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Adipose cell-free DNA in diabetes

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ABSTRACT

Cancer-associated necrosis is a well-known source of cell-free DNA (cfDNA). However, the origins of cfDNA are not strictly limited to cancer. Additionally, dietary exposure induces apoptosis-induced proliferation in adipocytes, leading to the release of cfDNA. The genetic information derived from cfDNA as a result of apoptosis-induced proliferation contains specific methylation patterns in adipose tissue that can be used as a marker to detect the risk of developing Type 2 diabetes Mellitus (T2DM) in the future. cfDNA is superior to peripheral blood leukocytes (PBL) and whole blood samples for reflecting tissue pathology due to the frequent use of PBL and whole blood samples that do not match tissue pathology. The difficulty of demonstrating that cfDNA is derived from adipose tissue. We propose several promising techniques by analyzing cfDNA derived from adipose tissue to detect T2DM risk. First, adipose-specific genes such as ADIPOQ and Leptin were utilized. Second, MCTA-Seq, EpiSCORE, deconvolution, multiplexing, and automated machine learning (AutoML) were used to determine the proportion of total methylation in related genes.

1. Introduction

Consumption of high-carbohydrate [1], high-fat [2], or a combination of these diets [3] and high-MSG diets [4] induces de novo lipogenesis (DNL), which causes adipose tissue remodeling with mechanism hypertrophy and hyperplasia in adipose tissue. Hypertrophy has a limit, which encourages apoptosis-induced proliferation followed by hyperplasia [5,6]. Proinflammatory cytokines such as TNF-α are produced during the apoptosis process. Obesity is thus caused by the accumulation of adipose tissue in large quantities. Obesity is a risk factor for T2DM; for each 2 kg/m^2 decrease in BMI, men have a 23 % lower risk of diabetes, and women have a 27 % lower risk of diabetes [7]. The apoptosis will further activate the apoptotic cascade, and the secretion of proinflammatory cytokines in a low-grade and chronic manner will disrupt glucose transporter type 4 (GLUT-4) translocation, resulting in insulin resistance and T2DM.

The interaction of genes and the environment can impact an individual's health. In the case of daily food consumption, such as a high carbohydrate diet [8], a high-fat diet [9], a combination of both diets [10], and a high MSG diet [11], Genetic interactions affect the body's metabolism and lead to diseases such as type 2 diabetes (T2DM). Unlike genetic alterations, epigenetic changes do not impact on the DNA sequence and are passed down to future generations. Epigenetics can be reversed by a balanced diet [12,13], physical activities [14], drugs [15,16], and surgical intervention [17]. Methylation is an epigenetic alteration that has distinctive patterns in each tissue. These patterns can predict the risk of T2DM in the future.

According to current understanding, cancer necrosis releases cell-free DNA (cfDNA). In addition, the development of adipose tissue has the potential of releasing genetically informative cfDNA into the bloodstream via the apoptosis mechanism. This tissue-specific cfDNA is detectable in the bloodstream and describes the molecular status. Moreover, excessive nutrients will alter the methylation pattern of adipose tissue, induce apoptosis-induced proliferation, and induce T2DM, resulting in the release of cfDNA.

Numerous studies on cfDNA methylation have been conducted concerning type 1 diabetes (T1DM). This marker is detectable in chronic conditions caused by pancreatic cell death in T2DM. Therefore, it cannot be used for early detection of T2DM. Differentiating the origin of cfDNA from adipose tissue and other tissues is a barrier to using cfDNA to detect T2DM. cfDNA usage markers are derived from adipose tissue because adipose tissue is the source of inflammation, which plays a crucial role in the onset of T2DM. Using methylated CpG tandems amplification and sequencing (MCTA-Seq) on cfDNA samples based on the proportion of

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methylation patterns in the same gene, a previous study successfully distinguished three cancers in asymptomatic patients [18]. This method can detect T2DM by translating methylation between adipose tissue and other tissue in cfDNA.

2. Dietary exposure effect on adipose tissue

2.1. Dietary exposure induces adipose tissue remodelling

High-carbohydrate [1], high-fat [2], or a combination of these diets [3] and high-MSG diets [4] induce de novo lipogenesis (DNL), leading to adipose tissue hypertrophy and hyperplasia. In a previous study [19], a high-MSG diet did not affect body weight compared to the diet of the control group. Instead of comparing body weight, this condition arose as a result of the fact that the majority of previous studies did not use BMI to determine obesity.

