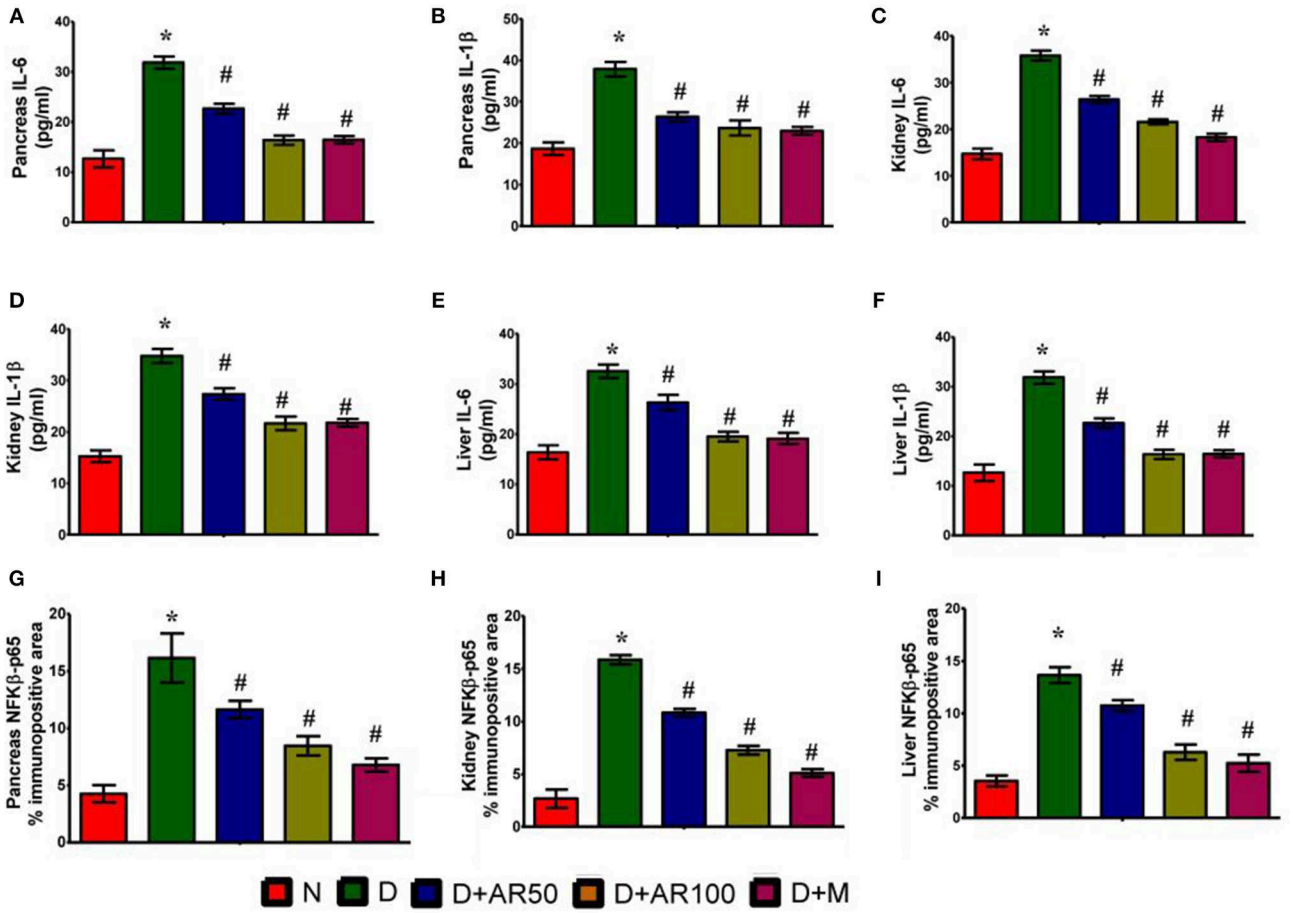

FIGURE 8 | Effect of *Annona reticulata* L on **(A)** ELISA pancreatic IL-6 levels **(B)** ELISA pancreatic IL-1β levels **(C)** ELISA kidney IL-6 levels **(D)** ELISA kidney IL-1β levels **(E)** ELISA liver IL-6 levels **(F)** ELISA liver IL-1β levels **(G)** Pancreas NF-Kβ p65% immunopositive area **(H)** Kidney NF-Kβ p65% immunopositive area **(I)** Liver NF-Kβ p65% immunopositive area.

reticulata or metformin treatment to diabetic rats markedly improved the kidney functions as evidenced by reversal of all kidney functionality markers, demonstrating the renoprotective of *A. reticulata* ethanolic seed extract.

The hepatic damage in the diabetic rats was evident from increased levels of liver enzymes, such as ALT, AST, ALP, ACP, and GGT in serum. The augmented levels of these marker enzymes are an indicator of hepatocellular damage, which allows these liver functional enzymes to escape from cytosol into the bloodstream (54). The present observations corroborate with the earlier findings of an increased serum levels of ALT, AST, ALP, ACP, and GGT in diabetic rats (55). The alterations in these marker enzymes were supported by deranged liver functional markers, such as steatosis, hepatocyte ballooning and necroinflammation, along with an increased liver index and decreased glycogen content in diabetic rats. Further, liver damage was evidenced by histopathological studies where distorted morphology with hepatocellular necrosis and vacuolization were observed in diabetic rats. The structural alterations in the present study might be due to observed alterations in liver markers (56). While, diabetic rats, treated with either 50 or 100 mg/kg *A. reticulata* and metformin, significantly prevented the histopathological changes and restored the liver enzymatic and functional markers, suggesting the role of *A. reticulata* seed extract in restoring the liver damage caused by diabetes.

The oxidative stress induced by reactive oxygen species is considered to be a common pathophysiology in diabetes-mediated complications (57). Oxidative stress in cells overwhelms when there is a loss of balance between oxidants and antioxidant defenses (58). In this study, the markers of oxidative status, such as MDA levels (measure of lipid peroxidation) and NO levels, were significantly elevated with a significant diminution in activities of SOD, CAT, and GPx in pancreas, kidney, and liver of diabetic rats. The present findings are in par with earlier observations (59). While the administration of 50 or 100 mg/kg ethanolic seed extract of *A. reticulata* to diabetic rats caused a significant improvement in all five markers of oxidative stress, suggesting the anti-oxidant effect of *A. reticulata* seed extract on diabetes-induced oxidative stress. Regulation of cellular redox homeostasis is mainly governed by an endogenous

anti-oxidative stress pathway, the Nrf2/Keap1/ARE pathway. Under normal biological conditions, cytoplasmic Nrf2 binds to Keap1, which restrict its access to nucleus to bind ARE, in order to transcriptionally activate Nrf2-target genes such as NQO1 and HO-1 (60). In the current study, diabetic rats have shown significantly down-regulated mRNA expression of Nrf2, NQO1, and HO-1, with a significant up-regulation in the Keap1 mRNA expression and Nrf2 protein expression in pancreas, kidney and liver. While 50 or 100 mg/kg ethanolic seed extract of *A. reticulata* to diabetic rats significantly attenuated all these gene and protein expressions to protect oxidative damage in pancreas, kidney, and liver through its anti-oxidant potential (60). The anti-oxidant effect of *A. reticulata* is almost similar to the effect observed by metformin.

Earlier research has pointed out the involvement of DM-induced oxidative stress in the activation of NF-kβ (61). NF-kβ is known to activate pro-inflammatory cytokines, which are considered to be important in an inflammatory reaction. In this study, an up-regulated expression of NF-κβ p65, along with elevated levels of NF-κβ p65, IL-6, and IL-1β in pancreas, kidney, and liver, were observed in diabetic rats. The results are in par with earlier observations (62, 63). After treatment of diabetic rats with 50 or 100 mg/kg *A. reticulata* ethanolic seed extract or metformin has resulted in a significant improvement in levels and expression of these inflammatory markers, suggesting the anti-inflammatory effect of *A. reticulata* ethanolic seed extract to protect diabetes-mediated complications.

Inflammation has a vital role in the progression of DM and is therefore related to increased insulin resistance and decreased response in insulin target tissues (64). NF-kβ and TNF-α are critical mediators of insulin resistance and beta cells dysfunction in the pancreas, contributing to the development of diabetes mellitus, and also anomaly in lipid metabolism (65). Persistent hyperglycaemia results in increased production of free radicals and other inflammatory cytokines, which activate NF-kβ (66). In normal conditions, NF-kβ is known to exist in an inactive state in the cytoplasm and then binds via p50 and p65 units to Ikβ, which then acts as an inhibitory protein (67). Upon stimulation, it becomes separated from Ikβ, and the active p65-NF-kβ translocates to the nucleus where it binds and activates the expression of pro-inflammatory cytokines (68, 69). Therefore, measurement of the accessibility of active p65-NF-kβ will serve as an important factor indicating the translocation of NF-kβ to the nucleus. Increased levels of the active form of NF-kβ, along with an increased level of inflammatory cytokines, were observed in liver, kidney and pancreatic tissues. Treatment of the diabetic animals with the *A. reticulata* seed extract resulted in a significant reduction in inflammatory cytokines, thereby increasing the insulin sensitivity in diabetic rats, suggesting a putative role of seed extracts of *A. reticulata* in alleviating diabetes mellitus through its anti-inflammatory effect.

In conclusion, the treatment of *A. reticulata* seed extract to diabetic rats significantly improved the metabolic alterations, dyslipidemia, oxidative, and inflammatory status through its anti-diabetic, anti-hyperlipidemic, anti-oxidative, and anti-inflammatory effects. Therefore, the seed extract of *A. reticulata* could be as an ideal treatment option for diabetes mellitus.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of ethical guidelines provided by the Animals Ethics Committee of Changzhi Medical College. The protocol was approved by the institute's animal ethical committee following the guidelines of National Institute of Health.

AUTHOR CONTRIBUTIONS

WW: data acquisition and manuscript preparation. YL: data acquisition and analysis. ZT: experimental design and manuscript preparation.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00716/full#supplementary-material

Supplementary Figure 1 | Effect of *Annona reticulata* L. on **(A)** HbA1c **(B)** Islets area/total pancreatic area (%). **(C)** Insulin % immunopositive area. **(D)** Immunohistochemistry of insulin in pancreas. Each value is mean ± SD of 6 rats in each group. *Significant compared to normal control group $p < 0.01$. #Significant compared to diabetic group $p < 0.01$.

Supplementary Figure 2 | Effect of *Annona reticulata* L. on **(A)** Immunohistochemistry of pancreatic Nrf2. **(B)** Immunohistochemistry of kidney Nrf.2 **(C)** Immunohistochemistry of liver Nrf2. Each value is mean ± SD of 6 rats in each group. *Significant compared to normal control group $p < 0.01$. #Significant compared to diabetic group $p < 0.01$.

Supplementary Figure 3 | Effect of *Annona reticulata* L. on **(A)** Immunohistochemistry of pancreatic NF-Kβ p65. **(B)** Immunohistochemistry of kidney NF-Kβ p65. **(C)** Immunohistochemistry of liver NF-Kβ p65. Each value is mean ± SD of 6 rats in each group. *Significant compared to normal control group $p < 0.01$. #Significant compared to diabetic group $p < 0.01$.

Supplementary Figure 4 | Effect of *Annona reticulata* L. on **(A)** ELISA pancreatic NF-Kβ p65 levels. **(B)** ELISA kidney NF-Kβ p65 levels. **(C)** ELISA liver NF-Kβ p65 levels. Each value is mean ± SD of 6 rats in each group. *Significant compared to normal control group $p < 0.01$. #Significant compared to diabetic group $p < 0.01$.

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Maternal Intake of n-3 Polyunsaturated Fatty Acids During Pregnancy Is Associated With Differential Methylation Profiles in Cord Blood White Cells

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A healthy diet during pregnancy is pivotal for the offspring health at birth and later in life. N-3 polyunsaturated fatty acids (n-3 PUFAs) are not endogenously produced in humans and are exclusively derived from the diet. They are pivotal for the fetus growth and neuronal development and seem beneficial in reducing the risk of cardiometabolic diseases and preventing later allergic disorders in the offspring by modulating the inflammatory immune response. In the present study, we investigated the association between maternal intakes of n-3PUFAs, profiled on maternal erythrocyte membranes at pregnancy term, and offspring DNA methylation on cord blood mononuclear cells in a sample of 118 mother–newborn pairs randomly drawn from the "Feeding fetus' low-grade inflammation and insulin-resistance" study cohort. N-3 PUFA content on erythrocyte membranes is a validated biomarker to measure objectively medium term intake of n-3 PUFAs. Based on distribution of n-3 PUFA in the whole cohort of mothers, we identified mothers with low (n-3 PUFA concentration <25th percentile), medium (n-3 PUFAs between 25th and 75th percentiles), and high n-3 PUFA content (>75th percentile). The HumanMethylation450 BeadChip (Illumina) was used for the epigenome-wide association study using the Infinium Methylation Assay. The overall DNA methylation level was not different between the three groups while there was significant difference in methylation levels at certain sites. Indeed, 8,503 sites had significantly different methylations between low and high n-3 PUFA groups, 12,716 between low and medium n-3 PUFA groups, and 18,148 between high and medium n-3 PUFA groups. We found differentially methylated genes that belong prevalently to pathways of signal transduction, metabolism, downstream signaling of G protein-coupled receptors, and gene expression. Within these pathways, we identified four differentially methylated genes, namely, MSTN, IFNA13, ATP8B3, and GABBR2, that are involved in the onset of insulin resistance and adiposity, innate immune response, phospholipid translocation across cell membranes, and mechanisms of addiction to high fat diet, alcohol, and sweet taste. In conclusion, findings of this preliminary investigation

suggest that maternal intake of n-3 PUFAs during pregnancy has potential to influence the offspring DNA methylation. Validation of results in a larger cohort and investigation of biological significance and impact on the phenotype are warranted.

Keywords: epigenetics, inflammation, insulin resistance, metabolic programming, pregnancy, polyunsaturated fatty acids

INTRODUCTION

Omega-3 polyunsaturated fatty acids (n-3 PUFAs) are not endogenously produced in humans but derived from diet. They play a pivotal role in the human health from conception through every stage of human development, maturation, and ageing. N-3 PUFAs are essential for neurodevelopment and modulation of inflammatory immune response, being protective against allergic disorders (De Giuseppe et al., 2014; Schindler et al., 2016).

Several lines of evidence suggest that individuals with a diet rich in n-3 PUFAs have lower incidence of cardiovascular disease and, in general, of chronic non-communicable diseases (Li, 2015; Abdelhamid et al., 2018).

The mechanisms underlying the protective effect of n-3 PUFAs from the intrauterine life to the adulthood are incompletely understood but are thought to include altered eicosanoid metabolism and subsequent changes in cell signaling, transcription factor activity, and gene expression. Evidence in animal models and humans suggests that n-3 PUFAs influence global DNA methylation patterns at any stage of life (Burdge and Lillycrop, 2014).

In the womb, n-3 PUFA supply to the fetus depends strongly on maternal consumption and metabolism, as well as on efficiency of the placental transport (Hornstra, 2000; Kilari et al., 2009). In controlled investigations, supplementation during pregnancy with docosahexaenoic (DHA) and/or eicosopentanoic (EPA) acids was associated with changes in the offspring methylation levels of various genes, including genes coding for inflammatory mediators (Lee et al., 2013; Amarasekera et al., 2014; Lee et al., 2014; Van Dijk et al., 2016). Animal and human studies demonstrated that the *in utero* environment shapes the offspring epigenome. The maternal diet and the hormonal milieu during gestation are claimed up- or down-modulating DNA methylation at global and region levels (Hochberg et al., 2011; Dominguez-Salas et al., 2014). The epigenetic effects of the intrauterine environment affect the phenotype throughout childhood and adulthood (Lee 2015). In an epigenome-wide association study (EGWAS), a specific pattern of CpG methylation in cord blood was significantly associated with birth weight in a large cohort of Norwegians (Engel et al., 2014).

A couple of studies reported that n-3 PUFA supplementation during childhood modulates DNA methylation at this age (Lind et al., 2015; Voisin et al., 2015).

Studies have investigated epigenetic effects of supplementation with selected n-3 PUFAs in pregnancy (Lee et al., 2013; Amarasekera et al., 2014; Lee et al., 2014; Lind et al., 2015; Voisin et al., 2015). No study has investigated the effect of n-3 PUFA deriving exclusively from the maternal diet during pregnancy in absence of supplementation. It is unknown whether the maternal dietary intake of n-3 PUFAs influences shaping of the epigenome during the intrauterine period when it has the greatest plasticity. It is also unclear whether different genomic regions show varying sensitivities to n-3 PUFA content during this vulnerable period.

Aim of the present study was to investigate the association between maternal n-3 PUFA erythrocyte content and DNA methylation profiles of offspring cord blood white cells at birth using whole-genome DNA methylation approaches. The study was designed as "hypothesis generating" investigation to identify candidate genes differently methylated in association with maternal n-3 PUFAs.

MATERIAL AND METHODS

Study Design

The primary aim of the "Feeding fetus' low-grade inflammation and insulin-resistance" cohort study was to investigate the association between maternal intake of lipids during pregnancy and offspring inflammation and insulin resistance at birth (Cinelli et al., 2016). Lipid content of erythrocyte membranes would reflect the maternal dietary fatty acid consumption in the prior 120 days before erythrocyte collection. For a full description of the parent study, see Cinelli et al. (2016). Briefly, healthy pregnant women (N = 1,000) were enrolled at the first trimester and followed up throughout the pregnancy according to guidelines of the Italian Society for Gynecology and Obstetrics (www.sigo.it). Inclusion criteria were ages 18–39 years old, planned pregnancy with folic acid supplementation starting 1 to 3 months before the conception, weeks 7–10 of gestation, on-going folic acid supplementation, singleton pregnancy, no alcohol or medications, no systemic, chronic, or autoimmune disease, no previous diagnosis of gestational diabetes mellitus or miscarriage, no conception through ovulation induction or *in vitro* fertilization, and planned delivery at San Camillo Forlanini Hospital.

During pregnancy, nutritional counseling was provided to all the pregnant women participating to the study (45 min in person meeting with an expert dietician). Pregnant women were encouraged to consume 60g/day of proteins, 45–64% of daily calories from carbohydrates with approximately six servings of whole grain and three servings of fresh fruit daily and 20–35% of daily calories from fats. Three servings per week of fish, particularly salmon, sardines, and anchovies were recommended. Frequencies of food consumption were investigated by a food frequency questionnaire at the study entry and around weeks 13–15 and 23–26.

Pregnant women were also encouraged to exercise regularly (i.e., 45 min walking) (Rasmussen and Yaktine, 2009; Kominiarek and Rajan, 2016).

For the purpose of this ancillary study, we excluded from the cohort of 847 mother–newborn pairs, mothers with history of smoking (N = 93), gestational diabetes mellitus and/or obesity (N = 41), small (N = 49) or large for gestational age (N = 68) newborns, and/or with distress at birth (N = 19, Apgar score at 1 and/or 5 min <7). We calculated quartiles of maternal n-3 PUFAs expressed as ng% in the sample of 577 mothers. We identified the three groups of low n-3 PUFA (N = 144, n-3 PUFA concentration <25th percentile), medium (N = 269, n-3 PUFAs between 25th and 75th percentiles), and high n-3 PUFA content (N = 144, n-3 PUFAs >75th percentile). Then, by using STATA command "sample" (STATA 13.1; StataCorp LP), we randomly drawn, in equal number for male/female, a sample of 39 pairs out of 144 belonging to the low and the high n-3 PUFA groups and of 40 out of 269 from the medium n-3 PUFA group.

The feeding study and the ancillary studies were approved by the Ethical Committee of the Bambino Gesù Children's Hospital and the San Camillo Forlanini Hospital. The study conformed to guidelines of the European Convention of Human Rights and Biomedicine for Research in Children as revised in 2008. Parents or responsible guardians provided written informed consent. All measures were taken to ensure participants' confidentiality.

Newborns' Anthropometrics

Newborns' body weight, length, and head circumference were evaluated at birth according to standardized procedures. Standard deviation scores (SDS) for infant weight and height were calculated following the Italian Neonatal Study Chart (Bertino et al., 2010).

Samples Collection, Lipid Profiling

Maternal blood was withdrawn at fasting, 12–24 h before giving birth, during the pre-partum fetal monitoring. Blood was placed in ethylenediaminetetraacetic acid tubes. Erythrocyte membranes were isolated within 2 h after collection: plasma was separated by centrifugation (980 rpm, 18 min), whereas erythrocytes were added with acid citrate dextrose, washed with distilled water (10:1), and centrifuged (4,000 rpm, 5 min) four times. Erythrocytes were frozen immediately at −80°C and stored until lipid extraction. Briefly, erythrocyte fatty acids were measured by gas chromatography as described previously (Cinelli et al., 2016).

Erythrocyte n-3 PUFAs were expressed as percent weight fraction of the total amount of fatty acids quantified on erythrocyte membranes (Hodson et al., 2008).

DNA Extraction and Methylation Analysis

Cord blood samples (2.5 ml) were collected at birth by venipuncture from the placental portion of the umbilical cord immediately after clamping. White cells were isolated from cord blood. DNA was extracted using QIAMP Blood Mini Kit (Qiagen, Hilden, Germany). Genomic DNA was extracted, followed by bisulfite conversion (EZ_DNA methylation Kit, Zymo Research, D5002), and methylation analysis (Illumina Infinium Methylation Assay; HumanMethylation450 BeadChip) was used for the epigenome-wide association study performed according to a standardized protocol (http://www.gqinnovationcenter.com/index.aspx) . Arrays were verified for quality control and submitted to the Gene Expression Omnibus. Commercially available custom Methyl Profiler PCR array from Qiagen SABiosciences is used for confirmation of a representative number of hypo- and hypermethylated genes (http://www.sabiosciences.com/dna_methylation_custom_PCRarray.php) using the standard 2-DDCt. Methylation profiles were compared between different samples.

Glossary

In the text, the terms "probe" or "site" refer to any "CpG site," a punctual CpG methylation genomic site. CpG is shorthand for 5'-Cytosine-phosphate-Guanine-3', that is, cytosine and guanine separated by one phosphate group. Cytosines in CpG dinucleotides can be methylated to form 5-methylcytosines. A "region" is a genomic DNA portion, belonging to any of the following categories: "gene body region" (Ensembl genes), "gene promoter region" (promoter regions of Ensembl genes), or "CpG islands" (CpG island track of the UCSC Genome Browser). A CpG island is a region with a high frequency of CpG sites. Hence, we refer to differential mutilated probes/sites (DMPs) and to differentially methylated regions (DMRs), and more specifically to differentially methylated genes (DMGs) as differential methylated gene bodies regions.

Statistics and Differential Analysis

Descriptive statistics were presented as mean ± SD, median (interquartile range), or frequencies and percentages, as appropriate. Normality of the data distributions was tested by using Kolmogorov–Smirnov's test with Lilliefors correction.

R packages ("RnBeads," "methylumi," "minfi") were used for the statistical analysis and to explore the chance of a strong differential methylation pattern among the three groups of samples. The background was subtracted using the "noob" method. The signal intensity values were normalized using the SWAN normalization method (Triche et al., 2013), as implemented in the "minfi" R package. In the filtering analysis, 10,119 sites were removed because overlapped with known single nucleotide polymorphisms. Data quality was assessed by using principal component analysis (PCA) and multidimensional scaling (MDS) using both Manhattan and Euclidean distances to identify differentially methylated sites.

