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Nutrient sensing pathway genes expression dysregulated in patients with T2DM and coronary artery disease

Esmaeil Rahimi [a], Amirhossein Ahmadi [b], Mohammad Ali Boroumand [c], Bahram Mohammad Soltani [a], Mehrdad Behmanesh [a,*]

[a] Department of Genetics, Faculty of Biological Sciences, Tarbiat Modares University, P.O. Box 14115-154, Tehran, Iran
[b] Department of Biology, Faculty of Sciences, Persian Gulf University, Bushehr, Iran
[c] Tehran Heart Center, Tehran University of Medical Sciences, P.O. Box 1411713138, Tehran, Iran

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ABSTRACT

Aims: Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder and its prevalence is rapidly increasing worldwide. Patients with T2DM suffer from an increased risk of vascular complications. Of these, the development of coronary artery disease (CAD) causes the most mortality in patients with T2DM, however, its underlying molecular mechanisms are not fully understood. Nutrient sensing pathways which play a key role in sensing cellular energy and nutrients levels are reported to dysregulated in metabolic disease like T2DM. The aim of this study was to investigate the expression levels of nutrient sensing genes including *SIRT1*, *PRKAB1*, *PRKAB2* and *mTOR* in CAD^+ versus CAD^-T2DM patients.

Methods: Sixty-five people with T2DM who referred to Tehran heart center were participated in this study. Based on coronary angiography data these individuals were classified into two groups: CAD^+ T2DM (n = 34) and CAD^-T2DM (n = 31). Peripheral blood mononuclear cells were isolated from these patients and the expression levels of genes were evaluated by RT-qPCR.

Results: Significant down-regulations of the *SIRT1* (3.1-fold, $p = 0.0013$) and *PRKAB1* (3.5-fold, $p = 0.0001$) mRNA expression were observed in CAD^+ T2DM group in comparison with CAD^--T2DM patients. Receiver operating characteristic (ROC) curve analysis showed that the area under the ROC curve was 0.8529 ($p = 0.0001$) and 0.7078 ($p = 0.004$) for *PRKAB1* and *SIRT1* respectively.

Conclusion: Our results suggest that the dysregulation of genes involved in nutrient sensing pathway may be associated with the pathogenesis of CAD in patients with T2DM. Furthermore, the expression levels of these genes could be consider as potential biomarkers.

* Corresponding author.
E-mail address: behmanesh@modares.ac.ir (M. Behmanesh).

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1. Introduction

Type 2 diabetes mellitus (T2DM) is a chronic progressive metabolic disorder and its prevalence has been rapidly increasing throughout the world during the past decades [1]. T2DM accounts for almost 90% of all diagnosed diabetes cases [2]. Based on an estimation the total number of patients with T2DM will raise to 439 million adults by 2030 [3]. T2DM increases the risk for the development of vascular complications including retinopathy, neuropathy, nephropathy and coronary artery disease (CAD) [4]. Of these, CAD, which is caused by the formation of atherosclerotic plaques within coronary arteries, accounts for about 80% of death among individual with T2DM [5]. Indeed, adults with T2DM have a 2- to 3-fold higher prevalence rate of CAD than people without T2DM [6]. Interestingly, many of the risk factors are common between T2DM and CAD, specially hypertension, dyslipidemia and obesity [7,8]. In addition, both diseases share common pathogenic features, including chronic inflammation [9]. Although increasing evidence supports an essential role for chronic inflammation in the pathogenesis of T2DM and CAD, its underlying mechanism is not fully understood [10].