In a separate study, MSG exposure led to massive fat deposition, but there was an insignificant amount of the MSG in body weight between the experimental and the control groups [4]. A negligible amount of research has been conducted on methylation induced by MSG. This small quantity may result in a dearth of MSG methylation studies. Due to permanent lesions in the arcuate nucleus of the hypothalamus, There was no statistically significant difference between the groups receiving the MSG-containing diet and the control group [20,21]. This nucleus is responsible for hormone production. Therefore, the growth hormone in male rats decreases, affecting their body length. This disorder was not detected in female rats because continuous growth hormone release occurs in female rats [20].

The reorganization of adipose tissue increases cell size (hypertrophy) and cell number (hyperplasia) [22,23]. The hypertrophy of adipose cells restricts their ability to store fatty acids. Thus, the body stimulates the proliferation of new adipose cells by inducing apoptosis [24] (Fig. 1).

2.2. Adipose tissue apoptosis-induced T2DM

Apoptosis will generate microparticles (MP) that act as chemoattractants for macrophages. MP functions as a Th17/Treg chemoattractant and dendritic cell, increasing macrophage-1 (M1) accumulation in adipose tissue. M1 attracts other immune cells. Infiltration of macrophages or polymorphonuclear (PMN) cells generates proinflammatory cytokines such as TNF-α (Fig. 1).

TNF-α binds to TNF-α related apoptosis-inducing ligand (TRAIL) to cause apoptosis both extrinsic (through the death receptor) and intrinsic (via mitochondrial pathways) [25]. TRAIL stimulates preadipocyte proliferation by activating extracellular signal-regulated kinase 1/2 (ERK 1/ERK2) [26]. ERK1/ERK2 increases the expression of Peroxisome proliferator-activated receptor gamma (PPARγ) during the maturation of preadipocytes into adipocytes and CCAAT enhancer-binding proteins (C/EBP) [27,28]. The fibro-inflammatory progenitor, also known as FIP, typically has a toll-like receptor 4 (TLR4) on its surface. The receptor is responsible for stimulating pro-inflammatory signaling. IkB binds to the p50 and p65 subunits in the inflammatory cascade due to TLR4 activation.

Inflammatory signals such as ROS degrade IkB, and activating p50-p65 at the transcription site of nuclear factor kappa-activated β-cell light chain enhancer (NF-κB). This process induces the release of pro-inflammatory cytokines [29,30]. However, in obese individuals, over-expression of zinc finger protein 423 (ZFP423) causes a decrease in the p65 subunit, induces recruitment of the NuRD corepressor complex to p65, and results in the loss of coactivator (p300), p65 acetylase, and p65 DNA-binding capacity. All of these things work together to suppress the expression of proinflammatory cytokines [31].

The up-regulation of ZFP423 suppresses FIP, thereby inhibiting the transformation of MSC into pre-adipocyte [32]. This effect induces low-level secretion of pro-inflammatory cytokines, including IL-1, IL-6, and TNF-α. Low and chronic levels of pro-inflammatory cytokines and high levels of circulating FFA cause insulin resistance by interfering with insulin-stimulated tyrosine phosphorylation insulin receptors (IR) or insulin receptor substrate (IRS), thereby reducing GLUT-4 translocation. Target cells that exhibit insulin resistance include adipose tissue, skeletal muscle, liver, cardiac, adipose tissue, and brain. T2DM is the result of insulin resistance.

The changes during adipose remodeling induce T2DM are epigenetic processes of hypermethylation and hypomethylation that affect gene expression. In a previous study using a mouse model of obesity, hypomethylation of the ZFP423 promoter was identified as the cause of ZFP423 overexpression [33]. Methylation alters the DNA cleavage pattern and influences the size of fragmented DNA in circulation. DNA segment length is proportional to the amount of methylation [34]. Hypomethylation will open chromatin, making DNA more easily cuttable than when chromatin is closed. Hypermethylation, in contrast, is the inverse.