Raw methylation level was expressed using the standard beta value (β). β was the estimate of methylation level using the ratio of intensities between methylated and unmethylated alleles. β was between 0 and 1 with 0 being unmethylated and 1 fully methylated.

P-values were calculated using limma t-test and then combined for each region using a generalization of Fischer's method, the Makambi Weighted inverse chi-squared test (Makambi, 2003). Combined p-value is a good method to avoid multiple

comparison issue and increase statistical power. Calculating statistical comparisons on larger genomic regions rather than on simple CpGs allow neighboring CpGs with similar differences in DNA methylation and providing more robust results (Bock, 2012; Müller et al., 2019).

RdBeads R package provides a combined rank number, which it is not intended as a statistical significance test *per se* but can be useful (especially for plots, see **Figure 1**) to have a rapid overall idea of each comparison's relevance, both from the statistical significance and the magnitude of differential methylation point of view. It is because combined rank is calculated as a combination of the combined p-value, the between group differences of methylation means, and the quotient between group methylation means. The lower the combined rank, the more interesting the comparison is expected to be.

Statistical significance was set at combined P value <0.05. False discovery rate (FDR) statistics was applied to test for type I error in null hypothesis.

Pathway Enrichment Analysis

Metabolic pathways associated with low or high methylated genes were identified with targeted questions by using Reactome (Fabregat et al., 2016). Reactome pathways over-represented (enriched) in the submitted data derived from the statistical test (hypergeometric distribution) analysis. This test produced a probability score, which was corrected for FDR using the Benjamani–Hochberg method. Venn diagrams showed the overlapping between the overrepresented Reactome pathways and the related different methylated genes obtained (http://bioinformatics.psb.ugent.be).

RESULTS

Study Population and Global Differential DNA Methylation Across Groups

One-hundred eighteen newborns were enclosed in the study following the criteria described in the material and method section being born to mothers with low erythrocyte concentration of n-3 PUFAs (concentration <25°percentile), medium n-3 PUFAs (concentration ≥25° percentile and ≤75° percentile), and high n-3 PUFAs (concentration >75° percentile).

Newborns' anthropometrics and maternal concentrations of n-3PUFAs in the three groups were reported in **Table 1**.

Methylation analysis revealed no significant difference in the overall methylation profiles of the three groups by using PCA and MDS both at the Manhattan and the Euclidean distances. Next, we performed a differential methylation analysis among groups. We found differential methylations both at site (DMPs) and region levels (DMRs). In **Table 2**, we reported statistically significant DMPs and DMRs at comparison among the three

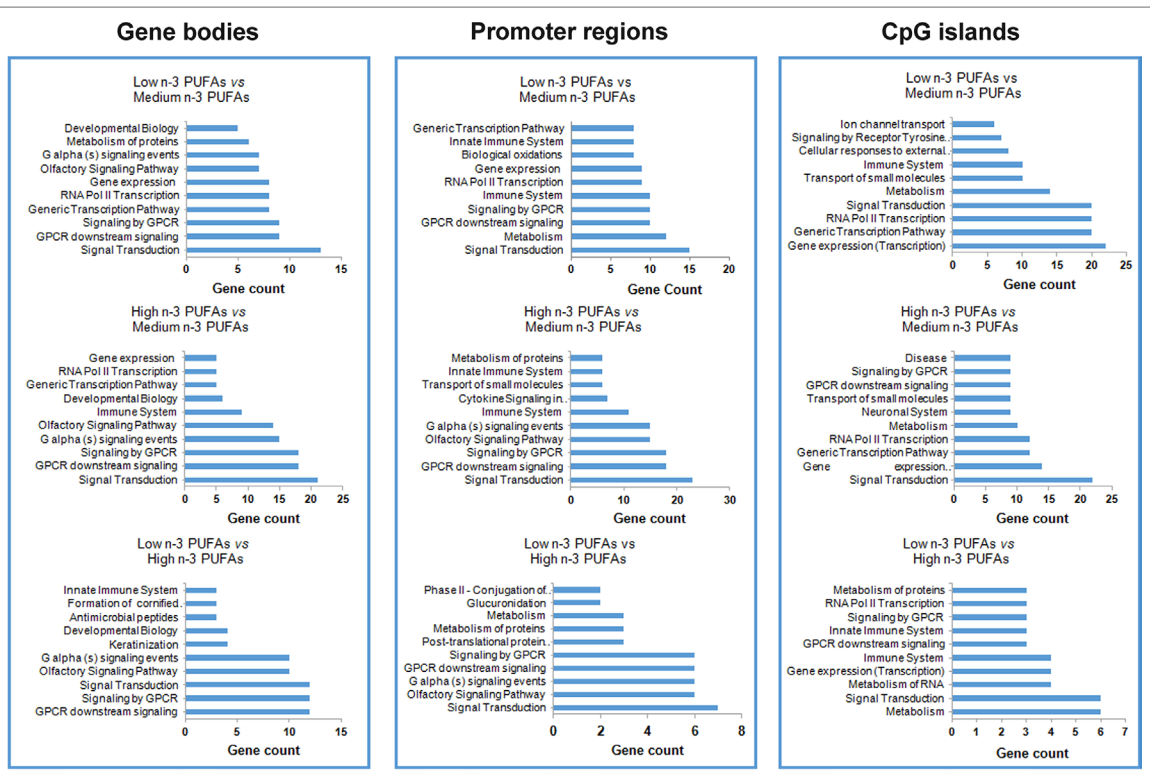

FIGURE 1 | Pathway enrichment resulting from differential methylation profiles associated to n-3 PUFA content. Bar plots of the 10 most abundant pathways resulting from differential methylation profiles at site and region level in low n-3 PUFAs *vs*. medium n-3 PUFAs, high n-3 PUFAs *vs*. medium n-3 PUFAs, and low n-3 PUFAs *vs*. high n-3 PUFAs.

TABLE 1 | Anthropometrics of mother–newborn pairs and n-3 PUFAs content (percent of total fatty acids) in maternal erythrocytes.

	Low n-3 PUFAs	Medium n-3 PUFAs	High n-3 PUFAs	*p*
	N = 39	N = 40	N = 39	
Newborns				
Gender (M/F)	19/20	20/20	19/20	0.2
Gestational age (weeks)	39.13 ± 1.05	38.8 ± 1.45	39.36 ± 1.16	0.35
Birth weight (g)	3292 ± 284	3357 ± 424	3354 ± 280	0.528
Birth length (cm)	50.2 ± 1.8	50.5 ± 2.2	50.7 ± 1.6	0.448
Birth weight z-score (SDS)	0.024 ± 0.62	0.024 ± 0.84	0.055 ± 0.62	0.529
Mothers				
Age (years)	33.0 ± 5.45	33.7 ± 4.8	32.6 ± 5.7	0.853
Pre-pregnancy BMI (kg/m^2)	21.9 ± 2.9	21.5 ± 2.5	21.8 ± 2.2	0.690
Gestational weight gain (kg)	15.0 ± 5.45	13.9 ± 4.3	12.95 ± 5.2	0.184
Erythrocytes n-3 PUFAs (%)	2.01 ± 1.26	4.34 ± 2.28	6.01 ± 2.34	<0.0001

TABLE 2 | Number of differential methylations among groups (combined p-value < 0.05).

	Differentially methylated sites	Differentially methylated regions		
		Gene bodies	Promoter regions	CpG islands
Low n-3 PUFAs *vs.* medium n-3 PUFAs	12,716	209	263	220
High n-3 PUFAs *vs.* medium n-3 PUFAs	18,148	317	369	205
Low n-3 PUFAs *vs.*high n-3 PUFAs	8,503	121	131	10,660

groups (combined p-value ≤ 0.05 for all the between-group comparisons). **Supplementary Figure 1** shows volcano plots (DMR distribution) of DMRs among the three groups, at gene level, promoter regions, and CpG islands. **Supplementary Tables 1–3** report data on DMRs in regions (i.e., gene bodies, promoter regions, and CpG islands) at comparison among the three groups. The analysis highlighted a region that was differentially methylated between low and high n-3 PUFA groups. It resulted in the most significant one by combined p-value (p=4.06^06, ~10^3 times lower than the next best one), and also, it has the best FDR statistics (FDR = 0.1, ~9 times lower than the next best one). It was a CpG island located on chromosome 7 (start 917633; end 917859) between two genes, COX19 (cytochrome c oxidase assembly factor COX19) and ADAP1 (ArfGAP with dual PH domains 1).

Differentially Methylated Genes and Pathway Enrichment

We identified DMGs in correspondence of DMRs ($p < 0.05$). They were located in gene bodies (low n-3 PUFAs *vs.* medium n-3 PUFAs, N = 109; high n-3 PUFAs *vs.* medium n-3 PUFAs, N = 130; low n-3 PUFAs *vs.* high n-3 PUFAs N = 59), promoter regions (N = 133; N = 181; N = 61, respectively), and CpG islands (N = 146; N = 135; N = 87) (**Supplementary Tables 4–6**). **Supplementary Figure 2** reports Venn diagrams showing number and list of DMGs for each site (gene bodies, promoter regions, and CpG islands) that were low or high methylated in low *vs.* medium and, conversely, high *vs.* medium n-3 PUFA groups. Interestingly, there were DMGs in common between the groups that were methylated either in the same or in the opposite direction.

Then, we performed pathway enrichment analysis of all of these DMGs. As shown in **Figure 1**, DMGs were overrepresented in 10 pathways. Most DMGs in gene bodies belonged to signal transduction—signaling by G-protein coupled receptor (GPCR) and GPCR downstream signaling pathways (**Figure 1A**). Most of DMGs in promoter regions belonged to signal transduction—signaling by GPCR, GPCR downstream signaling, and metabolism and olfactory signaling pathways (**Figure 1B**), and DMGs in CpG island belonged to signal transduction—signaling by GPCR and GPCR downstream signaling pathways (**Figure 1C**).

Figure 2 shows overlap of enriched pathways in gene bodies (**Figure 2A**), promoter regions (**Figure 2B**), and CpG islands (**Figure 2C**) after group comparisons.

We found three genes that were differentially methylated in both low and high n-3 PUFA groups as compared to medium n-3 PUFA group (**Figures 3A–C**): the *myostatin* gene (*MSTN*), the *interferon alpha* 13 gene (*IFNA*13), and the *ATPase phospholipid transporting 8B*3 gene (*ATP8B*3). The *gamma-aminobutyric acid type B receptor subunit* 2 (*GABBR*2) gene was differentially methylated between medium and high n-3 PUFA groups. Methylation levels of the four genes with different methylation profiles are reported in **Figures 3D–G**.

DISCUSSION

Findings of the present study support the notion that maternal dietary intake of n-3 PUFAs during pregnancy influences the offspring DNA methylation profile. Differently from previous studies (Lee et al., 2013; Amarasekera et al., 2014; Lee et al., 2014; Lind et al., 2015; Voisin et al., 2015), we explored the association of offspring DNA methylation patterns with content of n-3 PUFAs on maternal erythrocyte membranes in the absence of any n-3 PUFA supplementation. Pregnant women were, indeed, not supplemented but received nutritional counseling.

DNA methylation is an epigenetic mechanism that occurs on the carbon-5 position of cytosines within gene bodies, promoter regions, and, more frequently, CpG islands. DNA methylation

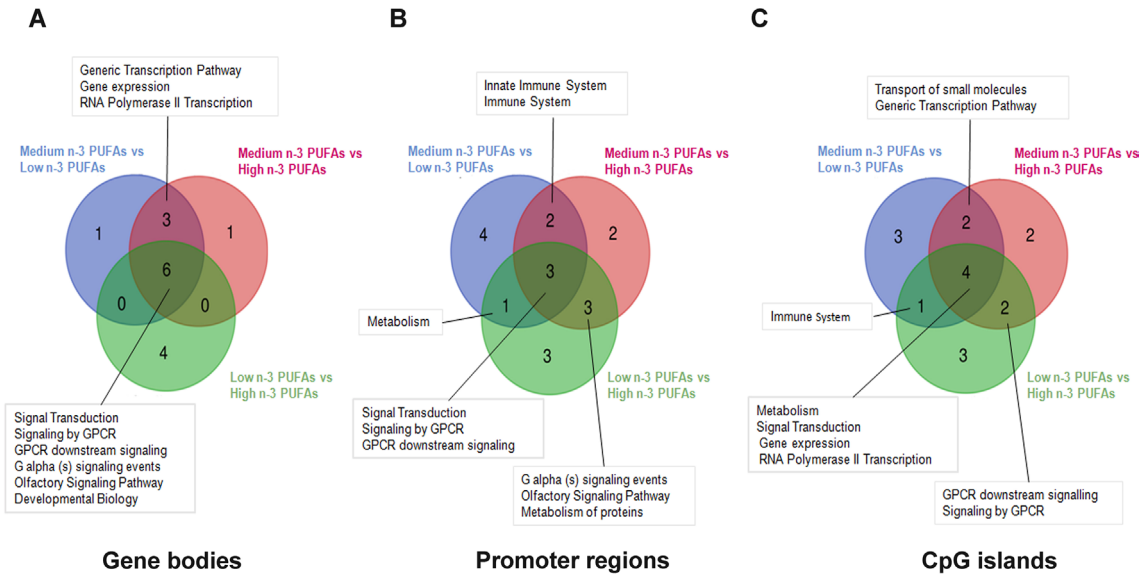

FIGURE 2 | Graphical representation of common inter-group over-represented pathways. Venn diagrams reported the overlapping relationship between the enriched pathways emerging from differential methylation profile at gene bodies **(A)** promoter regions **(B)** and CpG islands **(C)** in the three groups (Low n-3 PUFAs vs. Medium n-3 PUFAs; High n-3 PUFAs vs. Medium n-3 PUFAs; and Low n-3 PUFAs vs. High n-3 PUFAs).

induces typically a down-modulation of the gene expression (Manco and Dallapiccola, 2012).

The present study builds upon emerging literature suggesting dietary fats can exert an epigenetic regulation in humans influencing global DNA methylation patterns (Burge and Lillycrop, 2014; Tremblay et al., 2017). In a study of overweight and obese individuals supplemented with 3 g of DHA, 55 gene pathways were significantly overrepresented in supplemented people *versus* controls after 6-week treatment. Sixteen of these pathways were related to inflammatory and immune response, lipid metabolism, type 2 diabetes, and cardiovascular disease (Tremblay et al., 2017). In the Yup'ik Alaska Native people who have a diet rich in n-3 PUFAs from fish oil, the erythrocyte n-3 PUFA content was inversely related to the methylation of *interleukin*-6 (*IL*) gene, thus suggesting that n-3 PUFAs exert anti-inflammatory action also by imposing epigenetic changes (Ma et al., 2016). High n-3 PUFA intake was associated also with 27 other differentially methylated CpG sites at biologically relevant regions, including *FAS*, a key gene of the cell apoptosis (Aslibekyan et al., 2014a).

With regard to n-3 PUFA supplementation in pregnancy, a study of 261 Mexican pregnant women, daily supplemented with 400 mg docosahexaenoic acid (DHA) (N = 131) or placebo (N = 131) from 18 to 22 weeks of gestation to parturition, demonstrated no significant difference in promoter methylation levels of *Th*1, *Th*2, and *Th*17 and regulatory T-relevant genes between n-3 PUFA supplemented and not-supplemented controls. Nevertheless, in infants of mothers who smoked during pregnancy, supplementation was significantly associated with changes of methylation levels in LINE1 repetitive elements and of the promoter methylation levels in *IFN-γ* and *IL*13 genes (Lee et al., 2013). Furthermore, the DHA supplemented group of preterm infants had DNA methylation levels in *IGF*2 *P*3 significantly higher than controls. In addition, at H19 DMR, methylation levels were significantly lower in the DHA group than in controls of normal weight mothers (Lee et al., 2014). In a study of 70 mother–newborn pairs supplemented with 3.7 g of fish oil, association between methylation levels of cord blood CD4+ T with DHA, EPA acid, or total n-3 PUFA levels was suggestive of a dose-dependent effect (Amarasekera et al., 2014).

In the present series, we found DMGs that belonged prevalently to pathways of signal transduction, metabolism, downstream signaling of GPCR, and gene expression among others. Within these pathways, we identified four DMGs, namely, *MSTN*, *IFNA*13, *ATP8B*3, and *GABBR*2 that have potential to affect the infant's metabolic programming. They are related to the onset of insulin resistance and adiposity, to the innate immunity response that is ancestral response to insults including nutrients, to the fatty acid transfer across cellular membranes, and to the reward/addiction paths of the central nervous system (Folmer et al., 2009; Zhang et al., 2011; van der Mark et al., 2013; Aberg et al., 2018; Liu et al, 2018; Martins de Carvalho et al., 2018; Ramos-Lopez et al., 2018). They were low methylated in groups of newborns from mothers with either low or high content of n-3 PUFAs respect to those born to mothers with medium levels. Therefore, we expect these genes overexpressed in both conditions of low and high n-3 PUFA intake.

MSTN is a key protein in the regulation of energy metabolism and muscle insulin resistance. Its overexpression is associated with impaired glucose disposal mostly owing to inhibition of glucose transporter type 4 expression, integration into cytoplasmic membranes, and glucose uptake (Liu et al., 2018). MSTN overexpression leads to indirect suppression of 5' AMP-activated protein kinase activation and, as such, impacts on the overall energy metabolism (Zhang et al., 2011). Conversely, engineered

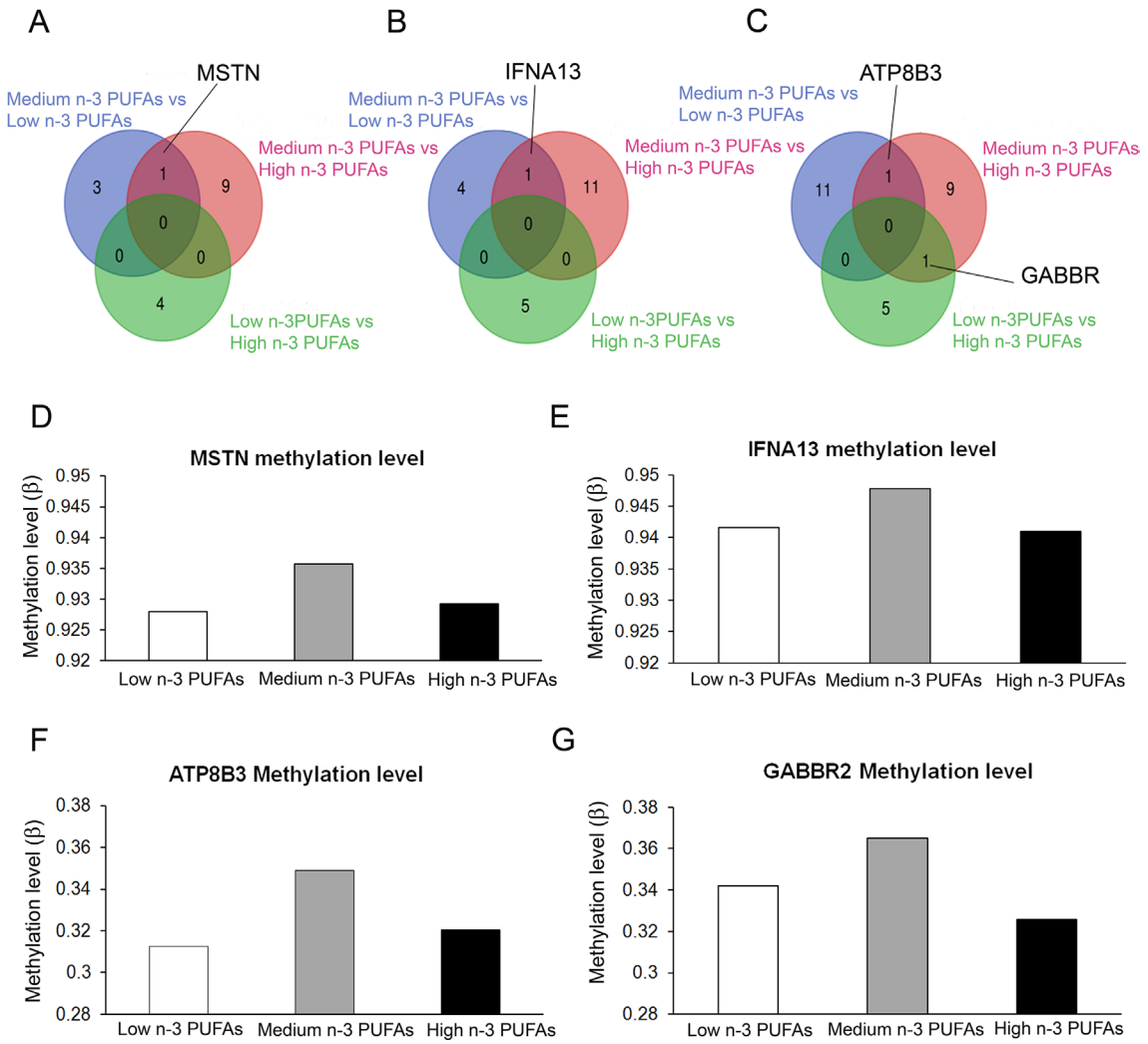

FIGURE 3 | Graphical representation of inter-group common genes. **(A–C)** Venn diagrams showing the overlapping of genes with different methylation level associated to the enriched pathways highlighted emerging from differential methylation profile at gene bodies **(A)**, promoter regions **(B)** and CpG islands **(C)** in the three groups (Low n-3 PUFAs vs. Medium n-3 PUFAs; High n-3 PUFAs vs. Medium n-3 PUFAs; and Low n-3 PUFAs vs. High n-3 PUFAs). **(D–G)** Histograms representing the methylation level of the four common genes resulting from the analysis among groups.

pigs (Mstn−/−) that lack MSTN present with enhanced insulin sensitivity reduced subcutaneous adipose tissue and increased browning of the latter tissue.

ATP8B3 belongs to the lipid flippases_P4 ATPases that are multispan transmembrane proteins implicated in phospholipid translocation from the exoplasmic to the cytoplasmic leaflet of biological membranes. ATP8B3 is highly expressed in testis, endometrium, spleen, and bone marrow (Folmer et al., 2009 van der Mark et al., 2013). Levels of the protein and its tight regulation by methylation mechanisms may be related to the fine regulation of maternal-fetus fatty acid transfer across the placenta.

GABBR2 seems to be involved in mechanisms of addiction to high fat and alcohol consumption (Martins de Carvalho et al., 2018). Reduced methylation of the gene has been associated with risk of major depression (Aberg et al., 2018) and enhanced sweet taste induced signaling in the 474 adults from the Methyl Epigenome Network Association project (Ramos-Lopez et al., 2018).

IFNA13 is involved in immune-regulatory activities and antiviral response and its relationship with fatty acids metabolism may deserve investigation.

In that, epigenetics may contribute to the fine regulation of fatty acid transfer across the placenta. N-3 PUFAs are essential for the embryo development and the maternal well-being. The placenta modulates the transfer acting as endocrine organ that regulates fetal health growth and maternal metabolism. Fatty acids are major player in the maternal-fetus cross-talk and insufficient or, conversely, excessive amount of these essential fatty acids are detrimental for the fetus' health (Larqué et al., 2014).

