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The Level of Anti-Oxidative Stress Marker Sestrin in Sera and the Expression of SESN1, 2, 3 Genes in T2DM with Dyslipidemia

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ABSTRACT

Background and Objective: Dyslipidemia is an important agent in Diabetes. Sestrin is considered an anti-oxidative stress that affects the metabolism homeostasis, and works as an anti-aging molecule through the regulation of AMPK-mTORC1 signalling. The aim of this study was to estimate the concentration of sestrin2 protein in patient sera, compared with control, and to measure the expression of SESN gene that encoded to 1, 2, and 3 isoforms.

Methods: This cross-sectional study includes 50 patients with Type 2 Diabetes mellitus (T2DM) and 50 healthy subjects. The clinical diagnoses of the patients included hyperglycemia with and without hyperlipidemia. The detection of them was performed by measuring HbA1C, FBS, and lipid profile. The expression of SESN1, 2, and 3 genes were evaluated by using the relative method.

Findings: The results suggest that the risk of developing Type 2 Diabetes Mellitus (T2DM) rises as individuals grow older, with a majority of patients being above 50 years old, which accounts for 52% of the population. The level of sestrin2 protein concentrations were decreased with T2DM and T2DMDL which recorded 11.18 and 12.23, respectively, when compared with control group (29.54). The expression of SESN genes was suppressed with T2DM, the gene SESN1 was recorded 0.801-fold in patients T2DM, 0.514-fold in those with dyslipidaemia (T2DMDL), and SESN2 gene was recorded 0.76-fold in patients suffering from T2DM and 0.70-fold with T2DMDL, the gene SESN3 was recorded 0.69 in T2DMDL, while in T2DM was upregulated to 1.31. The fold-change decreased with the worsening prognosis of the disease. The SESN gene expressions in the patient group were significantly lower than the control group ($p \leq 0.01$).

Conclusion: The results of the present study demonstrated that the gene expression was lower in the control group, but regarding SESN3, it was higher in patients with T2DM. On the other hand, it was downregulated in T2DMDL.

Keywords: *Diabetes, Sestrin, Dyslipideamia, ELISA, Gene Expression.*

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Introduction

Diabetes mellitus is a disease characterized by hyperglycemia. Type 2 diabetes mellitus involves the circumstances in which the pancreas does not produce enough insulin or the body cannot use it properly. Uncontrolled diabetes causes hyperglycemia (an increase in blood sugar) that causes serious damage to body systems, especially neurons, and blood vessels over time (1). It can be caused by various genetic and environmental conditions. Type 2 diabetes mellitus (T2DM) is a group of polygenic, metabolic, and endocrine diseases that cause various complications like atherogenesis (2). A common form of diabetes, affecting 85-95% of people, is type 2 diabetes mellitus. Type 2 diabetes mellitus has become one of the most pressing issues for the World Health Organization to address, especially concerning people in developing countries. In Arab countries, as in other developing countries, the prevalence of DM2 is increasing (3). The World Health Organization reports that approximately 415 million people are living with diabetes worldwide. Global trends indicate a steady increase in the prevalence rate, around 2.5% per year (4).

Hypertriglyceridemia, decreased serum HDL-cholesterol, and, intermittently, elevated serum LDL-cholesterol are reciprocally associated with relative insulin deficiency, insulin resistance, and obesity (5). T2DM results in the diminished activity of low-density lipoprotein LPL due to a deficiency of insulin and decrease in adiponectin leading to hyper-LDL, hypertriglyceridemia, and HLD. Qualitative LDL defects are also observed in type 2 diabetes, including atherogenic, glycosylated, or oxidized LDL, further increasing the risk of atherogenesis (6). Some other studies measured other parameters like vitamin D3 (7) and Dopamine 2 Receptors (8) that affect T2DM. Sestrins (Sesn1/2/3) are members of a small family of proteins with a variety of biological roles.

