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Metformin Increased Histone Deacetylases 1, 3, and 8 Expressions as Epigenetic Regulators in Type 2 Diabetic Patients

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Abstract

Background: Type 2 diabetes is a complex disease resulting from interactions between genetic, epigenetic, and environmental factors. Histone deacetylases (HDAC) are essential epigenetic-regulatory enzymes that affect gene expression and, through metabolic homeostasis and beta-cell function regulation, play significant roles in the development and treatment of diabetes. In this study, we specifically focused on the effect of metformin, the first-line therapy for type 2 diabetes on the expression of class I HDAC genes.

Methods: A total of 60 patients were equally allocated into two groups: those receiving metformin treatment and those without treatment. Also, 60 subjects with normal glucose tolerance were divided into two groups: non-obese (n=30) and obese individuals (n=30). All biochemical and clinical factors were estimated using standard methods, and RT-qPCR was used to quantify the expression levels of the candidate genes in peripheral blood mononuclear cells of different groups.

Results: The metformin treatment group exhibited increased expression of *HDAC1*, *HDAC3*, and *HDAC8* in comparison to the non-treatment group. Furthermore, the expression levels of *HDAC 1*, *2*, and *3* were higher in the obese group than the non-obese. Interestingly, evaluation of biochemical and clinical factors revealed significant association between the expression of class I HDAC genes and several diabetes-related risk factors.

Conclusions: The current findings suggest that *HDAC1*, *3*, and *8* genes expression are affected by metformin, and obesity has a substantial ability to increase the risk of diabetes. However, changes in HDAC expression may represent potential biomarkers and therapeutic targets for future clinical studies in diabetes, particularly in exploring combination therapies involving histone deacetylase inhibitors and metformin.

Keywords: Body Mass Index, Diabetes Mellitus Type 2, Gene Expression, Histone Deacetylases, Metformin.

Introduction

Diabetes mellitus is the ninth-leading cause of mortality (1), and its incidence has more than doubled between 1990 and 2019 (2). Diabetes is a metabolic disease characterized by hyperglycemia that may be caused by immune-mediated (type 1 diabetes), insulin-resistant (type 2 diabetes), pregnancy, genetic abnormalities, infections, or specific medications (3). Type 2 diabetes is the most prevalent type of diabetes mellitus and occurs

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through interactions among genetics, behavioral, and the environment factors (4, 5).

Epigenetics refers to the heritable modifications in gene expression that occur without changes in the underlying DNA sequence. Alterations in epigenomic regulation have been linked to numerous diseases, including type 2 diabetes (6). Histone acetyltransferase (HAT) and Histone Deacetylases (HDACs) are post-translational modification enzymes that regulate the acetylation status of histone lysine residues, influencing gene transcription. An imbalance in the ratio of HAT to HDAC activity has been observed in multiple cancers (7). Both HATs and HDACs regulate several transcription factors and are crucial for maintaining metabolic homeostasis and β-cell function, suggesting that they may play a significant role in the development or treatment of diabetes (8).

There are four distinct classes of HDAC enzymes. *HDAC* 1, 2, 3, and 8 are family members of Class I HDACs, which mostly exist in the nucleus. These enzymes are involved in the deacetylation, promoting a repressive chromatin state that limits access to the transcriptional machinery (9). HDACs control insulin expression, secretion and insulin resistance in Type 2 Diabetes Mellitus (T2DM) (10). Several studies have shown that HDAC inhibitors are potential therapeutic agents for diabetes mellitus (11), as they affect β-cell development, function, proliferation, and inhibition of β-cell destruction, as well as being involved in insulin signaling (12), insulin resistance, and protection of pancreatic cells from cytokine-mediated damage (13).

Metformin, a well-established first-line drug for the treatment of type 2 diabetes, reduces hepatic gluconeogenesis and glucose generation through different pathways (14). regarding metformin's mechanism of action is its ability to increase AMP-activated protein kinase (AMPK) phosphorylation (15), a master regulator of several metabolic pathways (16). However, its effect on epigenetic regulators such as Class I HDAC genes in patients with T2DM remain unclear.

The current study aimed to investigate the effects of metformin and body mass index (BMI) on the expression of histone deacetylases in patients with type 2 diabetes and in healthy subjects. Our findings suggest that the HDAC class I genes expression is affected by metformin and obesity and that their gene expression levels correlate with diabetes risk factors. These results indicate that alterations in HDAC expression may be valuable for future clinical studies on diabetes, particularly for the development of novel therapeutic strategies targeting epigenetic regulation.