We are aware of several caveats. We do not have genotype or transcription information at the differentially methylated loci, and thus, our understanding of the underlying biologic mechanisms

is limited. Differences in methylation levels reached statistical significance at p statistics but not at the FDR statistics probably because of both small-size effect and sample. Furthermore, DNA methylation levels are specific to the type of cell and tissue (Bell et al., 2012). These methylation profiles in cord blood leukocytes might not represent DNA methylation in other tissues even though patterns are globally conserved (Ma et al., 2014; Ronn et al., 2015; Guénard et al., 2016). Finally, annotation of genetic variants has been deemed as inconsistent across databases, incomplete, and subjective toward known genes and pathway analysis that is statistically unpowered (Aslibekyan et al., 2014b).

Strength of the study was measurement of n-3 PUFA content on erythrocyte membranes to estimate maternal intake. The life of a red blood cell is ~120 d; therefore, this analysis provided a biomarker to measure objectively n-3 PUFA intake in the pregnant women during the last trimester.

Future investigations based on our findings should include replication in independent large populations and functional analyses of the identified genes. As DNA methylation levels relieve functional effects in specific genomic contexts, there is need of functional studies that take into account effect sizes and genomic context.

In conclusion, this study provides evidence that the maternal intake of n-3 PUFAs during pregnancy influences DNA methylation levels in cord blood white cells of newborns. Studies are needed to investigate the clinical relevance and the biological significance of our findings and to inform about recommendable ranges of daily intake of n-3 PUFAs during pregnancy.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the Article/ **Supplementary Files**.

ETHICS STATEMENT

The feeding study and the ancillary studies were approved by the Ethical Committee of the Bambino Gesù children's Hospital and the San Camillo Forlanini Hospital. The study conformed to guidelines of the European Convention of Human Rights and Biomedicine for Research in Children as revised in 2008. Parents or responsible guardians provided written informed consent. All measures were taken to ensure participants' confidentiality.

AUTHOR CONTRIBUTIONS

MB and AA: data analysis (pathways enrichment) and interpretation and drafting of the manuscript. MF: DNA extraction and epigenetic profiling. CV and FS: cohort enrollment and follow-up. LR: sample stratification and data analysis. RG: bioinformatics analysis (raw methylation data) and contributed significantly to the revision of the entire manuscript. PV: lipid profiling on erythrocytes. MM: research fund acquisition, conception and design of the study, data analysis and interpretation, and drafting.

All the authors revised the manuscript for important intellectual content; approved the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work have been appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2019.01050/full#supplementary-material

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REVIEW
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The Role of Single-Nucleotide Polymorphisms in the Function of Candidate Tumor Suppressor ALDH1L1

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Folate (vitamin B9) is a common name for a group of coenzymes that function as carriers of chemical moieties called one-carbon groups in numerous biochemical reactions. The combination of these folate-dependent reactions constitutes one-carbon metabolism, the name synonymous to folate metabolism. Folate coenzymes and associated metabolic pathways are vital for cellular homeostasis due to their key roles in nucleic acid biosynthesis, DNA repair, methylation processes, amino acid biogenesis, and energy balance. Folate is an essential nutrient because humans are unable to synthesize this coenzyme and must obtain it from the diet. Insufficient folate intake can ultimately increase risk of certain diseases, most notably neural tube defects. More than 20 enzymes are known to participate in folate metabolism. Single-nucleotide polymorphisms (SNPs) in genes encoding for folate enzymes are associated with altered metabolism, changes in DNA methylation and modified risk for the development of human pathologies including cardiovascular diseases, birth defects, and cancer. ALDH1L1, one of the folate-metabolizing enzymes, serves a regulatory function in folate metabolism restricting the flux of one-carbon groups through biosynthetic processes. Numerous studies have established that *ALDH1L1* is often silenced or strongly down-regulated in cancers. The loss of ALDH1L1 protein positively correlates with the occurrence of malignant tumors and tumor aggressiveness, hence the enzyme is viewed as a candidate tumor suppressor. *ALDH1L1* has much higher frequency of non-synonymous exonic SNPs than most other genes for folate enzymes. Common SNPs at the polymorphic loci rs3796191, rs2886059, rs9282691, rs2276724, rs1127717, and rs4646750 in *ALDH1L1* exons characterize more than 97% of Europeans while additional common variants are found in other ethnic populations. The effects of these SNPs on the enzyme is not clear but studies indicate that some coding and non-coding *ALDH1L1* SNPs are associated with altered risk of certain cancer types and it is also likely that specific haplotypes define the metabolic response to dietary folate. This review discusses the role of ALDH1L1 in folate metabolism and etiology of diseases with the focus on non-synonymous coding *ALDH1L1* SNPs and their effects on the enzyme structure/function, metabolic role and association with cancer.

Keywords: folate metabolism, ALDH1L1, candidate tumor suppressor, SNPs, human diseases

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INTRODUCTION: FOLATE METABOLISM AND CELLULAR HOMEOSTASIS

Folate (vitamin B9) is a common name for a group of coenzymes that function as carriers of chemical moieties called one-carbon groups (OCGs) in numerous biochemical reactions. The combination of these folate-dependent reactions constitutes one-carbon metabolism, the name synonymous to folate metabolism. The intracellular folate pool consists of several major coenzyme forms, including tetrahydrofolate (THF) and its derivatives differing by the oxidation state of conjugated OCG (Fox and Stover, 2008; Tibbetts and Appling, 2010). Folate coenzymes and associated metabolic pathways are vital for cellular homeostasis due to their key roles in nucleic acid biosynthesis, DNA repair, methylation processes, amino acid biogenesis, and energy balance (Blom et al., 2006; Fox and Stover, 2008; Tibbetts and Appling, 2010; Locasale, 2013; Fan et al., 2014; Ducker and Rabinowitz, 2017). Folate-dependent biochemical reactions underlying these processes include *de novo* purine and TMP biosynthesis, re-methylation of homocysteine to methionine linked to the production of the universal methyl donor S-adenosylmethionine, degradation of histidine and glycine, interconversion of serine and glycine, and the final step of carbon oxidation to CO_2 linked with NADPH production (Tibbetts and Appling, 2010; Fan et al., 2014; Baggott and Tamura, 2015; Brosnan et al., 2015). Additional folate-dependent pathways include the clearance of formate (Brosnan et al., 2015) and the formylation of mitochondrial methionyl-tRNA, a process essential for translation initiation in eukaryotic mitochondria (Spencer and Spremulli, 2004; Tucker et al., 2011; Minton et al., 2018). Interestingly, a recent paper reported the direct involvement of one of folate coenzymes, 5,10-methylene-THF, in the methylation of mitochondrial tRNAs with the deficiency of this pathway likely being linked to defective oxidative phosphorylation in human cells (Morscher et al., 2018). This discovery not only extends the list of folate-dependent biochemical reactions and further underscores the indispensable role of the coenzyme but also emphasizes that precise molecular mechanisms underlying folate homeostasis are not completely understood.

Folate is an essential nutrient because humans are unable to synthesize this coenzyme and must obtain it from the diet (Cooper, 1986). Insufficient folate intake ultimately leads to deregulation of cellular homeostasis and is associated with increased risk of certain diseases, most notably neural tube defects (NTDs) (Rock et al., 2000; Fleming, 2001; Mitchell et al., 2004; Moat et al., 2004; Beaudin and Stover, 2007; Strickland et al., 2013; Newman and Maddocks, 2017). For example, periconceptional folate supplementation, in addition to preventing NTDs, has been associated with a significant reduction in the incidence of early spontaneous preterm births (Bukowski et al., 2009). Largely for NTD prevention, the FDA in 1996 approved a mandatory fortification of several types of grain foods in the US with a synthetic form of the vitamin, folic acid (FDA, 1996). The fortification resulted not only in a significant reduction of the incidence of NTDs in the US (Blom et al., 2006), but also improved folate status in the adult population (Jacques et al., 1999).

FOLATE ENZYMES, SINGLE-NUCLEOTIDE POLYMORPHISMS AND DISEASES

More than 20 enzymes are known to participate in folate metabolism (**Figure 1**) (Fox and Stover, 2008; Tibbetts and Appling, 2010). They bring OCGs into folate pool, interconvert folate coenzymes, or use OCGs in biosynthetic reactions (Tibbetts and Appling, 2010). Of note, folate enzymes are highly compartmentalized in the cell, being localized to either cytoplasm or mitochondria (Tibbetts and Appling, 2010). Several cytoplasmic folate enzymes can also translocate to the nucleus to enable TMP biosynthesis at specific sites (MacFarlane et al., 2011; Anderson et al., 2012; Field et al., 2014; Field et al., 2015). The nucleus and cytoplasm exchange folate through a simple diffusion, but the mitochondrial membrane is not permeable to folate and shuttling requires a special transporter (Titus and Moran, 2000). Thus, mitochondrial folate metabolism is distinct from cytosolic and uses its own set of enzymes (Tibbetts and Appling, 2010). Several folate reactions in mitochondria parallel those in the cytoplasm; these are catalyzed by homologous enzymes which are products of different genes (Tibbetts and Appling, 2010; Strickland et al., 2011). Folate mitochondrial pathways (i) provide one-carbon groups (in the form of formate) for the cytosolic folate pool, where they are utilized for biosynthetic reactions (Tibbetts and Appling, 2010); (ii) generate NADPH (Fan et al., 2014), or (iii)

Gene	Haplotype # common/rare	Gene	Haplotype # common/rare
ALDH1L1	**10/20**	MTR	2
ALDH1L2	0	MTRR	10/22
MTHFD1	6/9	TYMS	0
MTHFD1L	0	SARDH	3/7
MTHFD2	2	DMGDH	4/6
MTHFD2L	0	GNMT*	0
DHFR	0	GART	6/9
DHFRL1	3	ATIC	3
MTHFR	4/5	MTFMT	2
SHMT1	2	FOLH1	4/6
SHMT2	2	GLDC	6/11
MTHFS	3	GCSH	3/4
FTCD	4/5	AMT#	0
FPGS	3/4	DLD	2

FIGURE 1 | Numbers of common and rare haplotype alleles in genes of folate metabolism (human genome assembly GRCh37/hg19; rare haplotypes have frequency below 1%). *, GNMT is the enzyme regulated by folate. *Red box* indicates four enzymes of the mitochondrial glycine cleavage system, #, the folate dependent enzyme in glycine cleavage. Haplotypes were analyzed using UCSC Genome Browser (https://genome.ucsc.edu).

serve specific mitochondrial functions (Tucker et al., 2011; Morscher et al., 2018; Tani et al., 2018).

Changes in folate metabolism contribute to human pathologies (Stover, 2009), and recent studies underscore the role of several folate enzymes and associated pathways in NTDs and cancer (Jain et al., 2012; Narisawa et al., 2012; Momb et al., 2013; Nilsson et al., 2014; Pai et al., 2015; Piskounova et al., 2015; Ducker et al., 2016; Leung et al., 2017). Alterations in expression or activity of numerous enzymes of folate pathways can either enhance or impair folate metabolism. For example, the increased demand for nucleotides and methylation reactions in cancer cells commonly causes enhanced expression of folate enzymes to maintain the flux of folate-bound OCGs towards biosynthesis, thus supporting increased proliferation (Jain et al., 2012; Ducker and Rabinowitz, 2017; Rosenzweig et al., 2018). Accordingly, several of these enzymes were successfully targeted in cancer chemotherapy (Goldman et al., 2010; Visentin et al., 2012). Further links between the function of folate enzymes and onset of diseases have been clarified in studies using knockout mouse models. Thus, the loss of either MTHFD1L or the folate-dependent glycine cleavage (both localized to mitochondria) causes NTDs in mice (Momb et al., 2013; Pai et al., 2015). Another example is the knockout of folate-regulatory enzyme GNMT: the loss of this protein produces spontaneous tumors in the mouse liver (Martinez-Chantar et al., 2008). It has been also reported that the deficiency in the 10-formyl-THF synthetase activity of cytosolic trifunctional enzyme MTHFD1 is associated with increased incidence of congenital heart defects in mouse embryos (Christensen et al., 2015). Numerous studies also indicate strong gene-nutrient interactions in the folate metabolism regulation. For example, the loss of SHMT1 was insufficient to produce NTDs but caused exencephaly under conditions of maternal folate deficiency (Beaudin et al., 2011; Beaudin et al., 2012).

Single-nucleotide polymorphisms (SNPs) in genes encoding folate enzymes are associated with altered metabolism, changes in DNA methylation and modified risk for the development of human pathologies [reviewed in (Stover, 2011)] including cardiovascular diseases (Klerk et al., 2002), birth defects (Ou et al., 1996; Mills et al., 1999), and cancer (Sharp and Little, 2004; Lightfoot et al., 2010). The most investigated target in these studies was MTHFR (methylene-THF reductase) (Ueland et al., 2001; Hirschhorn et al., 2002; Klerk et al., 2002), which has two common SNPs in the coding region causing non-synonymous amino acid substitutions and creating enzyme variants with reduced activity (Frosst et al., 1995; Weisberg et al., 1998). Numerous SNPs in other key genes of folate pathways, including DHFR (dihydrofolate reductase) (Mishra et al., 2007), MTR (methionine synthase) (Harmon et al., 1999; Ma et al., 1999), TYMS (thymidylate synthase) (Pullarkat et al., 2001), and MTRR (methionine synthase reductase) (Wilson et al., 1999; Gaughan et al., 2001) were linked to human diseases. Of note, the effect of folate pathway gene polymorphisms on disease risk often depends on folate status (Friso et al., 2002; Ulrich et al., 2002; Philip et al., 2015).

ALDH1L1 FOLATE REGULATORY ENZYME

ALDH1L1, one of the folate-metabolizing enzymes, converts 10-formyl-THF to THF with simultaneous production of NADPH from $NADP^+$ (Krupenko, 2009). By oxidizing the formyl group to CO_2, this reaction clears the OCG from the cell, thus restricting flux through biosynthetic processes (**Figure 2**). In this way, ALDH1L1 regulates one-carbon metabolism and serves a catabolic function (Krupenko and Oleinik, 2002; Anguera et al., 2006; Krupenko, 2009). ALDH1L1 is active as a tetramer and has a complex structure and catalytic mechanism (**Figure 3**). The *ALDH1L1* gene originated from a natural fusion of three unrelated primordial genes (Strickland et al., 2011; Krupenko et al., 2015), and the resulting protein has a modular organization with three structurally and functionally distinct domains (Krupenko, 2009). The N-terminal folate binding/hydrolase domain structurally resembles methionine-tRNA formyltransferase (Schmitt et al., 1996; Chumanevich et al., 2004) and catalyzes the initial cleavage of the 10-formyl group from 10-formyl-THF (Krupenko et al., 1997a; Chumanevich et al., 2004). The C-terminal dehydrogenase domain forms the tetrameric core and is a structural and functional homolog of aldehyde dehydrogenases (ALDHs) (Krupenko et al., 1997b; Tsybovsky et al., 2007) [hence the assignment of ALDH1L1 to this superfamily of proteins (Marchitti et al., 2008)]. In humans, there are 19 genes encoding for aldehyde dehydrogenases (Marchitti et al., 2008; Koppaka et al., 2012). ALDHs catalyze $NAD(P)^+$-dependent irreversible oxidation of a wide variety of endogenous and exogenous aldehydes to corresponding acids, display distinct substrate specificity, and are generally regarded as detoxification enzymes (Marchitti et al., 2008; Koppaka et al., 2012). The ALDH domain of ALDH1L1 shares about 49% of its amino acid sequence with ALDH1, has a typical ALDH fold and by itself catalyzes the oxidation of short-chain aldehydes to corresponding acid using strictly $NADP^+$ (Krupenko, 2009). It is not clear whether ALDH1L1 is involved in the utilization of aldehyde substrates *in vivo*. As a part of the ALDH1L1 enzymatic machinery, this domain catalyzes the reduction of $NADP^+$ and the oxidation of formyl group to CO_2 (Krupenko et al., 1997b; Tsybovsky et al., 2007). The two catalytic domains communicate *via* the intermediate domain, which is a structural and functional homolog of acyl carrier proteins (Donato et al., 2007; Strickland et al., 2010).

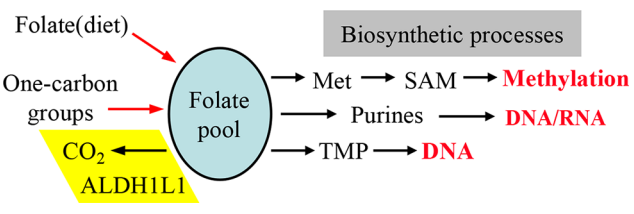

FIGURE 2 | One-carbon groups (derived from amino acid oxidation or formate) enter the folate pool and are directed towards three biosynthetic pathways (methionine, purines and thymidylate synthesis). Note that the enzyme ALDH1L1 diverts these groups from biosynthetic pathways thus serving a catabolic function. Input of folate from diet is required to support the intracellular levels of the coenzyme. SAM, S-adenosylmethionine.

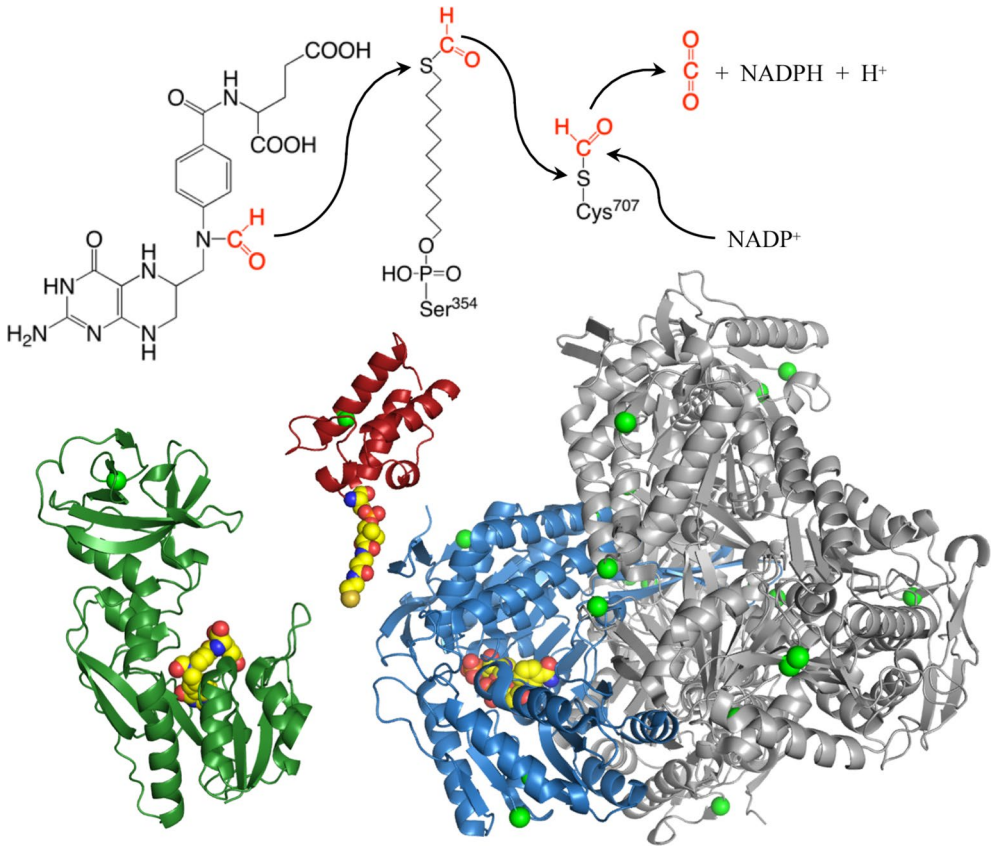

FIGURE 3 | Structures of the N-terminal formyltransferase (*dark green*), central acyl-carrier (*red*, with manually added phosphopantetheinyl moiety), and C-terminal dehydrogenase (*blue* and *gray*) domains of ALDH1L1. *Green spheres* highlight positions of amino acids corresponding to exonic SNPs discussed in the text, other colored spheres show positions of 10-formyltetrahydrofolate (N-terminal domain), phosphopantetheine (intermediate domain), and $NADP^+$ (C-terminal domain). Subunit A of the tetrameric dehydrogenase domain is *blue*, subunits B, C, and D are *gray*. The extended phosphopantetheine is critical to the reaction as both the formyl donor (10-formyltetrahydrofolate) and electron acceptor ($NADP^+$) are located at the bottoms of clefts in the protein surface. PDB structures are: 4tt8 (N-terminal domain); 2cq8 (intermediate domain); and 2o2q (aldehyde dehydrogenase domain).

Its prosthetic group, 4′-phosphopantetheine (4′-PP), functions as a flexible arm reaching into the catalytic centers on the N- and C-terminal domains (Horita and Krupenko, 2017) and transporting the reaction intermediate (formyl) from one center to the other (**Figure 3**). The three domains of ALDH1L1 work in concert to enable the conversion of 10-formyl-THF to THF and NADPH production linked to the oxidation of formyl group to CO_2. Thus, in the case of ALDH1L1 the recruitment of the folate-binding domain extended the substrate specificity of an aldehyde dehydrogenase. Of note, the ALDH family also includes ALDH1L2, the mitochondrial homolog of ALDH1L1 (Krupenko et al., 2010), which is the product of a separate gene [one of the 19 *ALDH* genes (Marchitti et al., 2008)].

That ALDH1L1 serves a regulatory role was determined by several reports that demonstrated the effect of the enzyme on folate and purine pools and on methylation (Champion et al., 1994; Oleinik et al., 2005; Anguera et al., 2006; Oleinik et al., 2006; Hoeferlin et al., 2011). ALDH1L1 is also a key component of the formate degradation pathway, which converts toxic formate to neutral CO_2, through 10-formyltetrahydrofolate as an intermediate (Strickland et al., 2011). In the cell, formate is directly produced not only from the degradation of 3-methyl-branched fatty acids and the shortening of 2-hydroxy long chain fatty acids (Casteels et al., 2007) but also from the oxidation of methanol present in juices and alcoholic beverages (Hang and Woodams, 2010) and from metabolism of artificial sweetener aspartame (Choudhary and Pretorius, 2017). The first step of the formate degradation pathway, the incorporation of formate into the folate pool, is catalyzed by MTHFD1 and the second rate-limiting step releasing CO_2 is catalyzed by ALDH1L1 (Neymeyer et al., 1997). It appears that the ALDH1L1-dependent pathway is the only pathway in humans to metabolize formate, and it is more prominent for the clearance of lower, physiological doses of formate (Cook et al., 2001). In further support of this role, decreased expression of ALDH1L1 was observed in cobalamin-deficient rats, likely as a mechanism to divert formate towards methyl group production (MacMillan et al., 2018). ALDH1L1 was also highlighted as a pan-astrocyte marker (Cahoy et al., 2008), but its importance for the astrocyte function is not clear. Interestingly, decreased levels of ALDH1L1 in cerebrospinal fluid were linked to neonatal hydrocephalus in a rat model (Cains et al., 2009).

Further studies of this model suggested a role for the enzyme in cerebral folate transport and regulation of folate availability in the brain (Naz et al., 2016; Jimenez et al., 2019). In line with such function, it has been also demonstrated that ALDH1L1 protects folate from degradation in zebrafish embryos, which is a defense mechanism against oxidative stress (Chang et al., 2014; Hsiao et al., 2014). Furthermore, the protective effect of ALDH1L1 on THF degradation has been recently observed in cancer cells (Zheng et al., 2018). These studies provide experimental support for the hypothesis that ALDH1L1 serves as folate depot (Krupenko and Krupenko, 2018).