Nutrient-sensing pathway, which is composed of the mammalian silent information regulator-two 1 (SIRT1), mammalian target of rapamycin (mTOR) and 5′-AMP activated kinase (AMPK) proteins, contributes to the pathogenesis of T2DM and atherosclerosis [11,12]. Also, this pathway is a master regulator of inflammatory responses [11]. Recent studies have shown that the cellular energy metabolism could suppress the inflammatory responses [13–15]. SIRT1 as an energy sensor regulates inflammation, mitochondrial biogenesis, autophagy and maintenance of chromatin structure through deacetylation of transcription factors and histones [16]. AMPK, a master regulator of energy metabolism, is a heterotrimeric complex composed of two regulatory subunits (β and γ) and one catalytic subunit (α) [17]. In mammals, each subunit is encoded by a distinct gene. The α1 and α2 isoforms are encoded by *PRKAA1* and *PRKAA2*, the two isoforms of β1 and β2 are encoded by *PRKAB1* and *PRKAB2*, and the γ1, γ2 and γ3 are encoded by *PRKAG1*, *PRKAG2* and *PRKAG3*, respectively [18,19]. AMPK has been considered as an attractive target to control the inflammatory responses [20]. Although NF-κB is not the direct target of AMPK, AMPK activation could decrease NF-κB-induced inflammatory cytokines in macrophages [13,20,21]. SIRT1 and AMPK interact with each other to regulate inflammatory responses in various cell types through transcription factors like NF-κB, PGC-1α and FoxO [13,21]. Increasing evidence has revealed that inactivation of SIRT1 and AMPK could increase inflammation and the formation of atherosclerotic plaques [22]. In contrast, accumulating evidence has shown that mTOR inhibition could ameliorate inflammatory responses and plaque formation [23,24]. mTOR is a member of phosphoinositide kinase-related kinase (PIKK) family which plays a significant role in cell proliferation and autophagy [23,25]. Inflammatory signals could activate mTOR kinase, which in turn regulates key transcription factors and expression of their downstream genes that are involved in atherosclerosis and other inflammatory diseases [26]. Many studies have shown that the activation of SIRT1 and AMPK could inhibit mTOR signaling and ameliorate its effect on inflammation and atherosclerosis plaque formation [27,28].

Given the important roles of SIRT1, AMPK and mTOR in the regulation of inflammatory responses and pathogenesis of atherosclerosis, the aim of this study was to evaluate of the mRNA expression profile of *SIRT1*, *AMPK* (*PRKAB1* and *PRKAB2*) and *mTOR* genes in the peripheral blood mononuclear cells (PBMCs) of CAD and non CAD T2DM patients.

2. Subjects

2.1. Patients and biochemical analysis

All participants of this study were patients with T2DM (fasting plasma glucose ≥ 126 mg/dl (6.9 mmol/L) and/or HbA1c ≥ 6.5%) who underwent coronary angiography at Tehran Heart Center (THC). Fasting blood glucose (FBS) and HbA1c were measured by glucose hexokinase method (Cobas Integra 400, Roche Diagnostics) and an enzymatic method (Diazyme Laboratories, USA) respectively. Based on angiographic results, two groups of patients with T2DM (34 CAD^+ patients and 31 CAD^- patients) were selected (Table 1). Patients with coronary artery stenosis (≥50%) were categorized as a CAD+ group. This group was classified further into a subgroup of patients with single-vessel disease (SVD) and a subgroup of patients with multi-vessel disease (MVD). Triglycerides and HDL-cholesterol levels were measured using an auto-analyzer (Cobas Integra 400, Roche Diagnostics) with enzymatic techniques. Friedewald's formula was used to estimate LDL cholesterol. This study was approved by the ethics committee of THC and Tarbiat Modares university and all patients gave written consent to participate in the study.

3. Materials and methods

3.1. PBMCs isolation and RNA extraction

Peripheral blood mononuclear cells (PBMCs) were isolated from 3 mL of whole blood using Ficoll-Paque™ (GE Healthcare) according to manufacturer's instructions. Total RNA was extracted from isolated PBMCs using acid guanidinium-phenol-chloroform method by RNX™-Plus reagent (Cinnagen, Iran). The integrity and quantity of extracted RNA were examined by agarose gel electrophoresis and spectrophotometry, respectively.

3.2. cDNA synthesis

To eliminate DNA contamination, the extracted RNA was treated by DNase I enzyme (Thermo Fisher Scientific, USA) at 37 °C for 30 min. 3 μg of total RNA was used to synthesize complementary DNA (cDNA). cDNA was synthesized by M-MulV reverse transcriptase (Thermo Fisher Scientific, USA) using random hexamer and oligo $(dT)_{18}$ primers in a

Table 1 – Clinical and demographic parameters of the patients with T2DM.

Characteristics	CAD^+ n = 34 (100%)	CAD^- n = 31 (100%)	*p*-values
Age (years)	61.23 ± 8.893	60.97 ± 8.547	0.891**
Gender (Male)	21 (61%)	15 (48%)	0.324*
BMI, kg/m^2	28.79 ± 5.10	27.53 ± 3.89	0.527***
HDL, mmol/L	41.21 [34–45]	42.76 [36–48]	0.497**
LDL, mmol/L	101.12 ± 36.38	96.78 ± 29.5	0.631**
TCH, mmol/L	173.29	160.97	**0.048****
Triglycerides, mmol/L	175.12 [117–213]	173.16 [103–197]	0.371***
HbA1C, mmol/mol	69 ± 5.42	59 ± 5.56	**0.014****
Hyperlipidemia, %	83	80	0.694*
Hypertension, %	77	75	0.838*
Diabetes duration (years)	8.98 [3–12]	8.34 [3–11.9]	0.732***
Smokers %	11.7	16.12	0.611*
Treatment:			
Metformin, %	93.54	92.71	0.894*
Statin, %	98.11	91.34	0.272*
Gelibenglamide, %	36.26	27.31	0.439*
Insulin, %	14.54	8	0.437*