Materials and Methods

Study Subjects

Study subjects were recruited from the Khatam AL-Anbia Hospital in Gonbad Kavous, Iran. In this study, the inclusion criteria for diabetic patients were a fasting blood glucose level of 140 mg/dL or higher, glycated hemoglobin (HbA1c) levels ≥ 8%, and age between 30 and 60. Furthermore, for type 2 diabetic confirmation, the HOMA-IR index (glucose × insulin / 405) and the presence of insulin were assessed.

Exclusion criteria for patients were insulin therapy and chronic illnesses. For normal subjects, the inclusion criteria were a fasting blood glucose level below 100 mg/dL, with no history of diabetes or other health problems.

Participants were categorized into four groups:

Group I: Normal non-obese subjects (BMI 18.5–24.9, n = 30)

Group II: Normal obese subjects (BMI ≥ 30, n = 30)

Group III: Subjects with type 2 diabetes mellitus (T2DM) receiving metformin treatment (n = 30)

Group IV: Subjects with type 2 diabetes mellitus without treatment (n = 30)

All participants were aged between 30 and 60 years. Gender distribution was balanced, with 15 males and 15 females in each group.

The same sampling design was applied to both diabetic and normal groups.

The study was approved by the Biomedical Research Ethics Committee of Golestan University of Medical Sciences (code IR.GOUMS.REC.1398.184) and was conducted according to the principles of the declaration. All methodologies of experiments were done according to the standards set by the (1975, revised in 2000). Written informed consent was obtained from all participants prior to enrollment.

Biochemical and clinical estimations

In this study, all biochemical and clinical factors were estimated using standard methods. Fasting Blood Sugar (FBS) and glycated hemoglobin A1C (HbA1c) levels were measured using the hexokinase method and ion-exchange chromatography resins, respectively. White blood cells count was obtained by using a hemocytometer. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured using an auscultatory Korotkoff-based sphygmomanometer and an oscillometer, respectively, and the serum triglyceride concentration was determined using a standard enzymatic triglyceride assay. Body fat percentage (BFP) was calculated by dividing the total mass by the total body mass of fat by the total body mass, multiplied by 100. Finally, insulin level and insulin resistance were assessed using HOMA IR for confirmation of type 2 diabetes. In addition, in this study, we calculated body mass index (BMI) using the formula weight (kg) / height (m) and then categorized the samples into two subgroups based on BMI18.5-25 (normal) and BMI>30 (obese).

Bioinformatics analysis

Forward and reverse primers were designed for all HDAC genes (1, 2, 3, and 8) using Gene Runner v6 software and Primer3 (https://primer3.ut.ee/) online tools, and then their specificity was confirmed with Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) online software. The primers used in this study are listed in Table 1.

Table 1. Primer's sequences.

Name	NCBI accession number	Sequence
HDAC1	NM_004964.3	Forward: 5'-GCACCATGCAAAGAAGTCCG-3'
		Reverse: 5'-ACCCTCTGGTGATACTTTAGCAG-3'
HDAC2	NM_001527.4	Forward: 5'-ATCCGCATGACCCATAACTTG-3'
		Reverse: 5'-TCATTTCTTCGGCAGTGGCT-3'
HDAC3	NM_001355039.2	Forward: 5'-CACCCGCATCGAGAATCAGA-3'
		Reverse: 5'-TCTGCAGGCACGTCATGAAT-3'
HDAC8	NM_018486.3	Forward: 5'-GGAATGTTGACCAGGGAGCA-3'
		Reverse: 5'-CGCTTAAAACCGTTCCGCAG-3'
GAPDH	NM_002046.7	Forward: 5'-GTGAACCATGAGAAGTATGACAAC-3'
		Reverse: 5'-CATGAGTCCTTCCACGTACC -3'

RNA extraction from PBMCs and cDNA synthesis

Five ml of peripheral blood was collected from the study subjects, PBMCs were isolated in less than 2 h using Ficoll (Sigma Aldrich, St. Louis, MO, USA), and total RNA extraction was performed according to the AccuZol Kit protocol (Bioneer Pacific, Australia). The RNA concentration was evaluated using a PicoDrop device (Pico 200, Quantica). Next, to remove possible DNA contamination, the RNA samples were treated with DNase I (TAKARA, Japan) according to the standard protocol. Briefly, for cDNA synthesis, 1 µg of total RNA was combined with PrimeScript RT Enzyme (TAKARA, Japan), 5X RT buffer, oligo dT primers, random hexamer primers, and RNase-free water according to the kit's protocol. First-strand cDNA synthesis was performed at 42 °C for 15 min and the enzyme

was then deactivated for 30 s at 85 °C. The cDNA samples were used for quantitative real-time PCR (qRT-PCR) analysis.