EVIDENCE THAT ALDH1L1 IS A CANDIDATE TUMOR SUPPRESSOR

ALDH1L1 is most abundant in liver, kidney and pancreas comprising about 1% of total cytosolic protein in hepatocytes (Krupenko, 2009). However, it is not a housekeeping gene and its expression is tissue-specific with some tissues lacking this protein expression (Krupenko and Oleinik, 2002). Furthermore, the enzyme is tightly regulated during mouse brain development (Anthony and Heintz, 2007) and during the progression of NIH3T3 cells through the cell cycle (Khan et al., 2018). In both cases, ALDH1L1 protein is dramatically decreased in proliferating cells but elevated in non-proliferating/resting cells. During mouse brain development, ALDH1L1 expression is likely controlled by transcriptional regulation (Anthony and Heintz, 2007) while in NIH3T3 cells it is rapidly degraded through the ubiquitin-proteasome pathway during the transition from G0/G1 to S-phase (Khan et al., 2018). Because the enzyme limits proliferation by diverting OCGs from biosynthetic to catabolic pathways, its down-regulation could be one of the mechanisms to maintain proliferative state.

In line with its antiproliferative function, ALDH1L1 is often silenced or strongly down-regulated in cancer cell lines and malignant tumors [reviewed in (Krupenko and Krupenko, 2018; Krupenko and Krupenko, 2019)]. This is in strict contrast to other folate enzymes, which are commonly up-regulated in cancer (Jain et al., 2012; Ducker and Rabinowitz, 2017). Several studies have established that the silencing of *ALDH1L1* in human cancers is driven by gene methylation (Oleinik et al., 2011; Dmitriev et al., 2012; Senchenko et al., 2013; Dmitriev et al., 2014; Beniaminov et al., 2018). Methylation takes place in the CpG island, which includes 96 CpG base pairs and covers the promoter, first exon and the part of the first intron in *ALDH1L1* (Oleinik et al., 2011; Beniaminov et al., 2018). Remarkably, a microarray-based global gene expression profiling of approximately 42,000 genes has found that ALDH1L1 was one of the most down-regulated proteins in primary hepatocellular carcinomas and in liver metastases (Tackels-Horne et al., 2001). Analysis of gene expression profiles across 33 human cancer types using The Cancer Genome Atlas (TCGA) data indicated that *ALDH1L1* is more strongly down-regulated in late-stage cancers (Li et al., 2017). Overall, the loss of ALDH1L1 protein positively correlates with the occurrence of malignant tumors and tumor aggressiveness [reviewed in (Krupenko and Krupenko, 2018; Krupenko and Krupenko, 2019)], hence the suggestion that the enzyme is a candidate tumor suppressor (Senchenko et al., 2013).

SNPS IN *ALDH1L1* AND THEIR ASSOCIATION WITH PATHOLOGIES

ALDH1L1 is located on the minus strand of chromosome 3, spans about 94 thousand nucleotides and may harbor numerous SNPs. Several reports have investigated the functional role of some of these SNPs as well as their associations with diseases. For example, genome-wide association studies (GWAS) revealed that SNPs in *ALDH1L1* are associated with serine to glycine ratio in serum (Dharuri et al., 2013) thus supporting the role of the enzyme as metabolic regulator. Another GWAS analysis identified an association between rs1107366, located about 3800 nucleotides upstream of the *ALDH1L1* transcription start site, and glycine to serine ratios (Xie et al., 2013). This study also indicated that the rs1107366-linked glycine to serine ratio is associated with insulin sensitivity but not with type 2 diabetes. *ALDH1L1* SNPs were also associated with NTDs in Dutch and Chinese Han populations (Franke et al., 2009; Wu et al., 2016).

An interesting study evaluated the effect of two intronic *ALDH1L1* SNPs, rs2276731 and rs2002287, on genome-wide DNA methylation as well as site-specific methylation in normal breast tissues from healthy women (Song et al., 2016). This study identified 57 CpG sites in human genome that were differentially methylated depending on SNPs in six genes of folate metabolism. The strongest association for differential methylation at these sites were with the *ALDH1L1* SNPs. Furthermore, rs2276731 was also associated with a significantly higher global DNA methylation as well as with differential methylation of CpGs within *ALDH1L1* itself. Of note, for both ALDH1L1 SNPs, the pattern of differentially methylated sites was different between whites and blacks (Song et al., 2016). Importantly, a modifying effect on breast cancer incidence of these *ALDH1L1* SNPs has also been reported (Stevens et al., 2007). Here, however, these SNPs have opposite effects: the rs2276731 allele was associated with increased risk whereas the rs2002287 allele was associated with decreased risk of breast cancer.

The rs2276731 SNP could also have a role in the host-gut microbiome interaction. This has been suggested from the 16S rRNA-based analysis of the gut microbiome in 1,126 twin pairs, which thought to calculate the heritability of specific components of the gut microbiota and to find associations between the abundance of specific microbes and host gene alleles (Goodrich et al., 2016). The study identified an association between the host gene *ALDH1L1* (via rs2276731) and the bacteria SHA-98 [unclassified genus of the order SHA-98, phylum Firmicutes (Goodrich et al., 2014)]. It further suggested that this association is linked to the metabolism of formate (as discussed above, ALDH1L1 is a key component of the formate clearance). In addition to the sources listed in the previous section, formate is also a fermentation product which acts as a major interspecies electron carrier promoting syntrophy (Goodrich et al., 2016). Of note, it has been shown that urinary formate excretion significantly correlated with blood pressure (Holmes et al., 2008).

Since a SNP in *ALDH1L1* was associated with incident ischemic stroke (Williams et al., 2014), the enzyme might link formate metabolism with the risk of cardiovascular diseases.

Interestingly, *ALDH1L1* has much higher frequency of non-synonymous exonic SNPs than most other genes for folate enzymes (**Figure 1**). Such SNPs cause amino acid substitutions, could affect the enzyme function, and thus could be relevant to the role of the enzyme in cancer. Curiously, a highly similar mitochondrial homolog, ALDH1L2, which is a product of a separate gene resulted from gene duplication (Krupenko et al., 2010; Strickland et al., 2011; Krupenko et al., 2015), does not have common SNPs (**Figure 1**). SNPs in *ALDH1L1* are common but their effect on metabolism and the etiology of cancer disease is not well understood. Notably, the frequency of exonic SNPs in this gene is highly different between ethnic populations [**Figure 4**; analyzed using UCSC genome browser (Mangan et al., 2014)]. While common SNPs at the polymorphic loci rs3796191, rs2886059, rs9282691, rs2276724, rs1127717 and rs4646750 in *ALDH1L1* exons characterize more than 97% of Europeans, additional common variants are found in African, Hispanic, and Chinese populations (**Figure 4**). Several studies indicated that coding SNPs in *ALDH1L1* are associated with altered risk of certain cancer types. Thus, *ALDH1L1* rs1127717 was associated with the increased risk of hepatocellular carcinoma in Chinese population (1500 cancer patients and 1500 controls were enrolled in this study) (Zhang et al., 2015). Another SNP, rs2276724, could be associated with the post-operative survival of patients with hepatitis B-related hepatocellular carcinoma (Zhu et al., 2017). This study indicates that the effect of the SNP is associated with the expression level of ALDH1L1 mRNA and also depends on the p53 status. An elevated risk of non-Hodgkin lymphoma (NHL) was observed among carriers of the *G* allele at *ALDH1L1 Ex21+31* (p.D793G; rs1127717) (Lee et al., 2007; Lim et al., 2007; Suthandiram et al., 2015). Furthermore, the protective effect of methionine on NHL was associated with *ALDH1L1* SNPs (Lim et al., 2007; Li et al., 2013) suggesting gene-nutrient interactions. Importantly, four exonic SNPs shown in **Figure 4** are associated with leukocyte telomere length (Pusceddu et al., 2017), implicating these polymorphisms in cancer (Sarek et al., 2015; Zhu et al., 2016). Of note, studies investigating *ALDH1L1* SNPs as a risk factor for prostate and renal cancers did not find any associations (Stevens et al., 2008; Gibson et al., 2011), which could suggest the cancer type-specific role of the SNPs. Additionally, the overall effect of *ALDH1L1* SNPs is likely ethnicity-specific (Marini et al., 2016; Wu et al., 2016) and could also be modified by the folate status.

Exonic SNPs in *ALDH1L1*	Haplo type	EUR %	AFR %	Hisp %	Han %
rs3796191 T/C; L254P	LVESDI	63.2	32.1	58.3	63.9
rs2886059 G/T; V330F	LVESGI	14.2	11.2	9.1	9.8
rs9282691 A/C; E429A	LFEGGI	6.3	1.4	1.5	0.5
rs2276724 A/G; S481G	LFEGDI	5.4	8.3	3.8	20.1
rs1127717 A/G; D793G	LFEGDV	5	2.4	5.3	1.0
rs4646750 A/G; I812V	PVESDI	3.3	7.5	18.9	3.1
	LVESDV	0.7	8.5	0	1.0
	LFESDI	0.3	6.5	0	0
	LFASGI	0	9.6	0	0
	LFASDI	0	9.8	3.85	0

FIGURE 4 | *Left panel*, SNPs in the exonic region causing non-synonymous amino acid substitutions are common in *ALDH1L1*. *Right panel*, SNP-associated haplotypes are markedly different between ethnic populations.

POTENTIAL IMPACT OF *ALDH1L1* EXONIC SNPS

The substitution of a single amino acid residue in the protein structure, caused by a SNP, could be mute or could cause significant alterations in protein properties. For example, one of the exonic SNPs in *MTHFR*, C677T, results in the A222V amino acid change in the FAD-binding catalytic domain of the enzyme. This substitution produces a less thermostable protein with reduced catalytic activity (Frosst et al., 1995). Another common exonic SNP in *MTHFR*, A1298C (Weisberg et al., 1998), exists in strong linkage disequilibrium with C677T (Stover, 2011) and results in the E429A enzyme variant. The effect on the enzyme activity of this substitution, which is in the regulatory domain of the protein, is less clear. Initial report indicated that this substitution decreases the enzyme activity though to a lesser extent than the A222V substitution (Weisberg et al., 1998). A later study of purified recombinant human MTHFR concluded that the E429A protein has biochemical properties that are indistinguishable from the wild-type enzyme (Yamada et al., 2001). *In vivo*, however, MTHFR is phosphorylated at multiple residues (Yamada et al., 2005), and both the A222V and E429A mutations are predicted to disrupt phosphorylation of neighboring Ser residues (Shahzad et al., 2013). Notably, the recently solved crystal structure of human MTHFR links the enzyme's phosphorylation state to its sensitivity to inhibition by S-adenosylmethionine (Froese et al., 2018).

Amino acid substitutions associated with common exonic *ALDH1L1* SNPs are found in each of the functional domains (**Figures 3** and **4**) but their effect on protein properties have not been studied. Analysis of the crystal structures of the ALDH1L1 domains identifies potential important structural roles for residues mutated by these polymorphisms. For example, Ser481 is an α-helix N-cap and its side chain makes a hydrogen bond with Gln549 in a different subunit, suggesting a role in protein oligomerization and stability. Two other residues affected by *ALDH1L1* SNPs, Asp793 and Ile812 (changed to Gly and Val, respectively) are strictly conserved through all species. Interestingly, these residues are adjacent on parallel β-strands and form backbone hydrogen bonds (**Figure 5**). This can be interpreted as a role in supporting protein conformation and stability. Of note, the co-occurrence of both SNPs is not found, suggesting that it perhaps would have too damaging a structural effect if both residues are changed. Our previous studies indicate that point mutations in the ALDH1L1 aldehyde dehydrogenase domain can significantly alter the protein conformation, with some of them impairing the protein's stability (Tsybovsky et al., 2007; Tsybovsky and Krupenko, 2011; Tsybovsky et al., 2013). Furthermore, a long-range communication between the aldehyde dehydrogenase catalytic center and the $NADP^+$-binding domain,

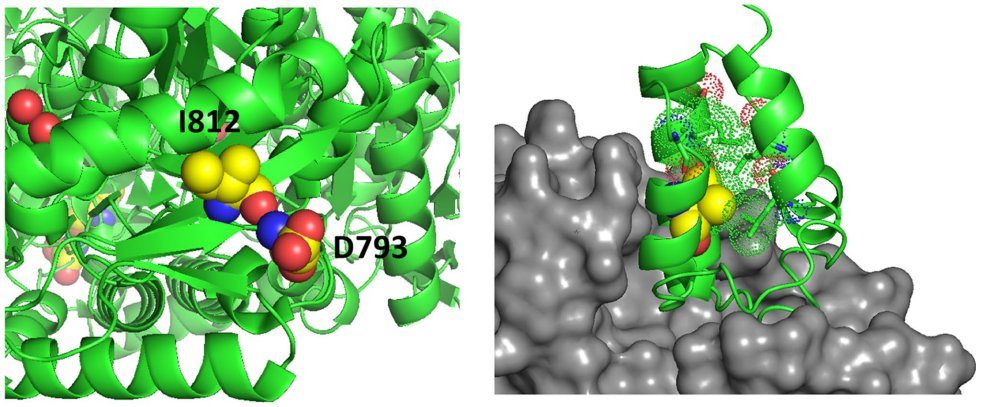

FIGURE 5 | *Left panel*, D793 and I812 are adjacent on parallel β-strands making backbone hydrogen bonds. *Right panel*, the structure of a phosphopantetheinyl transferase (*gray surface*) in complex with an ACP (acyl carrier protein) domain (*green ribbon*) shows that initial modification of the ACP domain serine (*spheres*) requires substantial access to the ACP surface. ACP helices 1 and 2 and the connecting loop lie on the surface of the transferase. The side chain of V330 (*yellow spheres*) packs in the interior of the ACP domain helical bundle. The substitution with Phe (rs2886059) will clash with surrounding residues (*dots*), likely causing a shift of the helix which contacts the transferase domain (*gray surface*) and interfering with binding.

observed previously (Tsybovsky and Krupenko, 2011), could transduce the effect of an amino acid substitution to distant domains with an unpredictable effect. In line with this notion, the structure of MTHFR suggests a long-range influence of S-adenosylmethionine binding in the regulatory domain of the enzyme on the catalytic domain some 300 amino acids away (Froese et al., 2018).

The SNP rs2886059 produces the V330F substitution in the intermediate domain of ALDH1L1, close to the modification site where the prosthetic group is attached (**Figure 5**). This substitution introduces a bulky side-chain in the core of the intermediate domain helical bundle which could interfere with the binding of phosphopantetheine transferase (PPTase) (Bunkoczi et al., 2007). PPTase appends the 4′-phosphopantetheinyl moiety to a serine in the intermediate domain and converts inactive apo-ALDH1L1 into active holo-ALDH1L1 (Strickland et al., 2010). Conformational changes associated with other SNPs could interfere with PPTase binding or hinder the ability of the intermediate domain to shuttle reactant between the catalytic domains. The SNP rs3796191 creates the L254P amino acid substitution in the C-terminal lobe of the N-terminal folate binding domain of ALDH1L1. In the structurally homologous enzyme, MTFMT, this sub-domain is responsible for the binding of methionyl-tRNA (Schmitt et al., 1996) but the role of this part of the ALDH1L1 molecule in the enzyme's function is not clear. It perhaps serves to properly align the folate-binding and the intermediate domains for the acceptance of the formyl group by the 4′-PP arm. Replacement of Leu with Pro will alter and restrict backbone conformation and loop flexibility, and perhaps cause a misalignment between the N-terminal and intermediate domains, impeding access to the folate-binding pocket. In fact, the role of this sub-domain for the proper ALDH1L1 function, likely through the proper orientation of the functional domains, has been demonstrated (Reuland et al., 2006).

Finally, as in the case with MTHFR, coding SNPs can affect ALDH1L1 stability and degradation rate. Towards this end, we have recently demonstrated that ALDH1L1 can be rapidly degraded through the ubiquitin-proteasome pathway (Khan et al., 2018). It is known that protein variants associated with non-synonymous SNPs can be differently degraded by the ubiquitin-proteasome pathway (Siegel et al., 2001; Bandiera et al., 2005). These findings raise the question of whether amino acid substitutions caused by coding SNPs will affect the ALDH1L1 degradation, which would affect the protein function as the proliferation regulator.

CONCLUDING REMARKS

While the phenomenon of ALDH1L1 silencing/down-regulation in cancer is now well recognized (Krupenko and

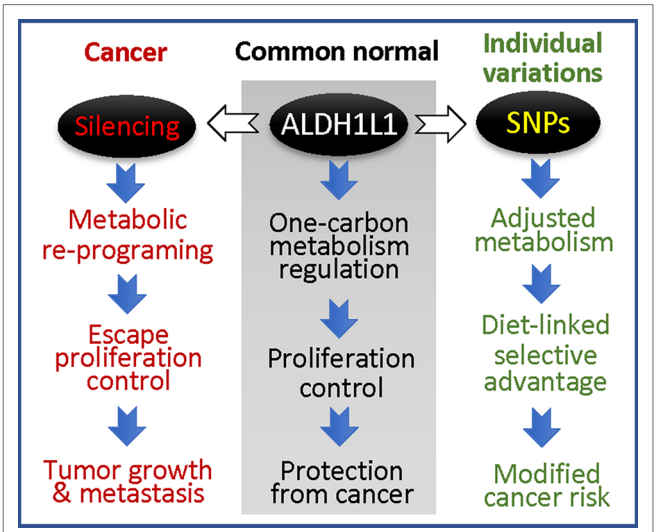

FIGURE 6 | ALDH1L1 is a main regulator of folate metabolism, and its gene is commonly silenced in cancer (the loss of the protein is linked to accelerated proliferation and tumor progression); coding SNPs in this gene are likely to modify cancer risk.

Krupenko, 2018; Krupenko and Krupenko, 2019), the effects of exonic SNPs on the protein function in tumorigenesis and tumor progression are not clear. It is also not known whether this gene is involved in tumor initiation or whether its loss provides selective advantage for tumor progression at later stages. The high prevalence of exonic SNPs causing non-synonymous amino acid substitutions in *ALDH1L1* raises the question of how these SNPs affect cellular metabolism and proliferation regulated by ALDH1L1. If ALDH1L1 polymorphic variants have altered activity or stability/half-life, they are likely to cause the imbalance of intracellular reduced folate pools with a consequent effect on *de novo* purine biosynthesis and amino acid metabolism. Overall, ALDH1L1-dependent metabolic reprogramming associated with functional exonic SNPs could be an important contributor to disease etiology with a more profound effect in populations with certain ALDH1L1 haplotypes (**Figure 6**). With regard to gene-diet interactions, the effect of dietary folate on the ALDH1L1 regulatory role is not clear, and the impact of functional SNPs is yet to be investigated. The understanding of how haplotype-specific effects are modified by folate supplementation could empower precision nutrition approach in disease prevention/treatment. Finally, since ALDH1L1 is involved in formate clearance, it could be an important component of the methanol detoxification pathway (Tephly, 1991). In this regard, it will be interesting to learn whether individuals with different *ALDH1L1* haplotypes have a different susceptibility to methanol toxicity.

AUTHOR CONTRIBUTIONS

SK conceived the project, performed analysis of ALDH1L1 gene for coding SNPs and wrote the manuscript. DH performed structural analysis of ALDH1L1 variants and participated in data analysis and manuscript writing.

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REVIEW
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The Epigenetic Connection Between the Gut Microbiome in Obesity and Diabetes

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Metabolic diseases are becoming an alarming health issue due to elevated incidences of these diseases over the past few decades. Various environmental factors are associated with a number of metabolic diseases and often play a crucial role in this process. Amongst the factors, diet is the most important factor that can regulate these diseases *via* modulation of the gut microbiome. The gut microbiome participates in multiple metabolic processes in the human body and is mainly responsible for regulation of host metabolism. The alterations in function and composition of the gut microbiota have been known to be involved in the pathogenesis of metabolic diseases *via* induction of epigenetic changes such as DNA methylation, histone modifications and regulation by noncoding RNAs. These induced epigenetic modifications can also be regulated by metabolites produced by the gut microbiota including short-chain fatty acids, folates, biotin and trimethylamine-*N*-oxide. In addition, studies have elucidated the potential role of these microbial-produced metabolites in the pathophysiology of obesity and diabetes. Hence, this review focuses on the interactions between the gut microbiome and epigenetic processes in the regulation and development of obesity and diabetes, which may have potential as a novel preventive or therapeutic approach for several metabolic and other human diseases.

Keywords: epigenetic, gut microbiome, diet, metabolic, obesity, diabetes

Abbreviations: SCFA, Short-chain fatty acid; TMA, Trimethylamine; FMO, Flavin monooxygenase; TMAO, Trimethylamine-*N*-oxide; ncRNA, noncoding RNA; HFD, High fat diet; miRNA, microRNA; siRNA, short-interfering RNA; piRNA, piwi-interacting RNA; lncRNA, long noncoding RNA; CpG, cytosine-guanine dinucleotide-rich; DNMT, DNA methyltransferase; SAM, S-adenosylmethionine; HAT, Histone acetyltransferase; HDAC, Histone deacetylase; Treg, regulatory T cells; ROS, Reactive oxygen species; LPS, Lipolysaccharide; TLR4, Toll-like receptor 4; GPR, G-protein coupled receptor.

THE GUT MICROBIOME

The gut microbiota consists of trillions of microorganisms including bacteria, archaea, viruses, and eukaryotes present in the intestine of humans (Allin et al., 2015). Generally, the gut microbiota is populated by *Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria, Verrucomicrobia, Tenericutes,* and *Lentisphaerae* as the predominant phyla. The major genera are *Pseudomonas, Streptococcus, Prevotella, Fusobacteria, Veillonella, Haemophilus, Neisseria, Porphyromonas, Bacteroides, Clostridium, Faecalibacterium, Eubacterium, Ruminococcus, Peptococcus, Peptostreptococcus, Lactobacillus, Streptomyces,* and *Bifidobacterium* (Marlicz et al., 2018; Neu and Pammi, 2018; Rea et al., 2018). Another similar term, gut microbiome, refers to the total genomes of gut microbiota and is often used to describe the entity of microbial functions encoded by gut microbiota (Schlaeppi and Bulgarelli, 2015). The advents in gene sequencing have revealed that the genome of human gut microbial communities (~3 million genes) is more than 100 times as large as the human genome (Gill et al., 2006), while the human:bacterial cells ratio is thought to be approximately 1:1 (Sender et al., 2016).

A neonate may procure its microbiota from the environment during delivery and also from its mother *via* breastfeeding (Dominguez-Bello et al., 2010). Studies have shown that neonates born through normal deliveries have an early and abundant composition of *Lactobacillus, Bacteroides*, and *Prevotella;* however, neonates born with cesarean deliveries have a delay in onset or lower levels of *Bacteroides, Bifidobacteria,* and *Lactobacillus* and predominately have colonization of *Clostridium difficile*, *Clostridium perfringens*, and *Escherichia coli* (Gronlund et al., 1999; Tsuji et al., 2012; Bokulich et al., 2016; Nagpal et al., 2016; Nagpal et al., 2017). These *Lactobacillus* species are known as probiotics due to their health-promoting properties and preventive properties in various metabolic diseases such as obesity and diabetes (Azad et al., 2018). However, *C. difficile* and *C. perfringens* bacteria are known for production of toxins, which can cause lethal diseases such as food poisoning, infection, diarrhea and colitis in humans (Noren, 2010).