Sestrins have oxidoreductase-independent activities such as AMP-activated protein kinase (AMPK) activation, mechanistic target of rapamycin complex 2 (mTORC2) activation, and inhibition of the mechanistic target of rapamycin complex 1 (mTORC1) in addition to its first reported oxidoreductase activity (9). Given the crucial role of these kinases in controlling metabolism and sestrin characteristics as high protein conserved in animals during stress such as radiation, hypoxia, starvation, DNA damage, and oxidative stress, sestrins can be considered as a biomarker and therapeutic target for diabetes and atherogenesis, and the level of sestrin in sera and expression of its genes had been evaluated in this study.

Methods

The present research is a case–control study and was conducted among 100 individuals who referred to Baghdad Teaching Hospital, between the period from December 2022 to March 2023. Their age range was 40 to 60 years, and 50 of them were diagnosed by physicians according to ADA (American diabetes association, 2021) with T2DM, and 50 were control. The study was conducted after being approved by the ethics committee of Middle Technical University, College of Health and Medical technologies with code 3/6613 on 21/11/2022. Both patient and control groups consisted of 25 males and 25 females. The volume collected from each individual was 7 mL of blood sample, 2 mL was dispensed into EDTA tubes for HbA1C and gene expression in patients and controls, then poured into a gel tube, allowing to coagulate. Then, using a centrifuge, it was then centrifuged at 3000 rpm for 10 minutes to obtain serum, followed by Eppendorf tubes, and stored at -20 °C. It was used for glucose, lipid profile, and Sestrin2.

Biochemical Tests: Biochemical analysis was done using Cobas c311 fully automated device for the determination of Cholesterol, Triglyceride, HDL, and fasting blood sugar in serum, while LDL and VLDL values were calculated by equation. HbA1c was done by TOSOH G8 device and the concentrations of Sestrin2 were estimated by using ELISA techniques based on the sandwich method.

RNA isolation and purification: The total RNA extraction was done by mixing 250 µl of blood with 750 µl of GENEzol, then extracted by using the GENEzol™ TriRNA Pure Kit (GENEzol Company). To determine the quality of samples for downstream applications by fluorescence method, a Qantas Fluorometer was utilized to detect the concentration and purity of extracted RNA. Using 1µl of RNA combined with 199 µl of diluted QuantiFluor Dye, the absorbance of the samples was measured at two distinct wavelengths to determine RNA purity (260 and 280 nm). The presence of an A260/A280 ratio of around 2.0 suggested that the RNA sample was pure. The total RNA was converted to cDNA by using TransScript® All-in-One by TransGen Company (Cat. No. AT341-01) which contain gDNA removal, Super mix (Easyscript), Enzyme, Oligo (dT) 18 primer, and RNase – free water. The steps are shown in Table 1.

Table 1. Thermal cycler steps for cDNA reverse transcription conditions

	Step 1	Step 2	Step 3
Temperature	25°C	42°C	85°C
Time	10 min	15 min	5 Sec.

Primer design: Specific primers for Sesrtin 1, 2 and 3 and Actin β (housekeeping gene) genes were designed by researchers for this study (Table 2).

Table 2. The sequences of the designed primers used in the present study

Primer	Sequence (5→3 direction)	Product size bp	Reference
SESN. 1 gene			
Forward	CTCTGAGAGCCATTACCCGC	117	This study
Reverse	CCATTGGTCCTGGGGCTTAG		
SESN. 2 gene			
Forward	ATCCAGGCCTTGCTGAAGAC	108	This study
Reverse	GCCAAACACGAAGGAGGAGA		
SESN. 3 gene			
Forward	GCCTTTGTTCTCACTCGGGA	155	This study
Reverse	AGACTTGACTGGGGAAAGCG		
Actine β (housekeeping gene)			
Forward	AAACTGGAACGGTGAAGGTGAC	70	(10)
Reverse	CTCGGCCACATTGTGAACTTTG		