Data are presented as median [interquartile range] for variables without normal distribution and mean ± SD for variables with normal distribution. CAD, coronary artery disease; BMI, body mass index; HDL, High density lipoprotein; LDL, Low density lipoprotein; TCH, Total cholesterol; HbA1C, Glycated hemoglobin.

Significant *p* values (<0.05) were presented by bold numerals.

* Fisher's exact test or Chi-square test was performed to compare variables between CAD^+ and CAD^- patients.

** Student's *t*-test was performed to compare variables between CAD^+ and CAD^- patients.

*** Mann–Whitney *U* test was performed to compare variables between CAD^+ and CAD^- patients.

total of 20 μl reaction mixture, according to the manufacturer's instructions.

3.3. *Real-Time PCR*

Genes expression was evaluated by ABI StepOne™ real-time PCR (Applied Biosystems, Foster City, CA, USA). Real-time PCR was carried out in final reaction volume of 20 μl with 4 μl 5X EvaGreen® qPCR Mix Plus master mix (Solis BioDyne, Tartu, Estonia), 10 ng cDNA template and 200 nM of each forward and reverse primer. The sequences of designed primers were as follows: forward: 5′-GGCGGCTTGATGGTAATCAG TATC-3, reverse: 5′-ACTCTGGCATGTCCCACTATCAC-3′ for *SIRT1*, forward: 5′-CCCACATCTCCTCCAGGTCATCC-3′; reverse: 5′-CGCTGAGCACCATCACTCCATCC-3′; for *PRKAB1*, forward: 5′-GCTCAGATGACCCTAGCCACACTG-3′, reverse: 5′-CATCCCAG CAAAACAACAATCCC-3′ for *PRKAB2*, forward: 5′-GGGACTGCT TTGAGGTTGCTATGACC-3′, reverse: 5′-TCGCAGCACCTCCAT CACTGTGTGG-3′ for *mTOR*, forward: 5′- AGCCTTCCTTC CTG GGCATGG-3′, reverse: 5′- AGCACTGTGTTGGCGTACAGGTC -3′ for *ACTB*. The thermal reaction condition was as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 20 s. Specificity of PCR products were verified using poly acrylamide gel electrophoresis and melt curve analysis. The expressions of *SIRT1*, *PRKAB1*, *PRKAB2*, and *mTOR* mRNAs were normalized to the *ACTB* mRNA as an internal control and ΔCts were calculated by subtracting the Ct value of *ACTB* from the Ct values of the target genes. The relative expression for each gene was calculated by $2^{-\Delta\Delta Ct}$ formula [29].

3.4. *Statistical analysis*

Statistical analysis was performed by SPSS (Chicago, IL, USA) and Graphpad Prism version 6.0 (Graphpad Prism Software, Inc., San Diego, CA) softwares. Data was analyzed for normal distribution by performing the Shapiro–Wilk test. Student *t*-test and Mann-Whitney *U* test was used to analyze parametric and nonparametric continuous variables, respectively. Fisher's exact test or Chi- square test was performed to compare categorical variables. Any *p*-value lower than 0.05 ($p < 0.05$) were considered statistically significant.

4. Results

4.1. *Down-regulation of* **SIRT1 and PRKAB1 mRNAs in PBMCs of patients with T2DM and CAD**

The expressions levels of *SIRT1* and *PRKAB1* genes was lower in PBMCs of CAD^+ T2DM patients as compared with CAD^- T2DM patients. Furthermore, as shown in Fig. 1, the expression level of *PRKAB1* was more down-regulated (fold change: 3.5, $p = 0.0001$) than the *SIRT1* expression (fold change: 3.1, $p = 0.0013$) in CAD^+ versus CAD^- patients. In addition, to find the influence of disease severity on the expressions of *SIRT1* and *PRKAB1* genes, their expressions were compared between single vessel disease (SVD, n = 13) and multi-vessel disease (MVD, n = 22) groups. The mRNA expression level of *SIRT1* was significantly decreased in MVD subgroups compared to SVD patients ($p = 0.026$) but there was no significant difference in the mRNA expression level of *PRKAB1* gene between these two subgroups (data not shown).