During the early developmental phase, dietary factors also play a pivotal role in shaping the microbiota (Cotillard et al., 2013). For example, the gut microbiota of breast-fed neonates is mainly dominated by *Bifidobacteria*, *Lactobacillus, Staphylococcus,* and *Streptococcus* as compared to neonates that were on infant formulas, who have higher numbers of *Bacteroides*, *Clostridia,* and *Proteobacteria* (Favier et al., 2002; Bokulich et al., 2016). Various other studies have also elucidated that diet has a strong impact on maturation as well as maintenance of the gut microbiome (De Filippo et al., 2010; David et al., 2014) and potentially on the health of an individual (Camilleri et al., 2019). In addition to dietary factors, other potential variables such as antibiotic intake and infections can also influence gut microbiota (Qin and Wade, 2018) and can result in the disproportion and reduction of microbial biodiversity which is known as gut dysbiosis. These factors influencing the development of the gut microbiota are depicted in **Figure 1**.

A newborn infant's microbiota starts to develop according to dietary and environmental factors, but lacks an abundant diversity of commensal microorganisms. Over the first few years of life, the young child's microbiota undergoes substantial alterations, ultimately transitioning to a more mature pattern by age 3 or 4, by which time the pace of change slows down (Koenig et al., 2011; Yatsunenko et al., 2012; Stewart et al., 2018). During adulthood, in the absence of major environmental perturbations, the microbiota appears to be stable for many years, perhaps the whole lifespan (Mehta et al., 2018). These microbiota and their functions represent a "core gut microbiome," which consists of particular types of gut microbial species, which resides in every

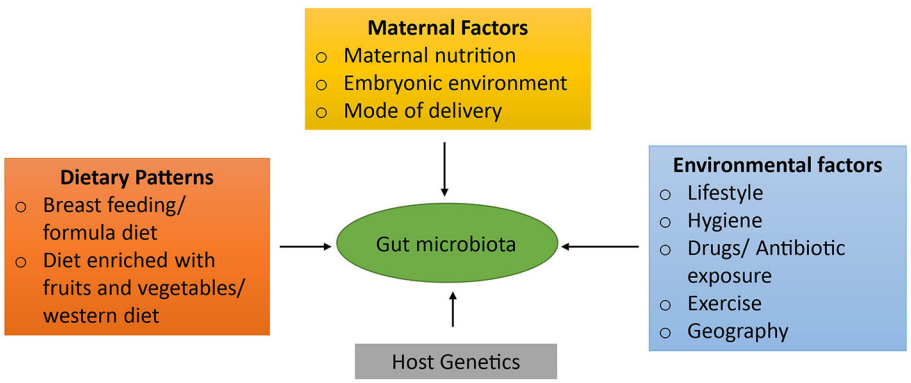

FIGURE 1 | Factors influencing the development of gut microbiota. Numerous factors play a paramount role in the development of gut microbiota. Initially, the gut microbiota is acquired from a mother during the pregnancy *via* maternal nutrition, embryonic environment, and the mode of delivery. In addition, dietary patterns plays the most crucial role in maturation as well as maintenance of the gut flora such as breast feeding, formula diet, the composition of diet: diet rich in fruits and vegetables or western diet. Other factors that also contribute to its shaping are individual's genetic factors and environmental factors such as drug or antibiotic intake, infections, life style patterns, migration to a different location, etc.

individual and is mainly responsible for the correct functioning of a gut microbial ecosystem (Turnbaugh and Gordon, 2009; Qin et al., 2010). Gut commensal communities impact several metabolic functions within the human body such as digestion, nutrients absorption, regulation of intestinal hormones secretion, modulation of intestinal immunity and inflammatory processes, synthesis of vitamins, amino acids, and various metabolites such as short-chain fatty acids (SCFAs), choline, and lipids (Nicholson et al., 2012; Allin et al., 2015; Devaux and Raoult, 2018). In addition to the metabolic functions, the gut microbiome also shapes gene expression in the host. Through various microbial-derived metabolites, gut bacteria can influence the host metabolism by inducing epigenetic alterations of key genes which modulate the initiation and progression of diseases (Yuille et al., 2018). Therefore, gut dysbiosis has been associated with an increasing incidence of conditions such as metabolic diseases such as type 2 diabetes (Allin et al., 2015) and obesity (Ley et al., 2006); inflammatory diseases such as inflammatory bowel disease (Pascal et al., 2017) and rheumatoid arthritis (Maeda and Takeda, 2017); liver diseases (Janssen et al., 2017); and cancers (Lofgren et al., 2011; Yu et al., 2017).

With the increasing prevalence of metabolic disorders such as obesity and diabetes in Western countries, it is essential to focus on the interplay between epigenetic mechanisms and gut microbial composition in the induction of these diseases that might provide a novel therapeutic approach for prevention and treatment. Hence, we will discuss the potential roles of the gut microbiome and epigenetics in the pathophysiology and pathogenesis of obesity and diabetes (type 1 and type 2).

THE GUT MICROBIOME AND EPIGENETICS

Epigenetics is the study of phenotypic changes secondary to alterations in gene expression that do not directly arise from changes in the underlying DNA sequence. In eukaryotes, epigenetic mechanisms primarily involve DNA methylation, posttranscriptional histone modifications, chromatin restructuring, and regulation of gene expression by noncoding RNAs (Paul et al., 2015). These epigenetic mechanisms can be regulated by cross-talk of microbial metabolites, external factors such as diet, antibiotics and also by other environmental factors (pH, oxygen, and temperature), resulting in the modulation of a large number of human metabolic diseases (Romano and Rey, 2018).

Noncoding RNA

The noncoding RNA (ncRNA) are RNA transcripts that are not translated into the proteins (Dempsey et al., 2018). The alterations in ncRNA have been known to contribute to various diseases such as obesity, diabetes, neurodegenerative diseases, liver diseases, and lung diseases (Devaux and Raoult, 2018). The major types of ncRNAs are microRNA (miRNA), short-interfering RNAs (siRNAs), piwi-interacting RNAs (piRNAs), and long noncoding RNAs (lncRNAs). lncRNAs modify gene expression *via* the formation of complexes with chromatin-modifying protein and by functioning as signaling and guide molecules (Devaux and Raoult, 2018). A few studies have focused on the role of the gut microbiome in regulation of lncRNA gene expression in the host. In order to obtain in-depth knowledge about their correlation, Liang et al. performed a bioinformatics study on characterization of lncRNA regulated by gut microbiome in intestinal epithelial tissues of mice (Liang et al., 2015). The comparison between germ-free mice with the conventional (re-colonized with mice microbiota) and gnotobiotic mice (recolonized with either *E. coli* or *E. coli*-expressing bile salt hydrolase) showed that only six lncRNAs were commonly upregulated. These lncRNAs were also found to be overexpressed in immune organs such as thymus and spleen, which may reflect their crucial role in immune processes. Furthermore, this study also elucidated that lncRNA expression profiles successfully differentiated gontobiotic mice from conventional mice based on their gut microbial composition. Despite the less available information on the association of lncRNA and gut microbiota, this study provided novel insight of microbial-regulated lncRNA expression. This study may also enhance understanding of the potential roles played by lncRNA expression that are regulated by gut microbiota and in-turn influence metabolic disorders such as obesity and diabetes. However, this study had a limitation with respect to determining whether the expression of lncRNAs was regulated by the gut microbial communities of the host (Liang et al., 2015). Another study by Dempsey et al. evaluated lncRNA expression in various tissues- liver, duodenum, jejunum, ileum, white adipose tissue, brown adipose tissue, colon, and skeletal muscle from germ-free and conventional mice (Dempsey et al., 2018). They found, in the absence of microbial communities (i.e., germ-free mice), the lncRNAs were differentially regulated in distal (liver, muscle, and fat) tissues as well as in proximal (intestinal) tissues. Most of the lncRNAs which were found to be regulated by gut microbiome were present in an essential metabolic organ, namely, the jejunum. Overall, this is the first study that demonstrated that the gut microbiome is important for the lncRNA expression in the various metabolic and other organs (Dempsey et al., 2018). These studies implicate a potential role played by the gut microbiome in regulation of lncRNA expression. However, more investigations in this context should be conducted to further clarify the role of this association of host-microbe interactions with the pathogenesis of obesity and diabetes.

Another type of ncRNA, miRNAs, also play crucial roles in maintaining metabolic homeostasis and in development of obesity and insulin resistance (Davalos et al., 2011; Trajkovski et al., 2011), This has also been shown in a recent study by Virtue et al. that focused on the interplay between miRNAs and development of obesity through gut microbial population in germ-free and conventionally housed specific pathogen-free mice (Virtue et al., 2019). The investigators found that the *miR-181a* and *miR-181b* expression were increased in epididymal white adipose tissues obtained from conventional mice as compared to germ-free mice, which implicates the importance of gut microbiota in the regulation of miRNA. In order to confirm this regulation, they colonized germ-free mice with conventional mice microbiota and found similar results,

that is, upregulation of *miR-181a* and *miR-181b* in epididymal white adipocytes. The investigators hypothesized that the potential mechanism behind the regulation of *miR-181* might be *via* gut microbial-produced metabolites. This leads to the finding that tryptophan-derived metabolites negatively regulate *miR-181* expression in white adipocytes, which further influence the pathways of adiposity, energy balance and insulin sensitivity. In addition, this study also revealed the imbalance of the microbiota—*miR-181* axis is vital for obesity and insulin resistance development (Virtue et al., 2019). Therefore, this novel study elaborates that the gut microbiota might serve as a key regulator for functioning of miRNAs especially in white adipocyte tissue, which have a major impact on the development of obesity and insulin resistance. However, this study has not provided the role of specific gut microbial species that may be critically involved in the regulation of *miR-181* expression. Further well-designed studies are required for elucidating the role of the gut microbiome on miRNA in the etiology of obesity as well as in diabetes.

DNA Methylation

DNA methylation refers to the inclusion of a methyl group ($-CH_3$) on the carbon-5 of the cytosine ring in cytosine-guanine dinucleotide-rich (CpG) regions (Romano and Rey, 2018). The enzymes, DNA methyltransferases (DNMTs) catalyze this process and also regulate the gene expression. These DNMTs are highly sensitive to the availability of nutrients that can also be affected by the metabolic activities of the microbial species present in the gut (Romano and Rey, 2018). The major metabolic activity involves synthesis of metabolites that can modulate the epigenome by participating in one-carbon metabolism (Mischke and Plosch, 2013). Metabolites such as folate, vitamin B12, betaine, and choline are potentially involved in the synthesis of 6-methyltetrahydrofolate, which is a methyl group donor, for the generation of S-adenosylmethionine (SAM) that participates in DNA methylation processes (Kovacheva et al., 2007; Crider et al., 2012; Kok et al., 2015; Zeisel, 2017; Mahmoud and Ali, 2019). These methyl donor nutrients are found to be regulated by specific gut microbial communities such as *Lactobacillus* and *Bifidobacteria* that are known for folate production (Strozzi and Mogna, 2008; Rossi et al., 2011). In order to understand the role played by *Lactobacillus* and *Bifidobacterium* in regulation of diabetes, a study by Murri et al. found their levels were reduced in type 1 diabetic healthy Caucasian children (Murri et al., 2013). These studies show an important association between gut microbial communities and DNA methylation mechanisms that may regulate diabetes.

Other crucial metabolites, such as SCFAs are synthesized in the gut by the fermentation of nondigestible carbohydrates by certain microbes. Some of the major SCFAs including butyrate can also influence DNA methylation processes by inducing phosphorylation of *ERK* (MAP kinase1), which results in down-regulation of DNMT1 and consequently demethylation of tumor suppressor genes including *RARB2*, *p21,* and *p16* (Sarkar et al., 2011). The amount of butyrate synthesis depends on both dietary intake and synthesis by gut microbial communities. Various bacteria are known for butyrate production including *Megasphaera, Odoribacter, Eubacterium, Peptoniphilus, Fusobacterium, Coprococcus, Porphyromonas, Faecalibacterium, Anaerotruncus, Clostridium, Subdoligranulum,* and *Roseburia* (Demehri et al., 2016). The butyrate-producing bacteria, *Clostridium,* was found to be present in abundant levels in p21-*p*-luc male mice, which were treated with a high-fat diet (HFD) diet for thirty weeks when compared with mice on a normal diet (Yoshimoto et al., 2013), indicating the potential role of diet in impacting the gut microbiota and downstream metabolic processes. Similarly, the number of *Clostridium* was increased in type 1 diabetic children (Murri et al., 2013). These studies suggest an important role by gut microbiota in regulation of obesity and diabetes *via* production of gut metabolites.

Several other crucial nutrients also play a vital role in the regulation of the DNA methylation process (Liu et al., 2019). For instance, choline is a water-soluble vitamin-like nutrient found in various food sources such as meat, grain, milk, egg, and their derived products. Gut communities can metabolize choline into several metabolites that impact human health, such as trimethylamine (TMA) (Romano et al., 2015). TMA can be further metabolized by flavin monooxygenase (FMO) enzymes into trimethylamine-*N*-oxide (TMAO) (Baker and Chaykin, 1960), which has been linked to obesity and diabetes (Fennema et al., 2016; Velasquez et al., 2016; Tang and Hazen, 2017). This choline derived metabolite, TMAO was also found to be involved in vast production of ROS (Sun et al., 2016), that in-turn can influence epigenetic programming as it can lead to deamination or depurination of nucleic acids, which may trigger DNA repair mechanisms and replacement with a nonmethylated cytosine (Avila et al., 2015). To investigate the relationship between choline and metabolic disorders *via* DNA methylation, a study was conducted on germ-free C57BL/6 female mice (Romano et al., 2017). These mice were divided into two different groups based on the colonization in gut; a) with choline-utilizing bacteria, or b) with bacteria unable to utilize choline and unable to produce TMA. Both mouse groups were kept on HFD supplemented with choline for several weeks, which resulted in lower methylation levels in heart, colon, brain, and liver tissues in the first group of mice as compared to the other group. In addition, the mice colonized with choline-utilizing bacteria displayed adiposity features. It was concluded that choline-utilizing bacteria compete for choline uptake with the host, resulting in lower levels of choline and methyl donor in the host, ultimately making the host more susceptible toward metabolic disorders (Romano et al., 2017). These studies implicate the association of choline in modulating epigenetic machinery that might act as a key player in the pathophysiology of metabolic diseases. However, more studies are required to confirm the exact mechanisms induced by choline on modulating the DNA methylation process.

Histone Modifications and Chromatin Remodeling

Histones are proteins that are wound by DNA in the nucleus to form the condensed chromatin and mainly comprise of four families: H1, H2A, H2B, H3, and H4. Histones are prone to

various modifications including acetylation, methylation, phosphorylation, SUMOylation, poly-ADP ribosylation, biotinylation, ubiquitination, citrullination, and proline isomerization (Bernstein et al., 2007; Paul et al., 2015). Out of all modifications, histone methylation, acetylation, and deacetylation are known to play key roles in the induction and progression of various disorders. The histone methylation process involves the addition of methyl groups to the histone proteins by histone methyltransferases (HMTs) enzymes. Histone methylation can either lead to transcription activation (e.g., H3K4) or transcription inactivation (e.g., H3K9, H3K27), depending on the specific residue and modifications (Orouji and Utikal, 2018). A recent study by Tateishi et al. showed a correlation of histone methylation with obesity and hyperlipidemia in mice. They found that impairment in Jhdm2a function (a H3K9-specific histone demethylase) in *Jhdm2a* knockout mice resulted in altered β-adrenergic-stimulated glycerol release and oxygen absorption in brown fat. This study also revealed that β-adrenergic activation induces Jhdm2a binding to the PPAR responsive element of the *Ucp1* gene, an important gene involved in energy balance and that leads to a decrease in H3K9me2, which contributed to obese phenotypes such as fat deposition and rise in lipid content (Tateishi et al., 2009).

Histone acetyltransferases (HATs) enzymes catalyze the transfer of acetyl groups from acetyl-CoA to the amino-terminal lysine residues on histone proteins (Roth et al., 2001). The histone acetylation process can be regulated by various gut-microbial derived metabolites such as SCFAs (Krautkramer et al., 2016; Qin and Wade, 2018). It has been found that supplementation with acetate raised the acetylation levels of brain histones H3 at lysine 9 and H4 at lysine 8 and 16 that resulted in neuroglial activation and decline in the cholinergic cell (a nerve cell) in a rat model of LPS-induced neuroinflammation (Soliman and Rosenberger, 2011). A recent study by Wang et al. investigated the impact of lentinan, a polysaccharide derived from mushroom, on the intestinal microbiota of piglets that were challenged with *E. coli* lipopolysaccharide-induced intestine injury. They found that lentinan supplementation increased SCFAs levels including butyrate, propionate, iso-butyrate, and isovalerate in the cecum, which further led to a rise in H3 histone acetylation and a decline in intestinal inflammation (Wang et al., 2019b). It has been evident that the chromatin state of several tissue constituents such as colonic cells, can be modulated by SCFAs produced in the gut (Krautkramer et al., 2016).

Histone deacetylases (HDACs) constitute a class of enzymes that remove an acetyl group from the amino-terminal lysine residues of histones resulting in compacted chromatin (Yuille et al., 2018). Histone deacetylation is primarily associated with transcriptional inactivation and overexpression of HDACs and has been linked to a number of neurological and inflammatory diseases. Overall, 13 HDACs have been found in humans, which are classified into four classes- Class I contains HDACs 1, 2, 3, and 8; Class IIa consists of HDACs 4, 5, 7, and 9; Class IIb contains HDACs 6 and 10; Class III is comprised of Sirt1-Sirt7 and Class IV consists of HDAC 11. Each of these plays an important role in cell survival, proliferation and differentiation which can also influence tumorigenesis (Yuille et al., 2018). HDAC inhibitors have well-known potential to act as therapeutic agents in various diseases. The gut microbiome can modulate the activity of HDACs *via* production of epigenetic metabolites such as the SCFAs. Butyrate and propionate have been identified as potential contributors to HDAC inhibition (Marlicz et al., 2018). Butyrate is essential for maintaining homeostasis in the gut and is also important in the regulation of many processes such as epigenetic mechanisms, lipogenesis, gluconeogenesis, and inflammatory conditions. Among various epigenetic modifications, butyrate is specifically known as a class I and class II HDAC inhibitor (Marlicz et al., 2018). An interesting study focused on 79 distinct commensal human gut bacteria to investigate the connection between SCFA profiles and HDAC inhibitory properties. These findings revealed that three butyrate-producing bacterial strains: *Megasphaera massiliensis* MRx0029, *Roseburia intestinalis* MRx0071, and *Bariatricus massiliensis* MRx1342, manifested the highest inhibition of HDAC activity. In addition, *M. massiliensis* produced significant levels of valeric acid and hexanoic acid, which are medium-chain fatty acids. It was also reported that valeric acid and butyrate cumulatively showed inhibition against Class I HDACs- HDACs1, 2, 3 8, particularly HDAC2 (Yuille et al., 2018).

GUT MICROBIOME, EPIGENETICS, AND OBESITY

Obesity is a leading disorder that involves accumulation of excessive fat in the body. Obesity has been found to be linked with multiple conditions including cardiovascular diseases, diabetes, metabolic disorders, and cancers (Kopelman, 2000). There are various factors that have been shown to play a key role in the pathophysiology and pathogenesis of obesity such as genetic susceptibility, dietary patterns, ethnic differences, antibiotic intake, and environmental factors.

Dietary Patterns

Diet is a vital factor in the establishment of the composition of gut microbiota, which cross-talks with the intestine, and participates in the generation of signals to communicate with distal organs such as the liver. It therefore plays an integral role in shaping the host's metabolism (Schroeder and Backhed, 2016). Differences in dietary patterns can lead to alterations in the composition and function of microbial communities, resulting in change in fermentation processes, energy consumption, sensation, and permeability in a manner that results in weight gain (Kumar et al., 2014; Devaux and Raoult, 2018). Beside these, recent evidence suggests that diets strongly influence epigenetic processes that are linked to obesity development (Qian et al., 2017). Numerous studies have indicated that the shifts in microbial communities mainly due to consumption of a "Western" diet, which is high in fat and carbohydrate, result in predisposition toward obesity. Specifically, a decrease of Bacteroidetes and an increase of Firmicutes levels have been

linked with the consumption of a western diet (Ley et al., 2006). These bacterial communities have been known to directly influence the epigenetic reprogramming *via* DNA methylation. As an illustration, Kumar et al. reported that the infants born from mothers who had a higher Firmicutes gut composition showed altered DNA methylation as compared to infants born from mothers with higher composition of Bacteroidetes in the gut. This study also reported that the differentially methylated genes of infants whose mothers had high levels of Firmicutes were positively correlated to cardiovascular diseases, inflammation, obesity, and abnormal lipid metabolism (Kumar et al., 2014).

In contrast, studies have shown that certain plant-based diets have been linked to a particular spectrum of bacteria that ameliorates development of obesity. For example, a recent study focused on the impact of ginger on gut microbiota and prevention of obesity in C57BL/6J mice (Wang et al., 2019a). The mice were divided into four groups based on the feeding of normal diet or HFD diet with or without ginger. It was found the mice on the HFD and ginger diets showed a decrease in weight, as well as diminished low-grade inflammation and insulin resistance. The microbiota profile associated with these changes included abundance of *Bifidobacterium* genus and major SCFA-producer bacteria such as *Alloprevotella* and *Allobaculum*. To address whether these gut microbial differences were responsible for the metabolic improvements, the investigators performed fecal microbiota transplantation into mice whose microbiota was depleted by antibiotics. The transplants were technically successful, insofar as they recapitulated the microbiota profiles of the donor mice. Importantly, the mice that received microbiota from the HFD and ginger diet group showed a decrease in body weight, body mass and improvement in glucose tolerance as compared to recipients of microbiota from the HFD group. This study confirms the association of particular microbial species with the ginger-supplemented diet and provides an underlying mechanism of prevention of obesity *via* these microbial species (Wang et al., 2019a). Multiple other species of bacteria may beneficially impact metabolic processes. For example, *Bifidobacterium* has been known to impact metabolic events such as insulin resistance, low-grade inflammation, and obesity in mice (Zhang et al., 2017). Similarly, the SCFA-producers *Alloprevotella* and *Allobaculum* have been found to be associated with improvement in obesity and insulin resistance (Zhang et al., 2015). Also, the SCFAs produced from these bacteria such as butyrate and propionate are known to strongly influence molecular pathways that impact obesity development (Lin et al., 2012). The major SCFAs such as butyrate, propionate and acetate bind to G-protein coupled receptors (GPRs) - GPR41 (Free Fatty Acid Receptor 3) and GPR43 (Free Fatty Acid Receptor 2) expressed on the intestinal mucus layer of immune cells, liver cells, and adipose tissue (Brahe et al., 2013), resulting in decrease in weight gain, less intake of food and liposysis inhibition in adipose tissues (Lu et al., 2016). In addition to these, various other studies have focused on changes in intestinal composition with obesity and its role in metabolic mechanisms and epigenetics as highlighted in **Table 1**. Therefore, diets may

TABLE 1 | The alterations in gut microbiota in obesity and its role in metabolic mechanisms and epigenetics.