RT-qPCR Analysis

RT-qPCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, USA).The reaction mixture included 10 µl of SYBR® Premix ex taq™ II (Takara, Japan), 500 ng of cDNA as a template, and 0.7 µL of each of the gene-specific primers with a concentration of 10 µM that made up to 12.5 µL with nuclease-free water. The average CT value was used for analysis. To prevent false-positive results, a no template control (NTC) was included in each experiment. Target gene expression was compared to the GAPDH level as an internal control.

Statistical Analysis

Real-time PCR data analysis was performed using R software (version 3.4.3). The relative expression of genes was calculated using the $2^{-\Delta\Delta Ct}$ formula. The hypothesis of normality of the data was investigated using the Kolmogorov-Smirnov (K-S) test. The Mann-Whitney U test was performed to compare gene expression between diseased and healthy subjects. Spearman's correlation analysis was performed to determine the correlation between gene expression and T2DM-related risk factors. Statistical significance was set at $P< 0.05$.

Results

Effect of metformin treatment on HDAC class I gene expression

To evaluate the effect of metformin treatment on the expression of circulating HDAC class I genes, we divided the patients into two groups: under treatment and without any drug treatment. RT-qPCR results showed a significant increase in the expression of HDAC1 (Fig. 1A) and HDAC8 (Fig. 1D) in the treatment group compared to the non-treatment and normal groups. Surprisingly, we observed switching from off to on in the expression level of HDAC3 after metformin treatment in diabetic patients (Fig. 1C). While results showed that there was no significant difference in the expression levels of HDAC2 (Fig. 1B) between treated and untreated patients. However, we illustrated a significant change in treated patients compared to normal individuals in the expression of all HDAC class I genes.

Effect of BMI on HDAC Class I Gene Expression

To investigate the effects of obesity on circulating levels of HDAC class I genes, we divided the normal sample into two groups: 18.5< BMI <25 as non-obese individuals and BMI > 30 as obese individuals. Our analysis showed that HDAC1 (Fig. 2A), HDAC2 (Fig. 2B), and HDAC3 (Fig. 2C) levels were significantly increased in patients with BMI>30 to those 18.5< BMI< 25, while HDAC8 did not change significantly (Fig. 2D).

Association between HDAC class I gene expression and type 2 diabetes risk factors

Spearman's correlation analysis was performed to investigate the relationship between the expression of HDAC1, 2, 3, and 8 and T2DM risk factors in patients with type 2 diabetes. The mean±SD of the risk factors is presented in Table 2. The correlation analysis results showed that BFP, FBS, HbA1c, and DBP were significantly correlated with the HDAC class I genes expression. Unlike other HDACs, HDAC3 has no significant association with age and triglycerides, while HDAC3 is the only HDAC associated with BMI and SBP in T2DM patients. In addition, HDAC2 and 3 were significantly correlated with white blood cells (WBC) (Table 2). However, there was no significant.