2.3. The superiority of cfDNA in predicting T2DM

Epigenetics plays a crucial role in the progression of diseases such as diabetes. Unsuitable nutrition is one of the epigenetic mechanisms responsible for modifying the methylation pattern of particular genes, whether increased methylation (hypermethylation) or decreased methylation (hypomethylation), which affects the body metabolism [35].

Methylation analysis typically involves invasive techniques such as

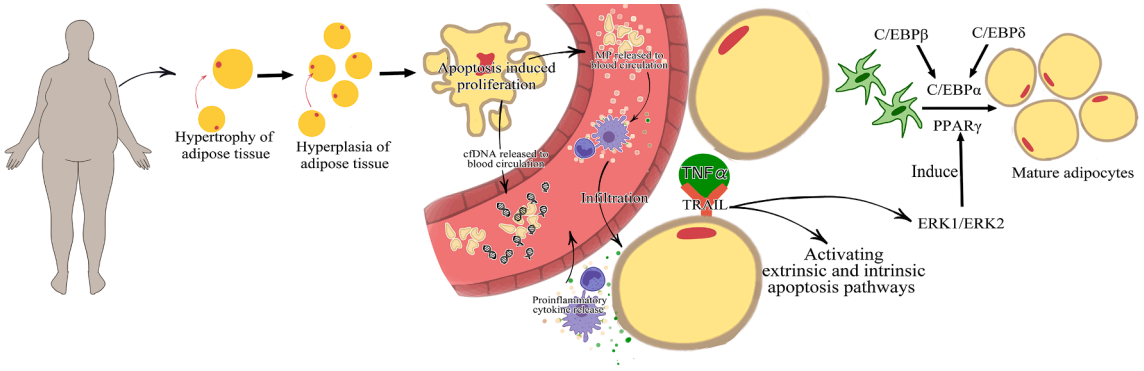

Fig. 1. Apoptosis-induced proliferation in adipose tissue release cfDNA. The excessive consumption of different diets stimulates hypertrophy to store FFA. The limit of hypertrophy stimulates apoptosis-induced proliferation, followed by hyperplasia. The process of apoptosis releases cfDNA into the blood and recruits immune cells. Inflammatory cytokines produced by immune cells, such as TNF-α, induce insulin resistance. TNF-α binds to TRAIL to induce apoptosis both extrinsically and endogenously. TRAIL also stimulates preadipocyte proliferation by activating ERK1/ERK2 signaling. In the proliferation of preadipocytes to mature adipocytes, ERK1/ERK2 increases the expression of PPARγ and C/EBP.

tissue biopsy. Due to its inability to perform multiple and repeated examinations, the invasive procedure poses a barrier. To overcome this obstacle, a noninvasive strategy involving blood samples is required. The methylation analysis utilized whole blood samples, peripheral blood leukocytes (PBL), and cfDNA.

Extensive research has been conducted using whole blood samples and PBL, but these samples do not represent the pathology that occurs in tissues. Frequently, the results of studies on the whole blood do not correlate with tissue pathology. In examining patients with colorectal cancer using methylation tests on the SEPT9 and SDC2 genes, for instance, the whole blood results are still inconsistent. Methylation levels in whole blood samples from cancer examinations were average, whereas methylation differences were found in cfDNA [36].

It is well known that cfDNA samples can be used for methylation testing to determine the presence of cancers such as breast, pancreatic, hepatocellular carcinoma, and colorectal cancer. This examination is used for cancer screening, diagnosis, prognosis, treatment evaluation, and relapse monitoring. Cell-free DNA (cfDNA) contains molecular status-describing genetic information, is detectable in the bloodstream, and originates from a specific tissue [37].

SIM1 hypermethylation manifests cervical cancer in cases of cervical cancer. For methylation that occurred in cervical tissue with a methylation cutoff of 80 %, 39 out of 41 people (95.6 %) had positive results. 15 out of 41 individuals (36.6 %) in the cfDNA sample returned positive results. None of the patients in the PBL sample had a positive result [38]. Another study confirms that methylation in cfDNA corresponds to particular tissues [39].