Study design	Method	Gut microbiota profile	Associated metabolic mechanisms	Association between microbiota and epigenetic modifications	References
Comparison of gut microbiota between obese and lean individuals. Furthermore, twelve obese individuals were randomly categorized to either fat-restricted or carbohydrate-restricted low calorie diet	16S rRNA gene sequencing of stool samples	Decrease of *Bacteroidetes* and increase of *Firmicutes* levels in obese individuals as compared to lean people After dietary treatments, increase in *Bacteroidetes* and decrease in *Firmicutes* levels were observed in both types of diets	*Bacteroidetes* are found to be correlated with intake of energy and fat (monounsaturated, polyunsaturated, and saturated fat)	Hypomethylation and upregulation of *HDAC7* and *IGF2BP2* in adipose tissue were associated with lower *Bacteroidetes* to *Firmicutes* ratio group when compared with high *Bacteroidetes* to *Firmicutes* ratio group	(Ley et al., 2006) (Mendez-Salazar et al., 2018) (Ramos-Molina et al., 2019)
Analysis of gut microbiome diversity and richness of undernourished (n = 12), obese (n = 12), and normal weight (n = 12) Mexican school-age children	16S rRNA gene sequencing of fecal samples	Decrease in bacterial richness and diversity in undernourished and obese with comparison to normal weight group. In addition, abundant levels of *Lachnospiraceae* family and Firmicutes phylum in undernourished children than obese children. Rise in levels of Proteobacteria phylum and *Bilophila* in obese group	*Lachnospiraceae* abundance associated with leptin and negatively associated with energy intake	*Firmicutes* dominant gut microbiota showed differential methylation profile of gene promoters, associated with obesity and lipid metabolism	(Mendez-Salazar et al., 2018) (Kumar et al., 2014)
The p21-*p*-luc male mice (n = 19) were treated with dimethylbenz(*a*) anthracene, and then fed either normal or HFD diet for thirty weeks	Meta 16S rRNA gene sequencing	Abnormal rise in the levels of gram-positive bacteria, specifically Clostridium genus in mice on HFD	Significant amounts of deoxycholic acid (DCA), a gut metabolite, were found in HFD-fed mice	DCA has been known to be involved in DNA damage. The enterohepatic circulation of DCA resulted in development of obesity-associated hepatocellular carcinoma in mice	(Yoshimoto et al., 2013)

act as a bridge that links the gut microbiome and host metabolism and that contributes to altered health outcomes through, at least in part, regulation of epigenetic mechanisms.

The direct association between diet, gut bacteria, and epigenetic factors that leads to obesity development have been highlighted in **Figure 2**. Epigenetic mechanisms such as histone modifications and DNA methylations play a key role in the development of obesity (Qian et al., 2017; Lieber et al., 2018). As a study found that loss of HDAC6 has been associated with a rise in acetylation of a protein called "cell death–inducing DNA fragmentation factor subunit α-like effector C," which further led to an increase in lipid droplet storage and ultimately body weight gain (Qian et al., 2017). DNA methylation also plays a promising role in regulation of obesity; this was demonstrated in a study involving administration of *Lactobacillus rhamnosus* and *Bifidobacterium lactis* to pregnant women, which resulted in a decrease in the methylation of the *FTO* and *MC4R* promoters in the women and their children (Vahamiko et al., 2019). The significance of these findings is that these genes such as *FTO* has been associated with weight gain and BMI; and is well-known risk factor of obesity (Claussnitzer et al., 2015; Qi et al., 2015). Also, *MC4R* gene involves in the key metabolic processes such as regulation of food consumption and energy balance; and the abnormalities in this gene can lead to decrease in satiety and onset of obesity (Fani et al., 2014; Rovite et al., 2014). Overall, this study has provided an important insight that supplementation of probiotics during pregnancy can modulate DNA methylation of promoter region of genes that are associated with weight gain and obesity in mothers and their children (Vahamiko et al., 2019). In the future, more research will be required to elaborate on the interaction between the gut microbiome and DNA methylation modifications that may play key roles in regulation of obesity.

Inflammation

Several studies have implicated the interplay between inflammation and obesity. For instance, the inflammatory cytokines such as interleukin 1, interleukin 6, tumor necrosis factor, and C-reactive protein were found to be associated with the obesity markers- BMI, waist circumference, or percentage body fat (Marques-Vidal et al., 2012). Obesity is also associated with inflammatory conditions, such as juvenile psoriatic arthritis. These individuals were found to have a greater risk of obesity as compared to healthy children as well as children with other forms of juvenile arthritis. (Samad et al., 2018). Low-grade inflammation may contribute significantly to obesity development as high levels of activated $CD8^{+}$T cells and aggravated immune response were observed in adipose tissue of HFD-fed mice (Nishimura et al., 2009). Regulatory T (T_{reg}) cells, a subpopulation of T-helper cells, maintains balance between proinflammatory and antiinflammatory immune responses. Studies have shown dependency of T_{reg} cell on the intestinal communities as they acquire signals from the gut microbiota (Romano-Keeler et al., 2012). Forkhead box P3 (Foxp3) is a transcription factor expressed by T_{reg} cells, that plays a crucial role in the development and function of T_{reg} cells (Hori et al., 2003). This Foxp3 protein is regulated posttranslationally by lysine acetylation by HDACs and HDAC inhibition (Wang et al., 2015). Therefore, the products of gut microbiota such as SCFA, which act as HDAC inhibitors, may be involved in T_{reg} cell differentiation and ameliorate inflammatory states such as obesity. For example, butyrate plays an essential role in the differentiation of T_{reg} cells *via* increasing the acetylation of noncoding regions of the *Foxp3* locus (Furusawa et al., 2013). It specifically acts as a HDAC inhibitor in intestinal epithelial cells and thereby alters metabolic functions. For example, HDAC3 knockout mice did not show obesity features (improvement in glucose tolerance and insulin

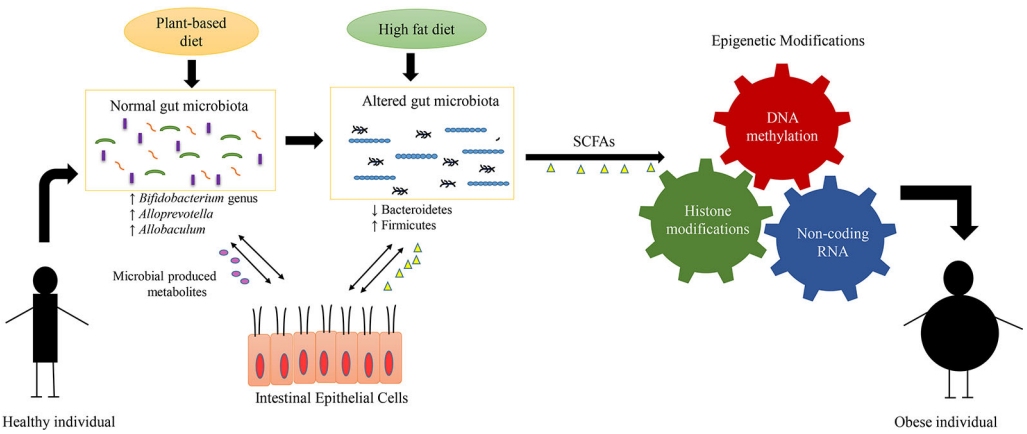

FIGURE 2 | Interplay between diet, gut microbiome, epigenetic mechanisms, and obesity. The alterations in composition of microbial communities mainly arise due to difference in dietary patterns. The plant-based diets have been known to be associated with diverse and particular gut flora such as *Bifidobacterium* genus, *Alloprevotella,* and *Allobaculum*. The diverse composition of gut microbiota results in production of various metabolites such as short-chain fatty acids (SCFA). These gut microbial-produced metabolites interact with the epithelial cells of the host and help to maintain the host metabolism. On the other hand, the diets enriched in fat and carbohydrate, result in lowered gut diversity and alterations in the composition of gut microbiota such as decrease in levels of Bacteroidetes and increase in levels of Firmicutes. This gut dysbiosis (alterations in composition and function of gut bacteria) produce metabolites that induce specific epigenetic alterations such as DNA methylation, histone modification and noncoding RNA, which in-turn regulate the development of obesity.

levels) despite being on the same HFD as wild-type C57BL6 mice. Further analysis of intestinal epithelial cells from HDAC3 knockout mice showed altered expression of *Chka*, *Mttp*, *Apoa1,* and *Pck1* (an enzyme involved in gluconeogenesis and glyceroneogenesis processes in adipose (Franckhauser et al., 2002)), which are associated with multiple metabolic processes in the intestinal epithelial cells. Thereafter, butyrate supplementation in control mice resulted in weight loss and refinement of metabolic functions (Whitt et al., 2018).

GUT MICROBIOME, EPIGENETICS AND DIABETES MELLITUS

From the past decade, there has been a significant increase in the incidence of Diabetes (Zhou et al., 2016). Type 1 diabetes is an autoimmune disease that arises when T-cell mediated destruction of insulin-producing β cells occurs (Gianani and Eisenbarth, 2005), whereas type 2 diabetes is a chronic disease which commences when insulin resistance develops in the body (Chen et al., 2011). Due to acquired insulin resistance, more insulin, which is produced by the pancreas, is required. However, the pancreas fails to generate enough insulin, which in turn increases the blood glucose level (Larsen et al., 2010). A large number of factors such as environmental, genetic and lifestyle factors influence both type 1 and type 2 diabetes (Qin et al., 2012). Various studies have provided evidence of a direct relationship between epigenetic mechanisms and the gut microbiome in the etiology of both types of diabetes (Allin et al., 2015).

Recent experimental data from human studies have shown that the gut microbial composition has a crucial role in the development of type 1 diabetes (Brown et al., 2011; Giongo et al., 2011). As also highlighted in **Table 2**, the study by Giongo et al. focused on the children at high risk for developing type 1 diabetes based upon at-risk HLA types (Giongo et al., 2011). They were followed prospectively for the development of antibodies associated with diabetes and the microbiota of children who ultimately developed antibodies was compared to that of the children who did not develop antibodies. They found that prior to disease onset, there were alterations in gut microbiota such as reduction in Firmicutes and increase in Bacteroidetes and decreased diversity in the infants who produced antibodies (Giongo et al., 2011). However, whether the observed microbial difference arose due to differences in dietary patterns of children is not yet known. Similarly, another study by Murri et al. focused on the gut microbial profile of children diagnosed with type 1 diabetes and healthy children (Murri et al., 2013). The results of this study revealed increased

TABLE 2 | Role of the gut microbiome in induction of type 1 diabetes and the association between gut microbiota, metabolic mechanisms, and epigenetic modifications.

Study design	Method	Gut microbiota profile	Associated metabolic mechanisms	Association between microbiota and epigenetic modifications	References
The fecal samples were obtained from Finnish children (n = 8), before the development of antibodies associated with type 1 diabetes, at three different time intervals	16S rRNA gene sequencing of stool samples	Reduction in levels of Firmicutes and increase of Bacteroidetes in diabetic children as compared to healthy children	Bacteroidetes play a role in polysaccharides metabolism	This ratio has been linked with lower levels of methylation in *TLR 2* promoter region and in the first exon of *TLR 4*	(Giongo et al., 2011) (Mahowald et al., 2009) (Remely et al., 2014)
Analyses of fecal bacterial composition of type 1 diabetic (n = 16) and healthy Caucasian children (n = 16)	PCR-denaturing gradient gel electrophoresis and qPCR	Increased levels of *Bacteroidetes*, *Clostridium* spp. and *Veillonella*; decline in *Lactobacillus*, *Bifidobacterium*, *Blautia*, and *Prevotella* in diabetic children	*Lactobacillus* and *Bifidobacterium* were found to be associated with excessive levels of plasma glucose in diabetic children *Bifidobacterium* have also been associated with improved metabolism of glucose, low-grade inflammation, and insulin resistance	*Bifidobacterium* was associated with metabolic mechanisms and improvement in insulin resistance and low-grade inflammation. It was also associated with lowering the incidence of autoimmune diseases in genetically predisposed individual	(Murri et al., 2013) (Philippe et al., 2011) (Eslami et al., 2019) (Zhang et al., 2017) (Mu et al., 2017)
Four cohort of participants with or without islet autoimmunity residing in the U.S.	16S rRNA gene sequencing	Elevation in *Bacteroides* and *Akkermansia* and decrease in abundance of *Prevotella*	*Akkermansia* is a known acetate and propionate producing bacteria *Prevotella* are SCFAs producing bacteria and associated with long-term consumption of dietary fiber	*Akkermansia* were found to decrease the expression of *Gpr43* and *Pparγ* and increase the expression of *Hdac3* and *Hdac5*	(Alkanani et al., 2015) (Mojica and de Mejia, 2015) (Lukovac et al., 2014)
Fecal bacteria investigation of type 1 diabetic (n = 15) and healthy (n = 15) Chinese children	16S rRNA gene sequencing	Increased composition of *Blautia* and decreased composition of *Lachnospira*, *Dialister*, *Haemophilus,* and *Acidaminococcus* in diabetic children	*Blautia* breakdowns undigested proteins and carbohydrates into acetic acid, which may generate energy in the human body	Cpf1 proteins from *Acidaminococcus* and *Lachnospiraceae* showed significant genome-editing efficacy to Cas9	(Qi et al., 2016) (Li et al., 2017)

levels of *Bacteroidetes*, *Clostridium* spp. and *Veillonella* as well as decline in *Lactobacillus*, *Bifidobacterium*, *Blautia*, and *Prevotella* in diabetic children when compared with healthy children. These alterations in gut microbiota were independent of dietary patterns, as no significant difference between the dietary habits was observed between both groups of children (Murri et al., 2013). The resulting limited gut microbial diversity creates an imbalance of the microbial ecosystem and essential processes in the gut which may reduce the diverse diet digestion capacity that eventually leads to a decrease in energy levels in type 1 diabetes patients and cause them to be more prone toward diseases (Giongo et al., 2011). The knowledge acquired from this study may allow interventions that can cure or delay autoimmunity in patients by changing the gut microbiota through epigenetic regulations.

Additional evidence has shown that the intestinal microbiota community is also associated with type 2 diabetes. As highlighted in **Table 3**, a study in the Chinese population revealed a decrease in the *Roseburia* and *Faecalibacterium* species and increase in *Escherichia coli* in type 2 diabetes patients as compared to nondiabetic control subjects (Qin et al., 2012). Both *Roseburia* and *Faecalibacterium* species are SCFAs-producing bacteria and also possess antiinflammatory properties (Zhang et al., 2013; Remely et al., 2014). These microbial-produced SCFA metabolites such as butyrate are important in epigenetic modulations, such as HDAC inhibition (Donohoe et al., 2014). As aforementioned, butyrate also plays an essential role in the differentiation of T_{reg} cells through acetylation of noncoding regions of the *Foxp3* locus (Furusawa et al., 2013). Various other studies also investigated the relationship between alterations in gut microbiota in type 2 diabetes and their associated metabolic and epigenetic mechanisms which are detailed in **Table 3**. Over the past years, it has become increasingly apparent that epigenetic modifications such as DNA methylation are associated with type 2 diabetes. Genome-wide association studies have revealed the correlation of single nucleotide polymorphisms of type 2 diabetes with defect in secretion of insulin, which corresponds to abnormality in pancreatic islet

TABLE 3 | Interplay between gut microbiota, metabolic mechanisms, epigenetic modifications, and type 2 diabetes.

Study design	Method	Gut microbiota profile	Associated metabolic mechanisms	Association between microbiota and epigenetic modifications	References
Metagenome-wide association study on fecal samples of 345 Chinese individuals	16S rRNA gene sequencing	Less levels of *Roseburia* species and *Faecalibacterium prausnitzii* were found in type 2 diabetes patients Elevated levels of *Escherichia coli* (Proteobacteria phylum) in the type 2 diabetes	These are SCFAs – producing bacteria and possess antiinflammatory properties. Also, *Faecalibacterium prausnitzii* is also a metabolic modulator *Escherichia coli* is a proinflammatory and opportunistic bacteria	These produced SCFAs such as butyrate, causes HDAC inhibition. This resulting HDAC inhibition led to downregulation of mRNA expression of *p21* and *p27* (cell cycle regulators) and may ameliorate transcription of *Fas* (a proapoptotic gene) Transplantation of normal mice microbiota *with E. coli* into germ-free mice led to differential lncRNA changes	(Qin et al., 2012; Karlsson et al., 2013) (Zhang et al., 2013; Remely et al., 2014) (Donohoe et al., 2014) (Liang et al., 2015)
Sixty type 2 diabetes patients were recruited in Japan. They were divided into two groups- placebo or transglucosidase for 12 weeks. In addition, fecal bacterial composition was compared with 10 healthy participants	T-RFLP analysis	Significant decline in the level of *Clostridium* and rise in the levels of *Lactobacillales* and *Bifidobacterium* in type 2 diabetes patients as compared with healthy controls	*Clostridium* are SCFAs – producers and involved in maintenance of gut immune homeostasis	*Clostridium* were associated with dephosphorylation of H3 histone and deacetylation of H4 histone, which may led to decreased transcription of major genes involved in immunity	(Sasaki et al., 2013) (Hamon et al., 2007)
145 women of European origin, with normal glucose tolerance, impaired glucose tolerance, or type 2 diabetes	Shotgun sequencing	Increase in the levels of *Lactobacillus* species in the type 2 diabetes group	Immunomodulating properties and some species of *Lactobacillus* are probiotic It also protects host from pathogenic microorganisms by production of lactic acid in vagina	*Lactobacillus* involved with upregulation of *miR-21-5p* in intestinal epithelial cells	(Karlsson et al., 2013) (Voravuthikunchai et al., 2006) (Nakata et al., 2017)
The investigation was done on three groups- insulin-dependent type 2 diabetes patients on glucagon like 1 peptide therapy (n = 24), obese individuals (n = 14) and lean individuals (n = 18)	High-throughput sequencing and fragment-length polymorphism analysis	Abundance of *Bacteroides vulgatus* significantly increased in type 2 diabetes patients	*Bacteroides vulgatus* exhibits proinflammatory properties	*Bacteroides fragilis* toxin have been known to cause colonic hyperplasia, colitis and tumor progression by signal transduction and activation of transcription 3 and a proinflammatory response	(Marlene et al., 2016) (Wu et al., 2009)

cells (Ruchat et al., 2009). Therefore, DNA methylation levels of CpG sites and the transcriptome in pancreatic islets were analyzed and compared between type 2 diabetes patients and normal individuals (Dayeh et al., 2014). This study detected 1,649 CpG sites and 853 genes, including *KCNQ1*, *TCF7L2,* and *FTO,* with differential DNA methylation in islets from type 2 diabetes patients (Dayeh et al., 2014). The findings also include 102 genes exhibiting differential DNA methylation as well as differential gene expression in islets from type 2 diabetes. Those genes include *CDKN1A*, *PDE7B*, *SEPT9,* and *EXOC3L2*, which were found to be key genes in regulating insulin secretion in β-cells and glucagon secretion in pancreatic α-cells (Dayeh et al., 2014). This study suggests that epigenetic mechanisms may contribute significantly to the regulation and developmental process of insulin resistance and type 2 diabetes *via* pancreatic cells.

The link between the gut microbiome and host metabolism is also shown in **Figure 3**. The gut microbiome can modulate the host metabolism in multiple ways and may result in insulin resistance and type 2 diabetes. First, with an abundance of gram-negative bacteria such as *Escherichia coli,* it has been hypothesized that the detachment of lipolysaccharide (LPS), the outer layer of gram-negative bacteria, may induce proinflammation in type 2 diabetes as well as in obesity (Allin et al., 2015). Secreted lipopolysaccharide binds with Toll-like receptor 4 (TLR4) and activates proinflammatory immune pathways, which leads to low-grade inflammation and further decreased insulin sensitivity (Cani et al., 2007). The decline in microbial gene richness was also found to be associated with low-grade inflammation (Cotillard et al., 2013). Second, three main types of SCFAs, butyrate, propionate, and acetate, produced from the fermentation of dietary fibers by gut bacteria, can influence glucose and energy metabolism of the host. Acetate and propionate are known to participate in essential metabolic processes such as glyconeogenesis and lipogenesis in the liver. Butyrate acts as an essential energy substrate for colonic mucosal

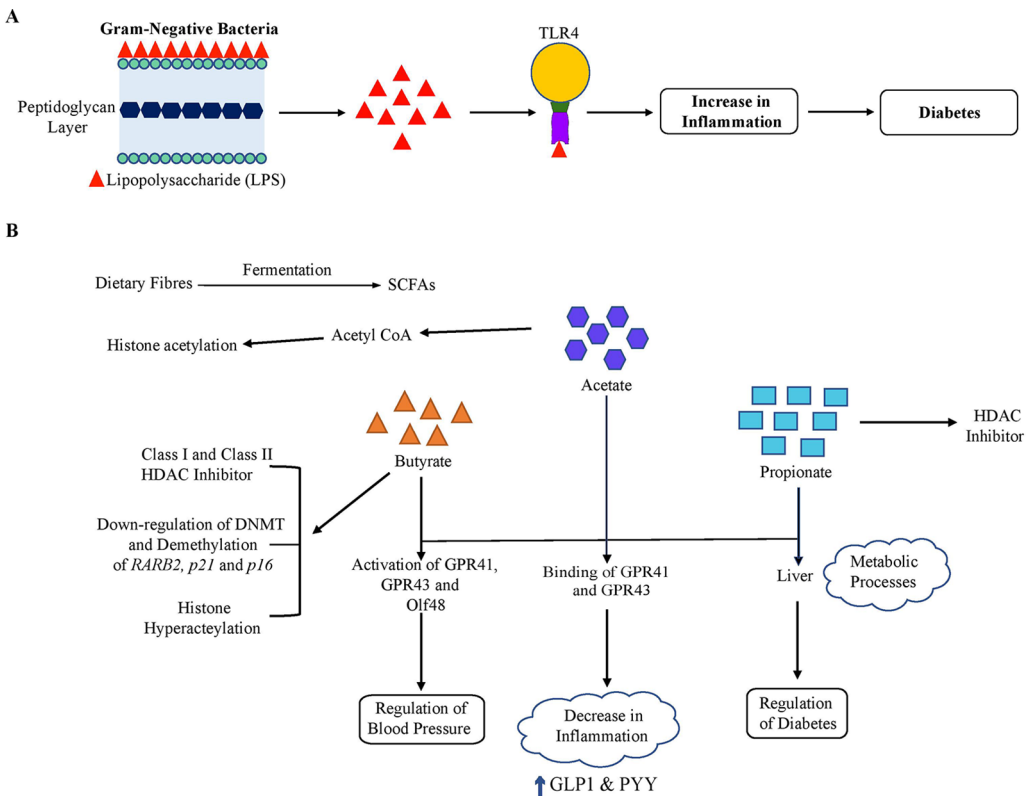

FIGURE 3 | Gut microbiome and host metabolism. The gut microbiome may alter host metabolism through many mechanisms. Of these, two important mechanisms are illustrated. **(A)** Lipopolysaccharide (LPS). LPS originates from the outer membrane of Gram-negative bacteria and binds to Toll-like receptor 4 (TLR4), which results in low-grade inflammation and thus a decline in insulin sensitivity. **(B)** Short-chain fatty acids (SCFA). The gut microbiota ferments dietary fibers to SCFAs, including acetate, butyrate, and propionate. Acetate and propionate participate in essential metabolic processes such as gluconeogenesis and lipogenesis in the liver. In addition, SCFAs bind to the G protein-coupled receptors GPR41 and GPR43 resulting in various effects depending on the cellular types affected. In immune cells, this signaling led to a decrease in the inflammation and resulted in an increase in GLP1 and PYY levels in enteroendocrine L-cells, which improve insulin sensitivity overall. Also, these SCFAs activate GPR41, GPR43, and Olfr78 expressed in the kidney. Olfr78 induces SCFA-mediated release of renin which leads to rise in blood pressure. On the other hand, GPR43 resists this change in blood pressure by vasodilatory action. SCFAs are involved in the induction of epigenetic alterations. Butyrate is a known class I and class II HDAC inhibitor. Butyrate can also affect DNA methylation and demethylation of some tumor suppressor genes (*RARB2, p21,* and *p16*) and is involved in acetylation of histone H3. Propionate is also a contributor to HDAC inhibition. In addition, acetate has been found to be involved in increasing histone acetylation *via* transferring an acetyl group from acetyl-CoA.

cells and exhibits a positive effect on insulin sensitivity (Gao et al., 2009). These SCFAs bind to GPR41 and GPR43, leading to various effects depending on the types of cells affected (Brahe et al., 2013). In immune cells, the binding of SCFAs with GPRs results in lesser development of inflammation. However, it contributes to an increase in the secretions of GLP1 and PYY from L-cells (an enteroendocrine cell) in the colon, which improves insulin sensitivity (Lin et al., 2012). It has been demonstrated that enhancing the levels of GLP-1 by altering the composition of the gut microbiota with exposure to antibiotics leads to improved glucose tolerance, insulin resistance and a rise in beneficial metabolites such as succinic acid (Hwang et al., 2015). In addition, SCFAs are found to regulate glucose metabolism by intestinal gluconeogenesis.