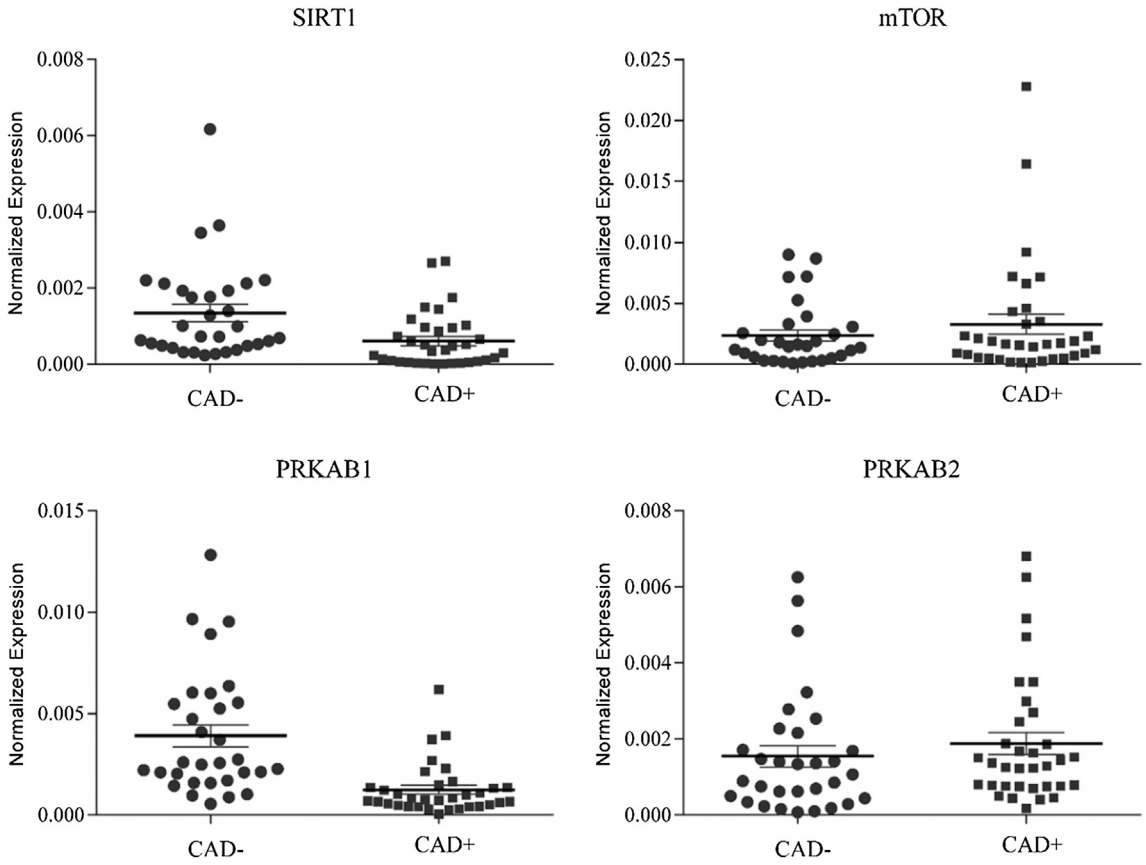

Fig. 1 – Normalized expression of *SIRT1* ($p = 0.0013$), mTOR ($p = 0.787$), *PRKAB1* ($p < 0.0001$) and *PRKAB2* ($p = 0.123$) genes in T2DM patients with CAD$^+$ (n = 34) in comparison with CAD$^-$ (n = 31). The mRNA expression of *SIRT1* and *PRKAB1* were down-regulated significantly in CAD$^+$ patients (Student's *t*-test, $P < 0.05$) but the mRNA expression of *PRKAB2* and *mTOR* was not significantly changed. The gene expression values of each sample were normalized against *ACTB* mRNA expression. Error bars indicate means ± standard errors of the mean.

4.2. *The expression levels of mTOR and PRKAB2 were not significantly changed*

Statistical analysis showed that there was no significant difference in the expression levels of *mTOR* and *PRKAB2* between CAD$^+$ and CAD$^-$ patients with T2DM (Fig. 1). To find whether or not the severity of CAD affects the expression levels of *mTOR* and *PRKAB2*, we further compared their expressions between the SVD (n = 13) and MVD (n = 22) subgroups. our data showed that there was no significant difference in the expression levels of these genes between SVD and MVD subgroups.