CPT1A is hypomethylated in PBL samples with metabolic disorder, whereas it is hypermethylated in whole blood samples [40–42]. The difference in adipose tissue methylation patterns is not reflected in the whole blood and PBL samples, according to previous research [43–45]. The inconsistent results render the whole blood and PBL samples unreliable for diagnostic purposes.

gDNA will be extracted from the two DNA sources: leukocyte DNA and cell-free DNA, using whole blood. The commingling of leukocyte DNA and cfDNA resulted in the difference in methylation pattern observed in whole blood samples. This is problematic due to the frequent use of PBL and whole blood samples that are unrepresentative of tissue pathology. cfDNA is superior to PBL and whole blood samples for tissue pathology evaluation. In light of this, analyzing the methylation pattern on cfDNA is a potential biomarker for detecting T2DM at an early stage.

Extensive research utilizing cfDNA in T1DM patients has been conducted. This study is based on pancreatic damage that results in the release of cfDNA. Known methylation studies have been reported for INS [46,47], CHTOP [46,48], amylin [49], LENG8, FBXL19, ZC3H3, MTG1 [50], and GCK [51] in the T1DM cfDNA sample. In T2DM, however, methylation of cfDNA has been performed to analyze cell death, including INS, IAPP, GCK, and KCNJ1148. INS, IAPP, GCK, KCNJ11, and CHTOP are genes that can be used as T2DM markers and have been detected in cfDNA samples. This marker cannot be used for early detection of T2DM because pancreatic cell death makes it detectable in chronic conditions.

2.4. Promising marker from adipose tissue to detect T2DM

Adipose tissue is the first organ implicated in the development of T2DM [52,53]. Early diagnostic is conducted to investigate the pathology of adipose tissue and diagnose it before T2DM. There are many sources of cfDNA from other tissue. Thus, the problem is to prove that cfDNA comes from adipose tissue. On cancer, a previous report using MCTA-Seq on cfDNA samples from the percentage of methylation patterns in the same gene successfully distinguished between gastric cancer, colorectal cancer, and hepatocellular carcinoma in asymptomatic patients. Surprisingly this methylation examination is superior to conventional examinations such as carcinoembryonic antigen (CEA) serum [18]. This approach can detect methylation patterns in cfDNA from adipose tissue in T2DM.

The well-known source of cfDNA is necrosis, which is typically seen in cancer, but it is now known that this process is not exclusive to cancer. Considering that the adipose development process release cfDNA via apoptosis-induced proliferation, particularly in obese conditions with insulin resistance. Obesity occurs due to increased adipose deposition. The adipose deposition process releases cfDNA via apoptosis-induced proliferation. The higher the deposition of adipose tissue is, the higher the release of cfDNA and insulin resistance. It proves that T2DM fragments that are dominant in size indicate more significant apoptosis (~160 bp) [54]. In previous research, obese mothers (pregravid BMI > 30) have higher cfDNA compared to lean mothers (pregravid BMI < 25), and no increase in cell-free fetal DNA was found [55]. These results were validated in preclinical studies with obesity models that had increased cfDNA and clinical studies that showed higher cfDNA in obese models [56].

Insulin resistance is an initial condition before the occurrence of T2DM. Thus, the Apoptosis process releases cfDNA use as early detection of T2DM. Based on the unique methylation pattern occurring in specific tissues and diseases, we suggest two methods to assess pathology occurring in adipose tissue. First, specific genes in adipose such as ADIPOQ and Leptin were examined. Second, the percentage of global methylation in related genes (global DNA methylation) was assessed) (Fig. 2).

2.5. Proving cfDNA origin from adipose tissue

Proving that cfDNA originates from a specific tissue requires effective, time-efficient, pure, and high DNA yield cfDNA extraction. The use of the kit must always be uniform within a study to avoid research biases or inaccuracies. In general, extraction of cfDNA is challenging due to its low concentration in plasma and short half-life, so it is necessary to isolate it immediately from whole blood by an effective method.

Previous research compared the MagMAX Cell-Free DNA Extraction kit, JBS cfDNA extraction kit, and QIAamp Circulating Nucleic Acid (CNA) Kit [57]. The amount of yield within this study is insignificant among the three kits. Other studies include the QIAamp CNA Kit, Maxwell RSC plasma cfDNA, and Zymo manual quick cfDNA kit. QIAamp CNA consistently yielded the most significant amount of cfDNA and fragments of a short-size [58]. However, when compared to dimethyl suberimidate dihydrochloride (DMS), the QIAamp Circulating Nucleic Acid (CNA) Kit exhibited 56 % less extraction yielded efficiency. The DMS technique shortens processing time from 1 to 2 h to 10 min and is able to covalently and electrostatically bind DNA [59].