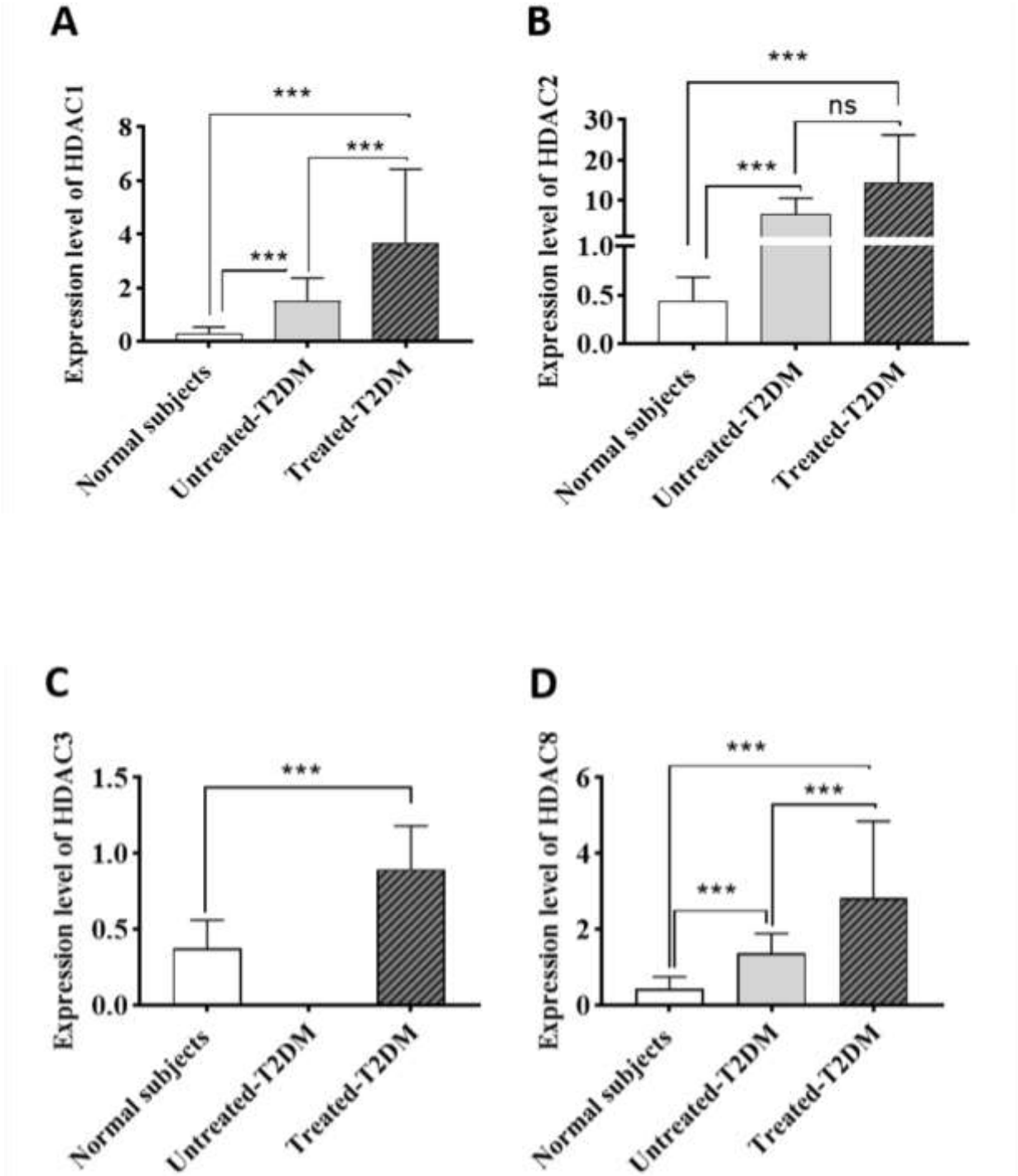

Fig. 1. A. HDAC1, B. HDAC2, C. HDAC3, and D. HDAC8 gene expression in untreated diabetic patient, and treated diabetic patient compared with normal subject. The mean ± SEM. *P< 0.05; **P< 0.01; ***P< 0.001; ns: non-significant.