SCFAs also play a crucial role in the regulation of blood pressure by renin secretion as also shown in **Figure 2**. The SCFAs induce activation of GPR41, GPR43, and Olfr78, an olfactory receptor expressed in the kidney. Olfr78 also participates in the secretion of renin, induced by SCFA from afferent arterioles that lead to a rise in the blood pressure. High blood pressure is a feature of metabolic syndrome, which includes metabolic alterations such as obesity, hypertension, glucose intolerance, dyslipidemia, diabetes, etc (Schillaci et al., 2004; Lin et al., 2009). However, GPR43 resists this change in blood pressure by vasodilatory action (Pluznick et al., 2013; Pluznick, 2014). In adipocytes, binding of SCFA with GPR43 contributes to enhanced metabolism by restricting fat accumulation in adipose tissue (Kimura et al., 2013). Thus, microbial-produced SCFAs may beneficially regulate glucose metabolism which correlate with a low risk of diabetes and also regulate the blood pressure.

CONCLUSION

The gut microbiome is regulated by multiple factors such as diet, environment, genetics and epigenetics. Several studies have implicated the interactions of the gut microbiome with the host epigenome, which shows a potential role of the gut microbiome in the regulation of host metabolism. The modulation by the gut microbiome to the host epigenome may be due to direct and frequent contact with the host as well as due to various microbial-derived metabolites produced in the gut. For instance, SCFAs produced in the gut, predominately acetate, butyrate, and propionate, interact with cell surface receptors and with the epithelial and submucosal layers of the colon thereby influencing obesity and diabetes outcome. Although SCFAs are crucial to alter the epigenetic processes of the host through DNA methylation as well as histone modifications, further studies are needed to elucidate the underlying molecular mechanisms and their biological properties in the hosts. For example, which bacterial species have symbiotic relationships, and in what manner do their metabolites participate in specific metabolic processes in humans? Hence, future studies focusing on specific gut microbial metabolites that affect the host epigenome will give new insights into the health and metabolic disease of humans. In addition, the studies focusing on epigenetic mechanisms of the gut microbiome and its influence in obesity and diabetes are emerging. This could hold a promising future in uncovering novel therapeutic mechanisms that may restore the altered intestinal microbiome to a healthy condition and assist in the prevention and treatment of obesity as well as diabetes.

AUTHOR CONTRIBUTIONS

MS and TT conceived of the review article and participated in all drafts of the manuscript. MS wrote the first draft of the manuscript with guidance from TT. YL and MLS participated in editing of several drafts. TT performed final editing and approval of the manuscript. All authors read and approved the final draft.

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REVIEW
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Epigenetics and *In Utero* Acquired Predisposition to Metabolic Disease

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Epidemiological evidence has shown an association between prenatal malnutrition and a higher risk of developing metabolic disease in adult life. An inadequate intrauterine milieu affects both growth and development, leading to a permanent programming of endocrine and metabolic functions. Programming may be due to the epigenetic modification of genes implicated in the regulation of key metabolic mechanisms, including DNA methylation, histone modifications, and microRNAs (miRNAs). The expression of miRNAs in organs that play a key role in metabolism is influenced by *in utero* programming, as demonstrated by both experimental and human studies. miRNAs modulate multiple pathways such as insulin signaling, immune responses, adipokine function, lipid metabolism, and food intake. Liver is one of the main target organs of programming, undergoing structural, functional, and epigenetic changes following the exposure to a suboptimal intrauterine environment. The focus of this review is to provide an overview of the effects of exposure to an adverse *in utero* milieu on epigenome with a focus on the molecular mechanisms involved in liver programming.

Keywords: intrauterine growth retardation, epigenetics, miRNAs, programming, cardiometabolic disease

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INTRODUCTION

Early life events are associated with susceptibility to chronic diseases in adult life (Gluckman et al., 2005). Several studies have shown a clear link between the exposure to a suboptimal *in utero* environment leading to intrauterine growth retardation (IUGR) and the development of cardiometabolic disease in adulthood (Barker and Osmond, 1986; Barker et al., 1989). During the last three decades, mounting evidence has linked early exposure to malnutrition, epigenetic changes, and diseases, leading to the formulation of the "developmental origins of health and disease" (DOHaD) hypothesis (Barker, 2007). According to DOHaD the organism exposed to *in utero* undernourishment diverts the restricted nutrients to preserve growth and function of vital organs, such as brain, at the expense of growth and organs, such as liver and pancreas. This intrauterine adaptation is favorable for survival if the fetus is born in conditions of inadequate nutrition (Hales et al., 2001). However, detrimental consequences of developmental programming arise if the fetus is born in an environment with normal or even increased nutrient supply. The mismatch between pre- and postnatal environment may predispose the offspring to the development of cardiometabolic disease in adulthood (Gluckman et al., 2009).

Intrauterine programming occurs at "critical time windows" of fetal growth, characterized by a high rate of differentiation and/or proliferation, and involve genes, cells, tissues, and even whole

organs (Fowden et al., 2006). Programming is an adaptive response of the organism to the surrounding environment: this capacity named "developmental plasticity" permits the development of a spectrum of phenotypes from a single genotype (Lucas et al., 1991; Gluckman and Hanson, 2004). When the resulting phenotype matches the environment, the organism will preserve a health status. On the contrary, if a mismatch occurs between the adaptive response and the environment, the organism is unable to cope with the environmental challenges, ultimately becoming susceptible to cardiometabolic disease (Godfrey et al., 2007).

One of the mechanisms of programming is the epigenetic change of genes involved in critical metabolic pathways. There is strong experimental evidence indicating that specific epigenetic hallmarks represent a sort of fingerprints of intrauterine programming. In this context, liver is a major target of *in utero* programming, undergoing structural, functional, and epigenetic changes as a result of early exposure to an adverse environment.

"Programming"

Epigenetic regulation is a mechanism of programming and is basically related to gene silencing, genomic imprinting and transcriptional regulation of tissue-specific genes during cellular differentiation (Schubeler et al., 2000). The epigenetic control of gene expression depends on the modulation of chromatin structure and accessibility to transcription factors. This control is obtained by multiple mechanisms, such as different methylation of cytidine-guanosine (CpG) islands in the promoter sites, acetylation-deacetylation of lysine residues of core histones in the nucleosome and production of miRNAs which bind to complementary sequences in the 3' end of mRNA and interfere with protein synthesis (**Figure 1**) (Goldberg et al., 2007).

Animal Evidence

Several animal models of fetal growth retardation have been developed to investigate the mechanisms of intrauterine programming, including maternal stress, hypoxia, glucocorticoid treatment, nutrient (protein) restriction, and utero-placental insufficiency.

The most commonly used animal model is based on fetal undernourishment, which may be induced by a complete maternal insufficiency of nutrients or by protein restriction in an isocaloric diet. Intrauterine malnutrition has been proven to change the expression of multiple genes involved in different metabolic pathways (Morris et al., 2009). These changes affect lipid and glucose metabolism, leading the organism to the preferential use of fatty acids as energy source in order to adapt the organism to a reduced nutrient supply.

Uteroplacental Insufficiency and Liver Programming

All organs may be affected by intrauterine programming thereafter permanently changing their structure and function. Liver is a major target of programming. Uteroplacental insufficiency alters the expression of genes encoding enzymes involved in hepatic

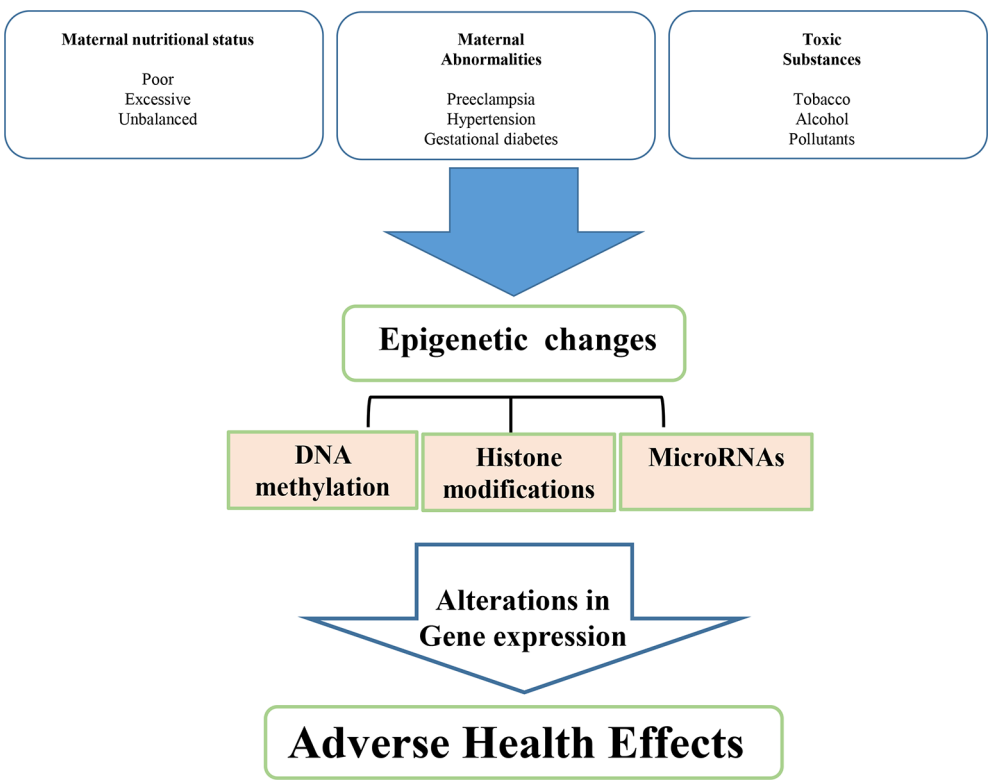

FIGURE 1 | Environmental determinants related to intrauterine development resulting in low birth weight and metabolic disease in adult life.

energy production (Lane et al., 1996), decreasing hepatic oxidative phosphorylation (Ogata et al., 1990) and affecting liver glucose transport (Lane et al., 1999).

We investigated the effects induced by an adverse intrauterine environment, using an animal model (Sprague-Dawley rats) of intrauterine growth restriction obtained through maternal uterine artery ligation on day 19 of gestation (Puglianiello et al., 2007; Germani et al., 2008; Puglianiello et al., 2009; Deodati et al., 2018). The animals exposed to uteroplacental insufficiency show an adaptive response in hypothalamic lipid sensing signaling, ultimately influencing food intake and endogenous carbohydrate production in post-natal life (Puglianiello et al., 2009; Deodati et al., 2018). Vuguin et al. (2004) have reported in the same animal model a change in the expression of the genes involved in gluconeogenesis (PEPCK and glucose 6-phosphatase), permanently influencing glucose metabolism in the offspring. These alterations were observed before the development of obesity and diabetes, suggesting that this dysregulation of hepatic glucose metabolism may represent an early defect that contributes to the subsequent onset of fasting hyperglycemia and ultimately diabetes.

The analysis of the liver expression profile of 84 gene showed that 26 genes were differentially expressed in IUGR *versus* SHAM rats. The functional analysis of these genes showed that most of them have a key role in metabolic signaling. In particular, glucose metabolism resulted to be affected by intrauterine malnutrition as indicated by the downregulation of Fbp1, Gpd, Pklr, and the upregulation of Gck, Hk2, and Slc2a1 genes (Cianfarani et al., 2012). Furthermore, in male IUGR adult animals, defective insulin signaling, liver focal steatosis, periportal fibrosis, and chronic activation of hepatic unfolded protein response (UPR) have been described (Deodati et al., 2018).

PGC-1 and CPTI

MacLennan et al., 2004 reported higher levels at birth of S-adenosylhomocysteine in the liver of animals exposed to adverse intrauterine environment. These levels were associated with reduced methylation and increased acetylation of histone H3 on lysine 9 (H3K9), lysine 14 (H3K14), and lysine 8 (H3K18) (MacLennan et al., 2004). These modifications persisted up to 21 post-natal day, suggesting a permanent effect on hepatic gene expression.

IUGR rats showed an increased acetylation on H3 in the liver associated with a reduction of nuclear protein levels of histone deacetylase 1 (HDAC1) and HDAC activity. These alterations influence the histone association with the promoter site of PPAR-gamma coactivator (PGC-1) and carnitine-palmitoyl-transferase I (CPTI), two genes whose expression is changed in IUGR rats (Liguori et al., 2010; Puglianiello et al., 2007), PGC-1 expression being upregulated, whereas CPTI expression being downregulated in IUGR rats predisposed to diabetes (Lane et al., 2001; Lane et al., 2002).

PGC-1 is a potent transcription factor that plays a key role in the regulation of cellular mitochondrial function, gluconeogenesis and glucose transport, glycogenolysis, fatty acid oxidation, peroxisomal remodeling, muscle fiber-type switching, and oxidative phosphorylation (Yoon et al., 2001; Lin et al., 2005; Corona et al., 2015). CPTI is a part of the carnitine shuttle and is considered to be a rate-limiting transporter in mitochondrial fatty acid β-oxidation (McGarry and Brown, 1997; Fu et al., 2004). Uteroplacental insufficiency affects the association between acetylated H3/K9 and the promoters of PGC-1 and CPTI, respectively, in IUGR liver, this effect persisting up to day 21 of life in male animals (Fu et al., 2004).

Intrauterine Programming and Pancreatic β-Cell

Adverse intrauterine environment may induce permanent and progressive alterations in the gene expression of susceptible organs, including pancreatic islets. DNA methylation of beta cell genome in animal models of uteroplacental insufficiency was investigated (Thompson et al., 2010). A genome-wide HpaII tiny fragment enrichment by ligation-mediated PCR assay (Oda M., et al., 2009 *Nucleic Acids Res*), generating DNA methylation map at almost 1 million loci in IUGR and SHAM animals, was used. In male IUGR rats a different cytosine methylation pattern in approximately 1,400 loci was found. The epigenetic changes mainly occurred in conserved intergenic sequences, located near genes regulating key processes, such as vascularization, β-cell proliferation, insulin secretion, and cell death, and were associated with concordant changes in mRNA expression. These findings suggest that epigenetic dysregulation may be a mechanism involved in propagating the biological memory of intrauterine events, leading to altered expression of nearby genes and ultimately affecting the susceptibility to type 2 diabetes (Thompson et al., 2010) (**Table 1**).

Pdx1

Pdx1 (pancreatic and duodenal homeobox 1) is a transcription factor involved in the regulation of pancreatic development and β-cell differentiation. Experimental evidence has documented that a decreased expression of Pdx1 is associated with β-cell dysregulation and impaired islet compensation in the presence of insulin resistance (Stoffers et al., 1997; Holland et al., 2005). Adverse intrauterine environment may lead to different epigenetic modifications of Pdx1 gene expression, such as histone modifications, DNA methylation, and chromatin remodeling (Park et al., 2008). In particular, decreased mRNA level of Pdx1 associated with normal β-cell mass was observed at birth in IUGR pups. In adult life, IUGR animals showed a progressive reduction of β-cell mass associated with undetectable mRNA level of Pdx1. These changes were associated with a significant decrease in H3 and H4 acetylation, a decreased methylation on H3K4, and a significant increase of methylation on H3K9 in Pdx1 proximal promoter of IUGR islets. Overall, these changes resulted in a chromatin silencing, with reduced USF-1 (a key transcription factor) binding and increased engagement of HDAC1 and its corepressor Sin3A.

Postnatally, these epigenetic changes and the reduction in Pdx1 expression can be reversed by HDAC1 inhibition. However, as H3K9me2 accumulates, DNMT3A (a DNA methyltransferase) is recruited to the promoter and initiates *de novo* DNA methylation, which silences pancreas function, eventually leading to diabetes (Park et al., 2008; Pinney and Simmons, 2010; Liguori et al., 2010) (**Table 1**).

TABLE 1 | The relationship between genes involved *in utero* programming and development of metabolic disease.

Ref	*Model*	*Organ*	*Gene*	*Epigenetic change*	*Gene function*
Fu et al., 2004	Rats Uteroplacental insufficiency	Liver	PPAR-y	H3K9	Transcriptional coactivator of key gluconeogenic enzymes
			Co CPT-I	Hypetacetylation affecting with gene promoter	Rate-limiting transporter in mithocondrial fatty acid β-oxidation
Park et al., 2008	Rats Uteroplacental insufficiency	Pancreatic islets	PDX-1	H3 and H4 Deacetylation, H3H4 demetlylation, H3K9 methylation	Transcription factor critical for β
Raychaudhuri et al., 2008	Rats Caloric restriction	Skeletal muscle	GLUT4	H3K14 deacetylation, H3K9 methylation	Glucose transporter
Thompson et al., 2010	Rats Uteroplacental insufficiency	Pancreatic islets	CGH-1	CpG hypermethylation in intergenic sequences (IS)	Role in endothelial dys function and β-cell development
			FGFR-1	CpG hyp omethylation in IS	Fibrobalst growth factor receptor
			PCSK -5	CpG hypmnethylation in transcription start site	Role in peptide processing and maturation
Suter et al., 2012	Rats HFD	Liver	SIRT-1	H3K14 Hyperacetylation	Regulation of glucose homesotasis insulin sensitivity, oxidative stress and anti- inflammatory activity
Heijmans et al., 2008	Humans AGA, periconceptionale famine	Blood	IGF -2	CpG hypomethylation	Fetal growth
Barres et al., 2009	Humans AGA Incubation with TNF alpha,FFA, glucose	Skeletal muscle	PPAR-y-C1 a1pha	Non CpG hypermethylation	Transcriptional Coactivator.regulator of mithocondrial genes
Brøns et al., 2010	Humans SGA/AGA HFD	Skeletal muscle	PPAR -y-C1 a1pha	CpG hypermethylation	Transcriptional Coactivator,regulator of mithocondrial genes

AGA, adequate gestational age; CGH-1, GTP cyclohdrolase1; CPT-1, carnitine-palmitoyl transferase I; FGFR-1, fibroblast growth factor receptor 1; FFA, free fatty acid; GLUT-4, glucose transporter 4; HFD, high fat diet; IGF-2, insulin-like growth factor 2; PCSK-5, proprotein convertase subtilisin/ketin type 5; PDX-1, pancreatic and duodenal homeobox1; PPAR-y coactivator, peroxisome proliferator-activated receptor-gamma coactivator; SGA, small for gestational age; SIRT-1, sirtuin 1 deacetylase; TNF alpha, tumor necrosis factor alpha.

Intrauterine Programming and Skeletal Muscle

GLUT4

In utero malnutrition induces histone modifications in skeletal muscle that directly influence the expression of GLUT type 4 (GLUT 4) (Raychaudhuri et al., 2008). GLUT4 is one isoform of a family of sugar transporter proteins containing 12-transmembrane domains with the function of glucose carriers and plays a key role in glucose homeostasis, depending on insulin action. In skeletal muscle of female rats at 450 days of post-natal life a significant reduction of GLUT4 expression was found. This gender-specific difference may originate from early alterations in pancreatic β-islet cell function, occurring during fetal development (Chamson-Reig et al., 2006). Young females showed lower post-natal insulin levels. This IUGR model showed deacetylation and di-methylation of amino acid residues in the N-tail of histone 3. These results suggest that epigenetic histone modification may influence GLUT4 transcription in skeletal muscle of adult female IUGR rats.

Maternal Protein Restriction and Offspring Programming

Intrauterine programming may be caused by maternal protein restriction during pregnancy. In pregnant rats, protein deficiency leads to structural and functional changes in liver of offspring, altering glucose homeostasis and insulin sensitivity (Burns et al., 1997; Desai et al., 1997). In young adult male rats, maternal nutritional restriction during pregnancy influences the expression of 249 genes, affecting the development of adiposity and insulin resistance (Morris et al., 2009). In particular, these animals show downregulation of genes involved in intracellular uptake of glucose and its metabolism *via* the glycolytic and tricarboxylic acid signaling, and upregulation of genes involved in the intracellular trafficking and oxidation of fatty acids (Morris et al., 2009). Multiorgan transcriptional modifications in the offspring are induced by maternal protein restriction causing a rearrangement of transcription factor-binding sites, especially in the liver (Vaiman et al., 2011).

Maternal Hypoxia and Programming

IUGR may also be the consequence of exposure to reduced oxygen supply *in utero* (Camm et al., 2011). Intrauterine hypoxia induces fetal adaptations in developing organs (kidneys, heart, and vascular system), leading to reduced nephron number and glomerular filtration and ultimately to hypertension in the offspring (Silver et al., 2003). Dysregulation also involves the function of the renin angiotensin system (RAS) *via* altered

expression and activation of angiotensin receptor subtypes which influence renal and vascular function (Vehaskari et al., 2001).

IUGR offspring, caused by fetal hypoxia, show altered vasoconstrictor and vasodilator mechanisms together with vascular remodeling (Morton et al., 2016).

These effects of *in utero* hypoxia on offspring cardiometabolic risk may be mediated by epigenetic changes. Increased DNA methylation subsequent to fetal hypoxia causes the reduction of protein kinase Cε (PKCε) gene expression in the heart of pups, resulting in higher cardiac susceptibility to ischemic injury (Patterson and Zhang, 2010).

In placentas of women with pregnancies complicated by IUGR, a vascular dysfunction was found, suggesting a key role played by placental oxidative stress (Schneider et al., 2015).