After selecting the appropriate cfDNA isolation from adipose tissue, the two suggested methods in the study were implemented. In the first method, specific genes were assessed in adipose tissue, namely ADIPOQ and leptin. Aberrant methylation of ADIPOQ and leptin contribute to the occurrence of T2DM. In clinical studies using a control group on obesity and T2DM adipose tissue samples, the ADIPOQ gene was hypermethylated, and leptin was hypomethylated gradually (Table 1). This method employs DNA extraction from plasma to obtain cfDNA, followed by methylation-specific PCR (MSP), Micro Arrays, denaturing high-performance liquid chromatography (DHPLC), ELISA-based MethylFlash Methylated DNA Quantification Kit (Epigentek), and Pyrosequencing.

Knowing the global methylation pattern makes this second method reliable. In lean, obese, and obese with insulin resistance and T2DM patients, the gene will experience gradual hypermethylation (Table 2). Given the established pattern in adipose tissue, methylation testing on cfDNA is a promising method, with two methods recommended for the early diagnosis of T2DM.

The second method can be assessed by several methods such as MCTA-Seq [70], EpiSCORE algorithm [71], deconvolution algorithm [72], multiplexing method [50], and automated machine learning

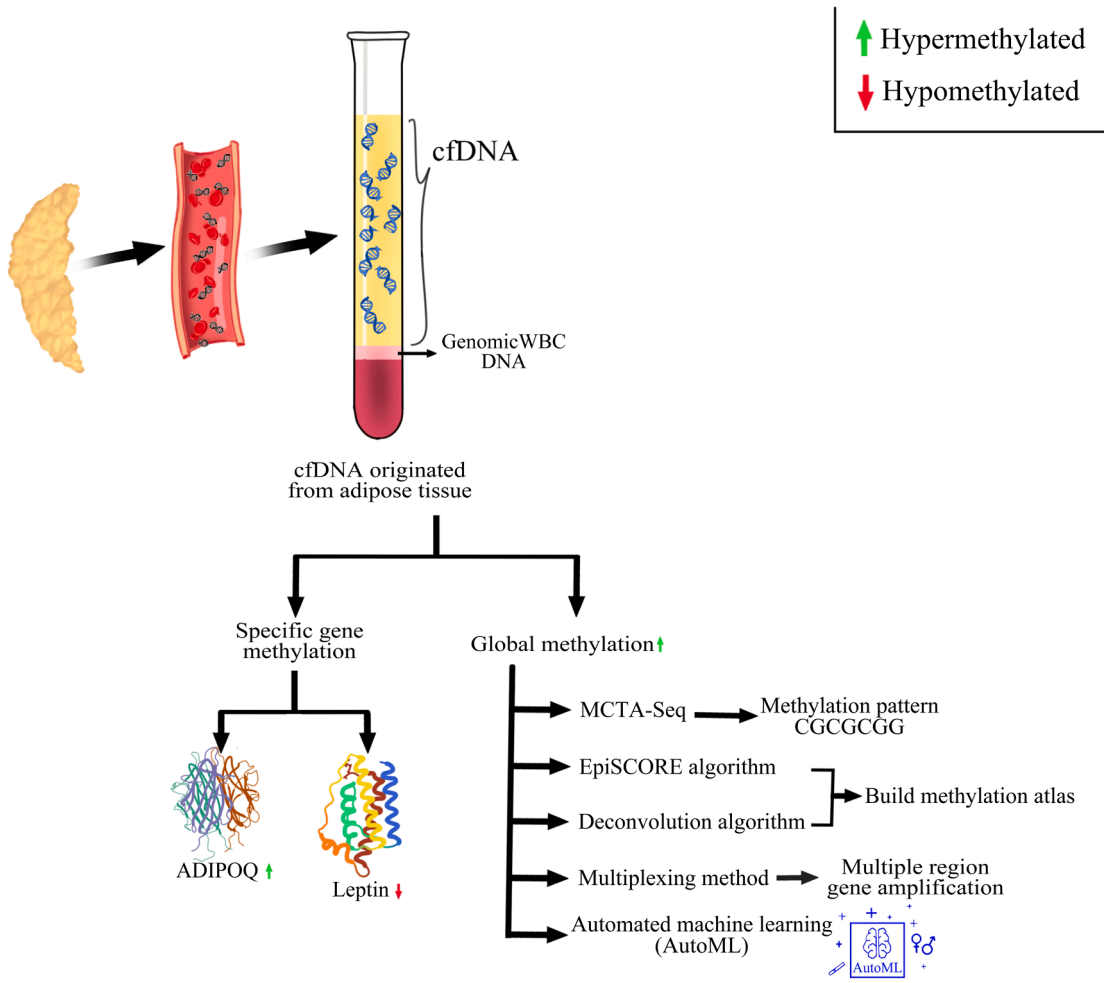

Fig. 2. Two Method examination cfDNA from adipose tissue origin. First, examining specific genes in adipose such as ADIPOQ and Leptin. Second, assess the percentage of global methylation using MCTA-Seq, EpiSCORE algorithm, deconvolution algorithm, automated machine learning (AutoML), and multiplexing DNA methylation.

(AutoML) [73]. This test is reliable since the MCTA-Seq method identifies the origin of cfDNA by using a specific CGCGCGG methylation pattern and is suitable for efficiently screening novel cfDNA methylation disease markers. The method itself is based on the fact that cancer is more hypermethylated than T2DM. As a result, this method still requires modifications for translation in metabolic disorders such as T2DM. The method outlines (a) identified significant differences in methylation between T2DM and non-T2DM patients and (b) similar methylation in cfDNA and adipose tissue. This pattern of methylation has previously been used to validate the methylation pattern in cfDNA. At the same time, a recent study has identified three types of gastrointestinal cancer.