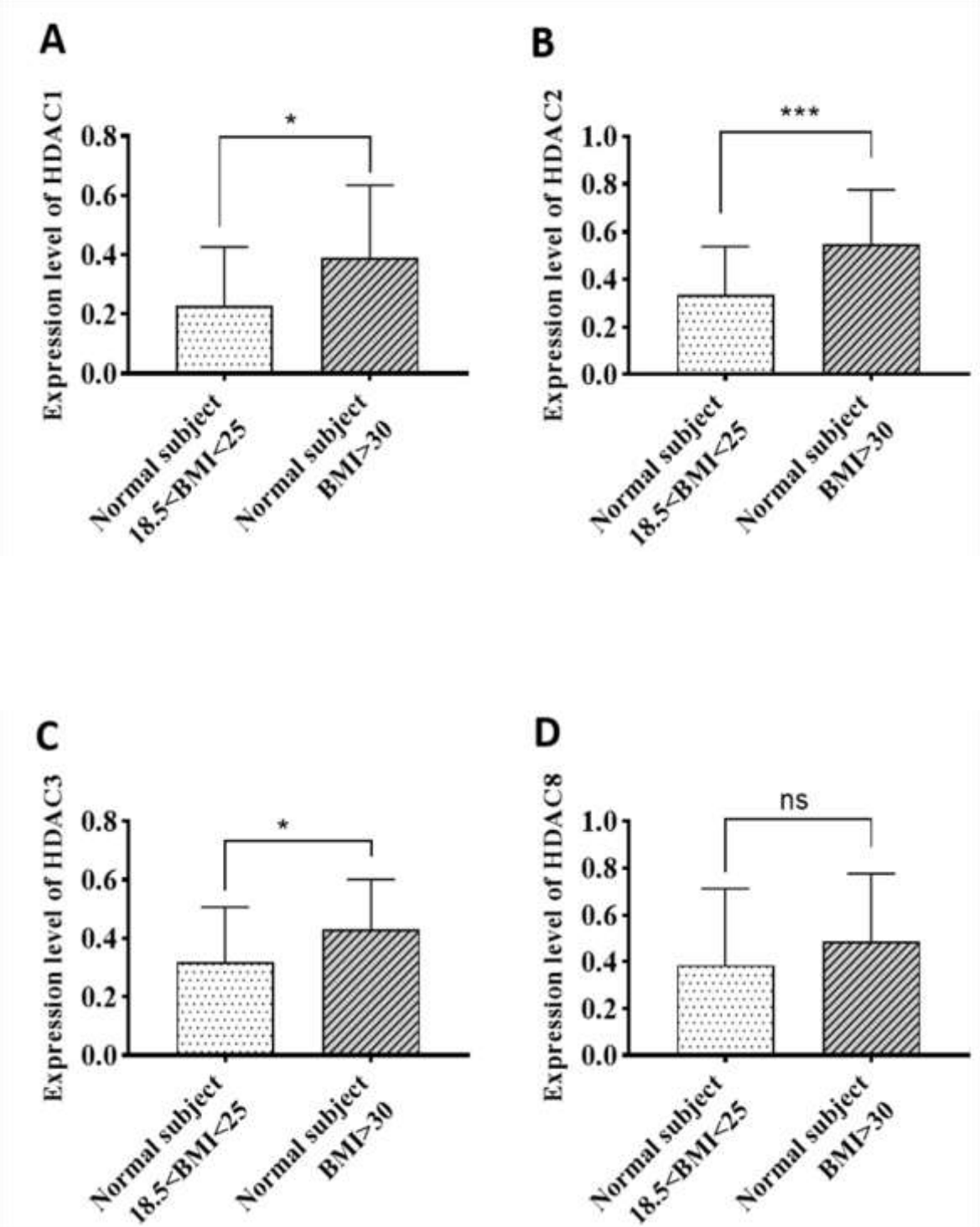

Fig. 2. A. HDAC1 **B.** HDAC2 **C.** HDAC3, and **D.** HDAC8 expressions in normal samples (N) with BMI>30 (obese) compared to normal samples with 18.5<BMI<25 **(**non-obese). The mean ± SEM. *P< 0.05; ns: non-significant.

Table 2. Spearman's correlation between T2DM risk factors and expression of HDAC class I genes in T2DM patients.

Risk factors	Mean ± SD	P value			
		HDAC1	HDAC2	HDAC3	HDAC8
Gender (male/female)	N=30/N=30	-	-	-	-
Age (years)	45.41±9.46	<0.01	<0.001	0.1	<0.01
BMI (Kg/m2)	27.93±4.65	0.2	0.3	<0.01	0.2
BFP (%)	35.81±8.65	<0.001	<0.01	<0.01	<0.001
FBS (mg/dl)	187.05±52.42	<0.001	<0.001	<0.001	<0.001
HbA1c (mIU/mL)	9.39±1.31	<0.001	<0.001	<0.001	<0.001
SBP (mmHg)	11.95±0.96	0.2	0.3	<0.05	0.8
DBP (mmHg)	8.63±0.75	<0.01	<0.05	<0.05	<0.05
Triglyceride (mg/dl)	156.73±58.63	<0.05	<0.001	0.2	<0.05
WBC (10*3/µl)	7.68.3±1.70	0.2	<0.01	<0.01	0.1

Discussion

Type 2 diabetes is a chronic disease that requires continuous medical care. Although important environmental factors in type 2 diabetes, such as diet and activity level, have been identified, identifying the genetic and epigenetic components that contribute to the development of type 2 diabetes remains a challenge (17). In the present study, for the first time, we investigated the effect of metformin treatment, a common diabetic drug, on the expression of histone epigenetic regulators in T2DM patients and compared it with those without drug treatment or normal subjects in the Iranian population.