Quantitative Real-time PCR (qRT–PCR): The sestrin 1, 2 and 3 gene expression was determined using the QIAGEN Rotor gene Real-time PCR System (Germany). The expression levels and fold changes of sestrin 1, 2, 3 genes and Actine β genes were determined using TransStart® Green qPCR Super Mix kit from TransGen company. The kit used consisted of 10µl of 2xTransStart® Green qPCR Super Mix, 4 µl from Nuclease-free water, Forward and Reverse Primers (10 µM) 1 µl and 4 µl from cDNA. Then, the threshold cycle (Ct) values were measured and every reaction was performed twice. The cycling protocol is presented in Table 3 and the results were calculated according to the $2^{-\Delta\Delta Ct}$ method (11).

Table 3. The thermal profile of Actine β and sestrin 1, 2, 3 genes expression

Step	Temperature (ºC)	Time (sec.)	Cycles
Enzyme activation	94	30	1
Denaturation	94	5	40
Annealing	56	15	40
Extension	72	10	40
Dissociation	55-95		1

Relative Quantification of the Expression: The Cycle Threshold (Ct) for both the target and housekeeping genes resulted from Rotor-Gene. In case of any result over 35, non-specific outcome was concluded and not taken into consideration. The Cts value were normalized to the Ct value of the housekeeping gene as an internal control relation (ΔCt= Ct gene of interest - Ct reference gene) for each sample. They were then normalized to the calibrator sample ΔCt calibrator= the three highest Cts (target gene in the control group) - mean Ct (reference gene in the control). After that, the 2^ (-ΔΔCt) value was calculated according to Schmittgen (11). Finally, the results were expressed as the relative expression of SESN 1, 2, and 3 genes to ß-Actin gene.

SPSS version 26 was used to revise, analyse, and code data. The difference between the two means was evaluated using an independent sample. ANOVA was used to determine statistical significance ($p \leq 0.05$), highly significant ($p \leq 0.01$), and non-significant ($p > 0.05$).

Results

This study included 100 individuals, 50 patients and 50 controls, with an age range of 40 to 60. Forty-eight percent of the patient group were under 50 and fifty-two percent of them were above 50 years of age, while sixty percent in the control group were under 50 and forty percent were over 50 years of age. The incidence of diabetes in males and females was the same according to the results obtained from this study and increased with age based on the sample groups. All of the patients had a family history of diabetes, while it was only six percent in healthy subjects as shown in Table 4.

Table 4. Age, sex, family history, smoking, and BMI distribution in diabetes patients and control

Criteria	Control Number(%)	T2DM Number(%)	p-value
Age			
<50	30(60)	24(48)	0.420
>50	20(40)	26(52)	
Sex			
Female	25(50)	25(50)	1.00
Male	25(50)	25(50)	
Family history			
No	47(94)	0(0)	0.00001
Yes	3(6)	50(100)	
Smoking			
No	6(12)	39(78)	0.00001
Yes	34(68)	11(22)	
BMI			
18.5-25	3(6)	7(14)	0.182
>30	47(94)	43(86)	

*BMI is body mass index. P-value calculated by using Chi-Square Tests, control compared with patient's groups.

The patients with Type 2 diabetes mellitus (T2DM) were divided into two groups of Diabetes mellitus Dyslipidaemia (DMDL) and Diabetes mellitus Dyslipidaemia without lipidaemia (DM) depending on their lipid profile. All subjects were subjected to test their lipid profile, which included Cholesterol, Triglyceride, HDL, LDL, and VLDL. Table 2 illustrates that the T2DMDL group recorded the highest values among other groups followed by the T2DM group then control group as represented in Table 5. There were significant differences ($p<0.01$) in lipid profile values between the control and patient groups. Table 6 illustrates the concentrations of haemoglobin A1C (HbA1C) test and fasting blood sugar in the blood (FBS) for both patients and controls, which recorded the highest value of Hb1AC in patients with T2DM DM (10.5), followed by T2DM patients (9.8), then the control group (5.1), and the FBS was the highest value in T2DM group followed by T2DMDL then control group. This result is in agreement with the study of Chachanet al (12) as shown in Table 6.