EpiSCORE algorithm and deconvolution algorithm are methods used to compare specific tissues with cfDNA samples using the construct methylation atlas method. The tissue-specific origin of cfDNA was discovered using the deconvolution algorithm. In contrast to the complex EpiSCORE algorithm, this method is simpler because it uses a previously published DNA methylation human-specific tissue database. The deconvolution algorithm compares specific tissues with cfDNA using non-negative least squares linear regression to create a methylome atlas. To obtain precise results, a subset from CpG sites in the genome that are differentially methylated between tissues in this atlas was selected. This selection method is currently applied only on healthy patients but soon, it will also be applicable to T2DM.

The EpiSCORE algorithm constructs a DNA methylation atlas from the promoter DNA methylation data validated using tissue-specific mRNA to a specific type of cell using single-cell RNA sequencing. This is based on the fact that methylation promoter DNA and mRNA are anti-correlated. The EpiSCORE DNA methylation atlas has three criteria: (a) two high-quality single-cell RNA sequencing, (b) a marker for each gene in each cell type, and (c) an independent DNA methylation dataset to validate tissue-specific DNA methylation EpiSCORE uses the gene-expression-based ESTIMATE algorithm, the CNV-based ABSOLUTE algorithm, immunohistochemistry (IHC), and a method that combines all these three (consensus purity estimation; CPE). Accessible data sets in the online database include single-cell RNA sequencing, promoter DNA methylation, and tissue-specific mRNA. Therefore, this method is reliable for translating cfDNA samples into T2DM-specific methylation atlas.

The multiplexing method employs cfDNA from T1DM samples. This method employs six methylation patterns of the pancreatic-cell pattern of T1DM, which is typically unmethylated in this disorder. This method is based on cell pancreas death in T1DM, which results in the release of DNA from the pancreas into the bloodstream. Multiplexing DNA methylation is a method used on multiple targets amplified and sequenced from bisulfite-treated cfDNA. The protocol for multiplexing DNA methylation consisted of two steps. The first step was to use up to 30 primer pairs in a single PCR reaction for the amplification of target regions of bisulfite-treated DNA, regardless of methylation status. In the same reaction tube, all primers were mixed.

The first PCR reaction products were treated with Exonuclease I (ThermoFisher Scientific) for primer removal in the second PCR step, as directed by the manufacturer. The amplification products were

Table 1
Methylation Pattern ADIPOQ and Leptin in Adipose Tissue.