Fetal programming in hypoxic or complicated pregnancies may be avoided with the use of antioxidant treatments. For instance, the early use of resveratrol [4 g/kg diet, gestational day (GD) 0.5–21] reduced blood pressure in hypertensive rats (Care et al., 2016). The use of ascorbic acid (5 mg/ml in drinking water, GD 6–20) was shown to inhibit placental oxidative stress related to maternal hypoxia (Richter, et al., 2012). However, results from prenatal antioxidant treatments are conflicting (Giussani et al., 2012; Stanley et al., 2012).

Maternal Overnutrition and Programming

Intrauterine overnutrition could be associated with the development of metabolic diseases in the offspring (Lillycrop, 2011; Pasternak et al., 2013). A maternal high fat diet (HFD) may cause changes in hypothalamic gene expression in the offspring, such as leptin receptor, proopiomelanocortin, and neuropeptide Y (Chen et al., 2008). Maternal HFD has been reported to increase the acetylation of histone H3 in the offspring (Aagaard-Tillery et al., 2008) and modify methylation and expression of the genes of the mesocortico-limbic reward circuitry (dopamine and opioids).

SIRT1, a member of the HDAC family, influences fat metabolism in adipocytes by repressing PPAR-gamma; upregulation of SIRT1 increases lipolysis, thereby inducing fat loss. SIRT1 is also implicated in the control of glucose homeostasis, insulin sensitivity, oxidative stress, and anti-inflammatory activity. Maternal HFD has been shown to reduce SIRT1 expression in fetal liver and heart by increasing the acetylation of histone H3K14 (Suter et al., 2012).

Human Evidence

Epidemiological studies have linked a suboptimal intrauterine environment and low birth weight with cardiometabolic risk in adult life (Hales et al, 1991; Ravelli et al, 1998). Notably, metabolic risk is mainly related to the pattern of early growth. The relationship between early catch-up growth and several determinants of cardiovascular disease and type 2 diabetes in adulthood has been reported (Cianfarani et al., 1999; Leunissen et al., 2009; Mericq, et al., 2017). Infants born small for gestational age (SGA) show a decrease in absolute fat mass when compared with appropriate for gestational age (AGA) children, reflecting reduced intrauterine fat accumulation (Ibanez et al., 2010). On the contrary, children exposed to uteroplacental insufficiency show an increase in fat accumulation when these babies experience rapid catch up growth during early postnatal life. SGA infants who achieve normal weight and height within 2 years of age show an increase of total body and abdominal fat between 2 and 4 years of age compared with AGA children (Ibanez et al., 2006).

IGF2

The possible involvement of epigenetic mechanisms in the development of human diseases, such as type 2 diabetes, was first suggested by data collected from subjects who were prenatally exposed to famine during the Dutch Hunger Winter in 1944–45. During this period, due to the food embargo imposed by Germany, famine was experienced in the western part of the Netherlands with an average daily ration of 667 kcal. Analysis of data from these individuals revealed an increased risk of developing cardiometabolic disease in adulthood (Lumey et al., 2007).

Notably, a reduced DNA methylation of the imprinted IGF2 gene was reported in a cohort of these subjects exposed to the famine during the prenatal period and evaluated six decades later (Heijmans et al., 2008). Therefore, permanent epigenetic modifications may result from the early exposure to malnutrition during critical time windows of development (Heijmans et al., 2008; Tobi et al., 2012). Additionally, a higher risk of insulin resistance was found in the daughters of women exposed to the Dutch Hunger Winter, suggesting that programming might be transmitted to the following generations (Stein and Lumey, 2000).

The maternal intake of protein and folic acid before and during pregnancy has been related to the degree of IGF2 methylation. A recent study, conducted in 120 children (aged 17 months), evaluated the degree of IGF2 methylation according to exposure (n = 86) or not (n = 34) to folic acid supplementation to mothers in the periconceptional period. The children exposed to folic acid showed higher methylation degree of the IGF2 *differentially methylated region* (DMR) compared with those not exposed. Interestingly, IGF2 DMR methylation was associated with mother's S-adenosylmethionine blood levels. Additionally, IGF2 DMR methylation was inversely related to birth weight (Steegers-Theunissen et al., 2009).

In summary, the use of folic acid during the peri-conception period is correlated with epigenetic modifications of IGF2 in children, potentially inducing consequences in adult life related to intrauterine programming (Steegers-Theunissen et al., 2009; Haggarty, 2013).

PPAR-γC1-α

PGC-1 family of co-activators is influenced by multiple environmental and nutritional factors.

These coactivators play a key role in the regulation of glucose and lipid metabolism and body energy expenditure. Alterations in PGC-1 mediated mechanisms affect multiple metabolic pathways, leading to development of chronic disease (Finck and Kelly, 2006). Muscle and pancreatic islets of individuals with type 2 diabetes show an epigenetic regulation of the transcriptional coactivator peroxisome proliferator activated

receptor γ coactivator-1 α (protein PGC-1α; gene PPAR-γC1-α), characterized by increased DNA methylation in PPAR-γ-C1-α gene promoter (Ling et al., 2008). Barres et al. (2009) performed a genome-wide promoter analysis of DNA-methylation to assess genes differentially methylated in skeletal muscle of normal glucose-tolerant and T2DM subjects. In this study, they found cytosine hypermetylation of genes involved in mitochondrial functions, such as PPAR- γ-C1-α in diabetic subjects. Methylation levels were negatively associated with PGC-1α and mitochondrial DNA. Furthermore, the authors provided a mechanism by which high level of cytokines, glycemia, or lipids can induce non-CpG methylation of the PGC-1α promoter in skeletal muscle. To investigate whether these external factors could directly influence the methylation status, they incubated primary human skeletal muscle cultures with multiple factors known to induce insulin resistance, such as hyperglycemia, hyperinsulinemia, elevated free fatty acids (FFA), and elevated cytokines. In these conditions an increase of non-CpG methylation in human myotubes, exposed to fatty acids and TNF-alpha, were observed. Moreover, selective silencing of DNA methyltransferase-3B prevented palmitate-induced PGC-1α promoter methylation.

These findings suggest that environmental changes can alter epigenetic modulation of PGC-1α, which is involved in T2DM and metabolic disease.

Additionally, PPARγ-C1-α promoter methylation status is stable in the blood of 5–7 year old children, representing a marker of fat accumulation up to 14 years of age (Clarke-Harris et al., 2014), thus suggesting that epigenetic analysis may be helpful in identifying individuals at risk of later onset of obesity and cardiometabolic disease (**Table 1**).

miRNA

Animal and Human Evidence

miRNAs have been related to the development of metabolic disease. MicroRNAs are small, non-coding, highly conserved regulatory RNAs (Bartel, 2004). miRNAs can regulate gene expression at a post-transcriptional level, and are implicated in multiple human process, including proliferation, differentiation, development, metabolism, and apoptosis (Lynn, 2009).

Recently, miRNAs have emerged as important regulators of several physiological and pathological processes. For instance, miR-199a may play a role in the development of liver and neurologic disease, by regulating hypoxia-inducible factor-1 alpha (Jiang et al., 2014).

Different miRNAs are associated with several metabolic pathways, including lipid metabolism, glucose metabolism, food intake, body weight homeostasis, inflammation, oxidative stress, expression of several cytokines, and angiogenesis in obesity. Intrauterine programming can cause modifications in miRNA expression, inducing alterations of metabolic pathways in children born to mothers affected by obesity (Williams, 2012). Circulating microRNAs (c-miRNAs) may reflect these alterations in tissue expression and intracellular signaling, supporting their utility as informative biomarkers.

miR-141 resulted significantly up-regulated in both the placenta and plasma of women affected by uteroplacental insufficiency and may play a key role in the pathogenic mechanism of IUGR by suppressing pleomorphic adenoma gene 1 (PLAG1) (Tang et al., 2013). PLAG1 is expressed mainly in placenta during embryonic development. In animal model, the disruption of PLAG1 causes growth retardation (Braem et al., 2002). Microarray analyses identified genes that were induced or repressed by PLAG1. In particular, this analysis showed an upregulation of genes encoding growth factors, such as insulin like growth factor 2 (IGF2), bone-derived growth factor (BPGF1), vascular endothelial growth factor (VEGF), and placental growth factor (PGF) (Van Dyck et al., 2004).

These findings are consistent with data reported by Tang et al. (2013) who found a significant positive correlation between mRNA expression of PLAG1 and level of IGF2 in placental tissues of fetal growth retardation compared to controls. Moreover, a downregulation of miR-16 and miR-21 in the placenta was significantly related with lower birth weight (Maccani and Marsit, 2011).

miRNA serum profile in umbilical cord of SGA children with (SGA-CU, n = 18) and without catch-up growth (SGA-nonCU, n = 24) was investigated (Mas-Parés et al., 2018). In particular, 12 miRNA were differentially expressed between SGA-CU and SGA-nonCU (miR-128-3p, miR-222-5p, miR-300, miR-374b-3p, miR-501-3p, miR-548c-5p, miR-628-5p, miR-770-5p, miR-873-5p, miR-876-3p and miR-940). Among them, miR-501-3p, miR-576-5p, miR-770-5p, and miR-876-3p showed a significant association with weight, height, weight catch-up, and height catch-up at 1 year of age in all subjects. mir-576-5p was an independent predictor of weight, waist circumference, and renal fat at 6 years of life. Additionally, *in silico* analysis revealed that mir-576-5p was associated with key metabolic pathways, such as insulin, IGF1, PDGFR-B, and mammalian target of rapamycin (mTOR) signaling. These findings suggest that miR-576-5p could play a role for the risk of metabolic disease related to postnatal growth.

MicroRNA expression in newborns with different birth weight (normal birth weight *vs.* low birth weight *vs.* high birth weight) was also reported. miR-454-3p was upregulated in low and high birth weight newborns compared to normal birth weight newborns. The analysis of prediction target genes revealed that seven possible pathways could be regulated by this microRNA, such as endocytosis, transforming growth factor ß (TGF-ß), axon guidance, forkhead box O (FoxO), p53, proteoglycans in cancer, and Hippo signaling pathway (Rodil-Garcia et al., 2017).

miRNAs are associated to metabolic disease such as insulin resistance, obesity, and non-alcoholic fatty liver disease (NAFLD). In particular, an increasing body of evidence shows that almost 100 different miRNAs are differentially expressed in non-alcoholic steato-hepatitis (NASH) patients (Cheung et al., 2008).

A high fructose diet administered to induce IR in mice led to overexpression of several miRNAs such as miR-19b-3p, miR-

101a-3p, miR-30a-5p, miR-582-3p, and miR-378a-3p, and downregulation of miR-223-3p, miR-33-5p, miR-128-3p, miR-125b-5p, and miR-145a-3p.

Interestingly, IRS-1, FOXO-1, SREBP-1c, SREBP-2, SREBP-2, Insig-1, and Insig-2 (Ing-2a and -2b), which are involved in insulin pathway and synthesis of hepatic lipids, are target genes of these miRNAs (Sud et al., 2017).

Another study found 46 distinctively expressed miRNAs in patients affected by NASH. Twenty-three resulted overexpressed, such as miRNA-21, miRNA-100, and miRNA-34a, and others including miRNA-126 and miRNA-122 were down-regulated (Cheung et al., 2008). Offspring of mothers exposed to HFD during gestation and lactation showed an altered expression of 23 miRNAs in the liver (Zhang et al., 2009). Specifically, miRNA122, the main hepatic miRNA, resulted downregulated in subjects affected by NAFLD (Zhang et al., 2009) and its deletion in mice caused hepatic steatosis, inflammation, and hepatocellular carcinoma. Therefore, miRNA-122 has been suggested as a major contributor to the regulation of hepatic lipid metabolism (Wen and Friedman, 2012). Additionally, mir-146b, mir-143, mir-34a, and mir-23a resulted increased in NAFLD patients (Tian et al., 2013). MiRNA 370, 33, 103, and 104 have also been recognized to modulate lipid and cholesterol regulatory genes, contributing to the development of NAFLD. Mice with deletion of this liver-specific miRNAs developed hepatic steatosis, inflammation, and hepatocellular carcinoma (Wen and Friedman, 2012).

miRNAs 146b, 143, 34a, and 23a have been found upregulated in NAFLD (Tian et al., 2013). Other miRNAs have been reported to influence lipid and cholesterol regulatory genes (miRNA 370, 33, 103, and 104), eventually contributing to the development of NAFLD.

We investigated hepatic miR-122-5p RNA expression in an animal model of IUGR induced by uterine artery ligation. Twelve SHAM and 12 IUGR male rats from four different litters were randomly selected. IUGR pups showed significant upregulation of gluconeogenesis and lipogenesis genes. Significantly reduced glucose tolerance and liver focal steatosis with associated periportal fibrosis were observed in adult IUGR male rats (Deodati et al., 2018). However, no significant difference in hepatic miR-122-5p RNA expression was observed.

A recent study, comparing obese SGA and AGA children with normal weight SGA and AGA children has shown a specific profile of circulating miRNAs (Marzano et al., 2018). Twenty eight miRNAs resulted dysregulated in obese-SGA (OB-SGA) *vs.* normal weight-SGA (NW-SGA) and 19 miRNAs were altered in OB-AGA *vs.* NW-AGA. In particular, miR-92a-3p, miR-122-5p, miR-423-5p, miR-484, miR-486-3p, and miR-532-5p were up-regulated, and miR-181b-5p was down-regulated in both OB-SGA and OB-AGA compared with normal weight subjects. These miRNAs resulted implicated in insulin signaling, glucose trafficking, insulin resistance, and lipid metabolism (**Table 2**). More specifically, an association between miR-122-5p and obesity, lipid metabolism, insulin resistance, metabolic syndrome, and type 2 diabetes (Wang et al., 2015) was found whereas mir-486-3p is increased in pre-diabetes and obesity. A significant association was found between circulating levels of miR-486-5p, miR-486-3p and miR-423-5p and BMI, fat mass, waist circumference, regional fat distribution, homeostatic model assessment of insulin resistance (HOMA-IR), high-molecular-weight adiponectin, C-reactive protein, and circulating lipids (Prats-Puig et al., 2013). A selective deregulation of miR-26a-5p, miR-126-3p, and miR-143-3p was observed in preeclampsia and IUGR pregnancies (Hromadnikova et al., 2015).

TABLE 2 | List of target genes, involved in severe metabolic pathways, differentially influenced by microRNAs expression.

Metabolic Pathway	Target genes
Insulin signaling	AKTl, MAPKl, PDKl, TFRC, GRB2, SOSl, RAFl, EEF2,MTOR, IRSl, PIK3Rl, PTPNll
Glucose transport	AKTl, CDKNlA, PDPKl, TFRC, GRB2, SOSl, EEAl, MTOR, IRSl
Cholesterol and Lipid metabolism	SREBFl, HDLBP, MBTPS2, LDLR, HMGCR, INSIGl, HMGCSl, ERLINl, PRKAAl, ABCAl, LDLRAPl, SREBF2, DHCR24, CYB5R3, SCAP, FDFrl, NPC2, INSIG2
Insulin Resistance	SREBFl, CRTC2, IRS2, PTPRF,PIK3CB, SOCS3, CREBl, TRIB3, FOXOl, PPPICC, PTEN, PPP1CA,GSK3B, GFPTl, GFPT2, SLC2Al, MGEA5, CREB3L2, PIK3CA, MLXIP, PRKAAl, MAPK8, OGT, NFKBl,RPS6KBl, AKTl, PDPKl, PIK3R3, PIK3Rl, PIK3CG, PRKAB2, PIK3CD, PCK2,PRKCD, IRSl, STAT3, PTPNll, MAPK9, PTPNl, MTOR

AKT1, AKT serine/threonine 1; MAPK1-8-9, mitogen-activated protein kinase 1-8-9; PDK1, pyruvate dehydrogenase kinase 1; TFRC, transferrin receptor; GRB2, growth factor receptor-bound protein 2; SOS1, son of sevenless homolog 1; RAF1, Raf-1 proto-oncogene, serine/threonine kinase; EEF2, eukaryotic elongation factor 2; MTOR, mammalian target of rapamycin; IRS1-2, insulin receptor substrate 1-2; PIK3R1-3, phosphoinositide-3-kinase regulatory subunit 1-3; PTPN11, protein tyrosine phosphatase non-receptor type 11; CDKN1A, cyclin dependent kinase inhibitor 1A; PDPK1,3-phosphoinositide dependent protein kinase 1; EEA1, early endosome antigen 1; SREBF1-2, sterol regulatory element binding transcription factor 1-1; HDLBP, high density lipoprotein-binding protein; MBTPS2, membrane bound transcription factor peptidase, site 2; LDLR, low-density lipoprotein receptor; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; INSIG1-2, insulin induced gene 1-2; HMGCS1,3-hydroxy-3-methylglutaryl-CoA synthase 1; ERLIN1, ER lipid raft associated 1; PRKAR1A, protein kinase cAMP-dependent type I regulatory subunit alpha; ABCA1, ATP-binding cassette transporter; LDLRAP1, low-density lipoprotein receptor adapter protein 1; DHCR24, 24-dehydrocholesterol reductase; CYB5R3, cytochrome B5 reductase 3; SCAP, sterol regulatory element-binding protein cleavage-activating protein; FDFT1, farnesyl-diphosphate farnesyltransferase 1; NPC2, NPC intracellular cholesterol transporter 2; CRTC2, CREB regulated transcription coactivator 2; PTPRF, protein tyrosine phosphatase receptor type F; PIK3CB-A-G-D, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta-alpha-gamma-delta; SOCS3, suppressor of cytokine signaling 3; CREB1, CAMP responsive element binding protein 1; TRIB3, tribbles homolog 3; FOXO1, forkhead box O1; PPP1CC-A, protein phosphatase 1 catalytic subunit gamma; PTEN, phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase; GSK3B, glycogen synthase kinase 3 beta; GFPT1-2, glutamine–fructose-6-phosphate transaminase 1-2; SLC2A1, solute carrier family 2 member 1; MGEA5, meningioma expressed antigen 5; CREB3L2, CAMP responsive element binding protein 3 like 2; MLXIP, MLX interacting protein; PRKAA1, protein kinase AMP-activated catalytic subunit alpha 1; OGT, O-linked N-acetylglucosamine (GlcNAc) transferase; NFKB1, nuclear factor kappa B subunit 1; RPS6KB1, ribosomal protein S6 Kinase B1; PDPK1, 3-phosphoinositide dependent protein kinase 1; PRKAB2, protein kinase AMP-activated non-catalytic subunit beta 2; PCK2, phosphoenol-pyruvate carboxykinase 2; PRKCD, protein kinase C delta; STAT3, signal transducer and activator of transcription 3; PTPN1 (protein tyrosine phosphatase, non-receptor type 1).

Genome Wide Association and Intrauterine Programming

Birth weight is a complex trait influenced by environmental and maternal-fetal genetic factors.

To clarify the association between birth weight and development of metabolic diseases in adult life, genome-wide association studies were performed.

The first meta-analysis of GWA studies reported data on birth weight in 10,623 subjects. The first cluster associated with birth weight includes CCNL1 and LEKR1 genes (Freathy et al., 2010). This cluster of SNPs was correlated also to reduced ponderal index at birth and smaller head circumference and birth length, suggesting that these loci could influence soft tissue rather than skeleton. In literature, there is a strong evidence of relationship between these genes and birth weight but the underlying mechanism has not been elucidated yet. The second cluster of SNPs was centered on the ADCY5 gene. This locus is associated to lower birth weight and predisposition of type 2 diabetes. Strong associations have been reported between the type 2 diabetes risk allele and higher fasting glucose (Dupuis et al., 2010) and higher 2-h glucose level during oral glucose tolerance test (OGTT) with lower 2-h insulin level and HOMA in adults (Saxena et al., 2010). In addition, evidence from studies of other type 2 diabetes loci is accumulating for association between the fetal risk alleles at CDKAL1 and HHEX-IDE and lower birth weight (Andersson et al., 2010). In particular, both the CDKAL1 and HHEX-IDE have been associated with reduced pancreatic beta-cell function and reduced insulin secretion in studies on non-diabetic adults (Pascoe et al., 2007; Steinthorsdottir et al., 2007). Further studies are needed to show whether these loci may influence birth weight by altering intrauterine insulin secretion.

There evidences that all type 2 diabetes loci are not associated with birth weight. For example, TCF7L2, which confers the largest risk of type 2 diabetes in Europe, showed no link *via* the fetal genotype (Mook-Kanamori et al., 2009; Andersson et al., 2010). These findings are in contrast to the effects of ADCY5, CDKAL1, and HHEX-IDE, previously reported, and suggest that there is an overlap between the genetics of type 2 diabetes and intrauterine growth.

Sixty-five loci have been correlated with birth weight in genome-wide association studies (GWASs) (Hattersley and Tooke, 1999; Freathy, 2010; Horikoshi et al., 2016). Most of these studies did not discriminate between maternal and fetal genetic influences, producing overlapping signals due to the correlation between maternal and fetal genotypes.

A recent meta-analysis of GWASs of offspring birth weight, reporting maternal genotypes in up to 86,577 women of European descent from 25 studies identified 10 autosomal loci (MTNR1B, HMGA2, SH2B3, KCNAB1, L3MBTL3, GCK, EBF1, TCF7L2, ACTL9, CYP3A7) that were associated with offspring birth weight (Weedon et al., 2007; Beaumont et al., 2018). In addition, at least 7 of the 10 associations were consistent with effects of the maternal genotype acting *via* the intrauterine environment, rather than *via* shared alleles with the fetus. This study confirms previous results about the associations with birth weight at TCF7L2 and GCK (Freathy, 2010; Freathy et al., 2007).

Another study of GWA evaluated maternal and fetal genetic effects on birth weight and their impact on metabolic risk, using Mendelian randomization to distinguish the contributions of direct fetal and indirect maternal genetic effects (Warrington et al., 2019). They identified 64 SNPs with fetal effects, 32 SNPs with maternal effects, 27 SNPs with directionally concordant fetal and maternal effects, and 15 SNPs with directionally opposing fetal and maternal effects. Furthermore, the authors performed the analysis to estimate the effect of insulin secretion on birth weight, including SNPs previously associated with fasting glucose and insulin sensitivity. They confirmed previous results of GWA SNPs for fasting glucose, T2D, insulin secretion, and insulin sensitivity loci and supported the opposing contributions of fetal *versus* maternal glucose-raising alleles on birth weight.

In conclusion, the Mendelian randomization provided evidence about the influence of fetal insulin on intrauterine growth and the link between lower birth weight with reduced insulin secretion and higher T2D risk in adults (Hattersley and Tooke, 1999; Warrington et al., 2019).

Further experimental and clinical studies are necessary to clarify the link between events *in utero* environment and predisposition to metabolic disease in adult life.

CONCLUSION

Although the available data are not conclusive and even partly conflicting, an increasing body of evidence indicates that a suboptimal intrauterine environment induces epigenetic changes and eventually leads to programming. These epigenetic fingerprints are still present at birth and are related to specific structural and functional alterations in postnatal life. The question whether these epigenetic changes are causative or simply associated with the metabolic profile remains unanswered. Nevertheless, the epigenetic modifications, especially those detectable in the circulation, such as miRNAs or SNPs, may become predictive markers of cardiometabolic risks provided that a specific pattern is proven to be associated with long-term risk in controlled prospective longitudinal studies.

AUTHOR CONTRIBUTIONS

AD, EI, and SC wrote the article. SC critically reviewed the article.

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