4.3. *Influence of lipid profile and glycemic control on the expression profiles of SIRT1 and PRKAB1*

To detect any correlation between lipid profile, FBS, HbA1c and mRNA expressions of the studied genes, Pearson correlation analysis was performed in both CAD$^-$ and CAD$^+$ patients with T2DM. There was no significant correlation between expression levels of the studied genes with lipid profile, FBS and HBA1c except for *PRKAB2* that showed positive correlation with HDL level in CAD$^+$ patients with T2DM ($r = 0.445$, $p = 0.008$).

4.4. *Co-expression of SIRT1 with mTOR and PRKAB1 in T2DM patients with CAD*

To find out any pairwise correlation for mRNA expression of each possible gene pair in CAD$^-$ and CAD$^+$ patients with T2DM, Pearson correlation test was carried out. The results revealed that there was a correlation between the expression level of *SIRT1* with *mTOR* ($r = 0.480$, $p = 0.004$) and *PRKAB1* ($r = 0.583$, $p = 0.001$) in CAD$^+$ patients. A pairwise correlation was also found for the expression level of *mTOR* and *PRKAB1* ($r = 0.351$, $p = 0.041$) in these patients.

4.5. *The expression profiles of SIRT1 and PRKAB1 genes could be used as potential biomarkers of CAD in patients with T2DM*

Examining whether the expression profiles of *SIRT1* and *PRKAB1* genes can be used as potential biomarkers for CAD in patients with T2DM, receiver operating characteristic (ROC) curve analysis was performed and the area under the ROC curve (AUC) was calculated. This result showed that *PRKAB1* had higher AUC value than *SIRT1* to predict CAD in T2DM patients (Fig. 2). For *PRKAB1* at the cutoff value of

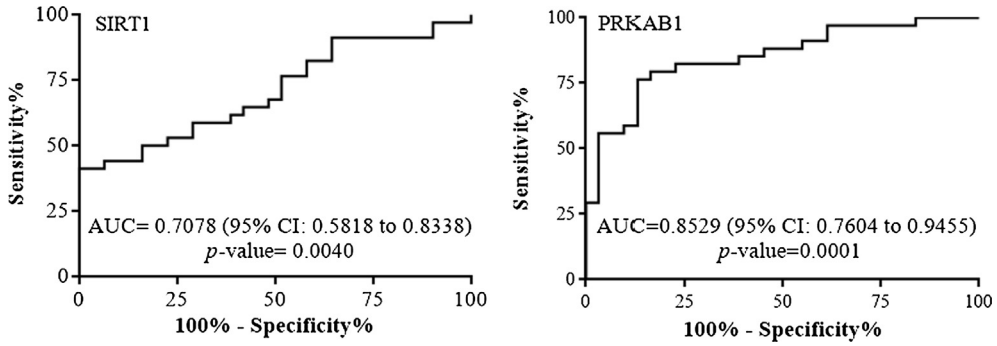

Fig. 2 – Results of ROC curve analysis for *SIRT1* and *PRKAB1* mRNA expression levels as potential biomarkers. *PRKAB1* was found to have the highest AUC value of 0.8529 (*p* value <0.0001).

9.483, the optimal sensitivity and specificity were 76.47% and 87.1%, respectively. For *SIRT1* at the cutoff value of 10.65, the optimal sensitivity and specificity were 61.76% and 61.29%, respectively.

5. Discussion

Molecular events underlying the development of T2DM complications, especially CAD has been the subject of many investigations [30,31]. Different hypotheses have been proposed for CAD development over the past years [32,33]. Among others, inflammatory hypothesis of CAD is of particular interest which knows CAD as a chronic inflammatory disorder [33,34]. Based on this hypothesis different types of inflammatory cells and molecules contribute in CAD development [35]. Although several pathways regulate inflammatory responses, nutrient sensing pathway is of particular interest due to its inevitable role in the pathogenesis of T2DM and its complications [11]. Possible genes involved inside this pathway are *SIRT1*, AMPK and *mTOR*. Although the dysregulation of these genes were reported to be involved in the pathogenesis of metabolic diseases [12,19,22,24], the question as to how CAD alters the expressions of these genes in T2DM patients remains unanswered. To the best of our knowledge, this is the first study to compare the mRNA expression levels of *SIRT1*, two regulatory subunits of AMPK (*PRKAB1* and *PRKAB2*) and *mTOR* in T2DM patients with and without CAD.