Experimental Design	Method	Methylation Result	Reference
Normal weight with overfeeding treatment	Arrays	ADIPOQ↑	[60]
Normoweight vs overweight	Arrays	ADIPOQ↑	[61]
Normal gestation vs gestational diabetes melitus	Pyrosequencing	ADIPOQ↑	[62]
Normal weight vs Obesity and T2DM	Denaturing high performance liquid chromatography (DHPLC)	ADIPOQ gradually↑	[63]
Normal weight versus overweight, and overweight with insulin resistance	Epigentek	ADIPOQ gradually↑	[64]
Normoweight vs obese and obese insulin resistance	Epigentek	ADIPOQ gradually↑	[64]
Normoweight vs obese	Pyrosequencing	ADIPOQ↑ and leptin↓	[65]
Overweight, obese and obese with insulin resistance	Methylation-specific PCR	Leptin gradually↓ and ADIPOQ gradually↑	[66]

↑ = Hypermethylated; ↓ = Hypomethylated.

Table 2
Global Methylation Pattern in Adipose Tissue.

Experimental Design	Method	Methylation Result	Reference
Lean vs obese and obese with insulin resistance	Epigentek	Gradually↑	[64]
Healthy vs T2DM	EpiJET DNA Methylation Analysis Kit	↑	[67]
Normal weight vs obese women with insulin resistance	BeadChips	↑	[68]
Normal weight with overfeeding treatment	Array	↑	[60]
Before and after gastric bypass surgery	Arrays	↓	[69]
Lean vs obese and obese with insulin resistance	Epigentek	Gradually↑	[64]
Healthy vs T2DM	EpiJET DNA Methylation Analysis Kit	↑	[67]
Normoweight vs obese women with systemic insulin resistance	BeadChips	↑	[68]

↑ = Hypermethylated; ↓ = Hypomethylated.

sequenced thousands of times (multiplexed) in the MiSeq/NextSeq platform to determine the fraction of molecules in plasma carrying the tissue-specific methylation pattern. By multiplying the fraction by the total concentration of cfDNA in a sample, the concentration of cfDNA molecules derived from a specific tissue was calculated. The multiplexing method successfully detected cfDNA from the cell pancreas in a cohort study.

To treat T2DM, AutoML is the only usable method and the AutoML used pancreatic-cell INS, IAPP, GCK, and KCJN11 markers. Unfortunately, the respective method used to release the gene via cfDNA is in a chronic state. This method employs artificial intelligence to aid in the accurate analysis of data by taking into account cfDNA parameters, methylation data, demographic data such as age, gender, and smoking habit to improve non-expert statistical analysis replicability and protect against common methodological analysis pitfalls such as overfitting.

The AutoML algorithm automatically performs data preprocessing (Mean Imputation, Mode Imputation, Constant Removal, and Standardization), serves featured selection using lasso or Statistical Equivalent Signatures (SES) algorithms, attempts thousands of algorithmic configurations, selects the best-performing model, and estimates the accuracy of the method. The five steps are recommendable to determine the combinable origins, such as the initial step of using MCTA-Seq, followed by creating a methylome atlas using the EpiSCORE algorithm or deconvolution algorithm, and analyzing data with AutoML.

3. Conclusion

Excessive daily intake of high-carbohydrate, high-fat, or a combination of these diets and high-MSG diets causes apoptosis-induced proliferation and interacts with genetics to change the methylation pattern in adipose tissue. Adipose tissue apoptosis produces cfDNA, which can be used to detect T2DM early. We propose methods for validating cfDNA extracted from adipose tissue. We suggest selecting a suitable DNA extraction method, such as dimethyl suberimidate dihydrochloride (DMS). The subsequent technique is MCTA-Seq or Multiplexing, followed by the construction of a methylome atlas utilizing the EpiSCORE algorithm or deconvolution algorithm and the precise analysis of data using AutoML. Thus, cfDNA a promising biomarker for future research into early T2DM detection.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. We want to acknowledge Indonesia Ministry of Research, Technology, and Higher Education for funding this research (058/SP-Lit/LPPM-01/KemendikbudRistek/Multi/FTB/V/2022 and 004/SP2H/PT/LL7/2022).

Data availability

No data was used for the research described in the article.

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