Histone deacetylase (HDAC) enzymes function as epigenetic regulators that modulate gene expression (7) and represent potential targets for the treatment of several disorders (18), including diabetes mellitus (11). The result of the current study demonstrated that HDAC class I, such as HDAC1, HDAC2, HDAC3, and HDAC8, was significantly increased in patients with type 2 diabetes mellitus. This increase in HDACs expression and their activity suggests a potential role of these enzymes in the progression or development of T2DM. Further studies also indicate that all Class I HDACs are related to insulin resistance. So that HDAC1 can prevent peripheral glucose absorption by suppressing glucose transport through glucose transporter (GLUT) 4 (19), HDAC2 involved in diabetes development (20, 21) and is up-regulated in the diabetic mice's aorta (22). As well as HDAC3 initiates hepatic gluconeogenesis (23), and single nucleotide polymorphisms in this gene are related to type 2 diabetes risk (24). In addition, Sathishkumar et al. showed that increased HDAC3 activity (25) and its overexpression in PBMCs were accompanied by the concurrent activation of pro-inflammatory markers and insulin resistance in T2DM patients. Tian et al. reported that HDAC8 expression is markedly elevated in individuals with type 2 diabetes and promotes insulin resistance and β-catenin activation in NAFLD-associated hepatocellular carcinoma (26).

Investigating the effect of metformin as a typical and best treatment of type 2 diabetes on gene expression (27) could be helpful for understanding gene networks and resistance to T2DM. Our previous study revealed that metformin influences MALAT1 and GAS5 lncRNA expression in diabetic patients (28). Moreover, the present investigation indicated that metformin could influence the expression of circulating histone deacetylases, and T2DM patients with metformin treatment had higher expression of HDAC1, HDAC3, and HDAC8 compared with other groups. These results align with Bridgeman et al. result that suggested in their research that metformin may influence the activity of several epigenetic

modifying enzymes, particularly by modulating AMP-activated protein kinase (AMPK). On the other hand, histone acetyltransferases (HATs) and class II histone deacetylases (HDACs) are two epigenetic enzymes that are phosphorylated by active AMPK (29). Furthermore, metformin is effective on histone deacetylase inhibitors and acetyltransferases in different cancers (30, 31). An interesting observation in our study was that HDAC3 was not expressed in any of the untreated samples, but its expression elevated in treated patients. This suggests a pivotal role of metformin for regulation of HDAC class I gene expression.

In the following, we observed that in normal individuals with high BMI (BMI>30), the expression levels of HDAC class I genes increased. This result is aligns with Abbasi et al. results that was shown the body fat percentage has a positive correlation with diabetes risk (32). As well as evidence suggests that weight reduction can significantly lessen the risk of diabetes in all persons (33). This result indicating that obesity has a substantial ability to increase diabetes risk.

This study also shows association of T2DM risk factors and HDACs genes expression in Iranian population. Analyses of body fat percentage indicate a positive correlation between BFP and diabetes risk. As well as the p-value of age and triglyceride suggest there is significant association with our study outcome and other studies. Yan et al. and Escobedo-de la Pena et al. revealed that older adults have a greater frequency of diabetes and prediabetes than middle-aged adults (34) and triglyceride level, as biochemical marker, has biologically correlated with diabetes (35). In addition HbA1c, systolic blood pressure (SBP), and diastolic blood pressure (DBP) that significantly were higher in T2DM patients (36) have shown a strong correlation with HDACs expression in our T2DM samples. This study confirmed an interaction between diabetic influencing factors and HDAC class I expression, which may be important in explaining the differences in risk factors for diabetes and prediabetes in different populations.

In the present study, we revealed that metformin in T2DM patients and BMI in normal subjects could elevate HDAC class I genes expression and that there is an association between the expression of all members of Class I HDACs and most diabetic risk factors. Overall, this finding may be important in selecting treatment methods based on histone deacetylase inhibitors in diabetic populations.

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Authors' contributions

Amin Izadi, Azam Zarourati and, Sohrab Boozarpour designed the experiments; Sohrab Boozarpour supervised the research; Amin Izadi, and Azam Zarourati performed the experiments; Mohsen Ghalandar and Mina Lashkarboloki performed data analysis. Mina Lashkarboloki wrote the manuscript; Masoud Fahimi sample preparation, Sohrab Boozarpour and Madjid Momeni Moghaddam revised and edited the manuscript. All the authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors have no competing of interests.

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