Since PBMCs are composed of different types of inflammatory cells, they were used in the expression analysis in this study. In addition, their availability makes PBMCs the suitable source of biomarkers in clinical studies [7,10].

Our results demonstrated that the mRNA expression levels of *SIRT1* gene significantly decreased in T2DM patients with CAD. This result was in line with the study of Li *et al* which found that SIRT1 expression decreased in PBMCs of patients with both T2DM and CAD [36]. Furthermore, our results were supported by other studies indicating that SIRT1 expression decrease in patients with CAD [16,37]. Such a downregulation in patients with T2DM may contribute to CAD development through the increase of inflammatory responses [36]. Indeed, SIRT1 exerts anti-inflammatory effects both in vitro and in vivo and its inactivation could induce the overexpression of inflammation-related genes through the activation of NF-κB signaling [12,38]. Our results also support this idea that SIRT1 activation could be a promising strategy for treating CAD in T2DM patients [39,40]. We also found that the level of SIRT expression was lower in patients with more sever CAD (MVD subgroup). This result is in contrast to a report by Li *et al*, in which SIRT1 expression was not correlated with the severity of coronary lesions [36]. This discrepancy may be due to different grading system for CAD severity used in that study [41].

We also evaluated the expressions of two β subunits of AMPK in this study. Although several genes encode different subunits of AMPK [19], the expressions of *PRKAB1* and *PRKAB2* were analyzed in the present study due to their higher prior probability of association with T2DM [42]. β subunits have both regulatory and structural functions and are necessary for proper activation and membrane localization of AMPK [43]. Our data showed that the expression of PRKAB1 decreased in T2DM patients with CAD but the mRNA expression level of PRKAB2 was unaltered. Downregulation of PRKAB1 may involve in the development of CAD through increase of inflammatory responses. Galic *et al*. revealed that *PRKAB1* deficiency in mice reduces the activity of AMPK in macrophage. They also showed that *PRKAB1* deficiency increases inflammatory responses in macrophages [44]. In addition, since AMPK is an attractive target to control inflammation [18,20,21], our data showed that *PRKAB1* may be a better target than *PRKAB2* to control the activity of AMPK in patients with T2DM.

The mTOR pathway lies at the core of nutrient sensing pathway and regulates numerous processes within a cell, including cell cycle, cell size, cellular growth, energy metabolism, translation initiation, ribosome biogenesis, transcription, autophagy and immune responses [11]. Our data showed that the level of *mTOR* expression was not different in T2DM patients with CAD in comparison with T2DM patients without CAD. This data is in contrast to a previous study showing that expression of *mTOR* increases in peripheral blood lymphocytes (PBLs) of patients with CAD [45]. This might be due to following reasons: 1) Different cell population (PBLs) were used in that study, and 2) The expression of *mTOR* were not compared between T2DM patients in that study.

This study showed that there was no significant correlation for expression levels of the studied genes with lipid profile, FBS and HBA1c. Since several studies have demonstrated that AMPK, mTOR and SIRT1 activity could be influenced by

lipids and glucose concentration [46], our result showed that their mRNA level might not be influenced by these nutrients. Furthermore, since co-expression analysis can be used to find genes with a regulatory role in disease, pairwise correlations were analyzed [47]. Co-expression of *SIRT1*, *mTOR* and *PRKAB1* in T2DM patients with CAD was further suggest their regulatory role in CAD development.

Several biomarkers have been reported for diagnosis of cardiovascular events in patients with T2DM. Since CAD accounts for a large fraction of death in these patients, finding new biomarkers of CAD is a vital clinical question [48]. The present study introduced new potential biomarkers of CAD in patients with T2DM and demonstrated that the expression levels of *PRKAB1* might have a higher diagnostic value than *SIRT1*.

In addition, this study also has some limitations. First, PBMCs are a mix population of T cells, B cells, NK cells and monocytes [7]. Although most of these cell types have shown to play a role in CAD development [49], the expression level of studied genes may be different in each cell type and may show a different effect on each cell physiology. In this way, further studies need to be carried out to find the expression level of studied gene in each cell type. Second, the sample size was relatively small and further studies with more patients need to be performed on the same topic.

6. Conclusions

Taken together, despite some limitations, our data provide important evidence for the association of *SIRT1* and *PRKAB1* mRNA expression with occurrence of CAD in T2DM patients. Furthermore, expression levels of these genes may serve as useful biomarkers for identifying T2DM patients with CAD.

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Conflict of interest

The authors declare that they are no conflict of interest.

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