Table 5. Lipid Profile for Diabetes and control groups

Group	Serum lipid profile (mg/dl)				
	Cholesterol Mean±SD	**Triglyceride Mean±SD**	**HDL Mean±SD**	**VLDL Mean±SD**	**LDL Mean±SD**
Control	175.7±29.04[a]	126.1±43.54[a]	38.3±4.01[a]	25.5±8.69[a]	101.7±32.6[a]
Patient T2DMDL	222.3±51.10[b]	317±150.3[b]	29.66±5.4[b]	61.87±30.5[b]	134.4±43.8[b]
Patient T2DM	174.4±31.7[a]	119.1±30.61[a]	38.2±6.5[a]	23.6±5.3[a]	104.7±32.7[a]
p-value	0.001	0.0001	0.0001	0.001	0.001

¥: One way ANOVA was used to test between groups Means that do not share a letter are significantly different. *(p≤0.01) is significant different. *(p≤0.01) is significant.

Table 6. The concentrations of HbA1c and FBS in patients and control groups

Group	HbA1c Mean±SD	FBS Mean±SD
Control	5.1±0.5608	99.9±11.5
Patient T2DM	9.8±1.5300	306±85.633
Patient T2DM DM	10.5±1.4374	283±72.348
p-value	0.0001	0.0001

¥: One way ANOVA was used to test between groups ©Means that do not share a letter are significantly different. *(p≤0.01) is significant.

The level of sestrin2 in sera: In the sera, the concentrations of sestrin2 were recorded as follows: the highest value recorded in the control group was 29.53±10.35, followed by DMDL (12.23±3.93) and DM (11.18±1.82). There is a highly significant difference ($p<0.0001$) between the control and patient groups and non-significant difference between DM and DMDL as shown in Figure 1.

The expression of SESN 1, 2, and 3 Genes in Patients with Diabetes: In this study, the researchers aimed to establish a reliable baseline of T2DM by closely examining the SESN1, 2, and 3 genes. To achieve this, the level of these genes and β-Actin mRNA were measured by using qPCR and an in-depth melt analysis to assess the primers. Furthermore, we conducted an extensive quantitation data analysis foe cycling A green, as illustrated in Figure 2. These findings showed that diabetes considerably suppresses the expression of SESN 1, 2, and 3 genes, with SESN1 recording 0.801-fold in DM samples and 0.514-fold in DMDL samples. Additionally, our results revealed that the folding decreases as the disease progresses, making our

study a crucial contribution to the understanding of T2DM progression. Moreover, our study also highlighted that the SESN2 gene resulted in the highest expression in the control group (1-fold). Followed by 0.76-fold in DM and 0.70-fold in DMDL samples. Interestingly, the SESN3 gene was upregulated in DM samples (1.31) but down-regulated in DMDL samples (0.69) (Figure 1). Overall, our study provides a comprehensive and detailed analysis of T2DM by examining the expression of SESN 1, 2, and 3 genes as shown in Tables 7-9.

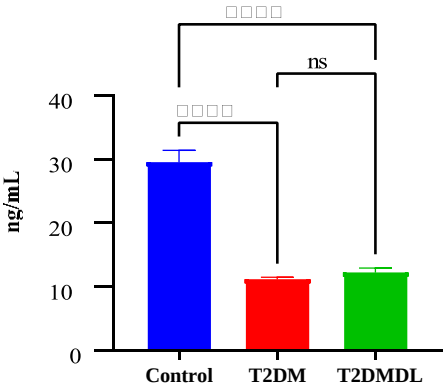

Figure 1. The level of sestrin2 in sera of control and two patient groups DM and DMDL. There is highly significant difference between control and both T2DM and T2DMDL (p=0.0001), and non-significant difference (p=0.8049) between T2DM and T2DMDL.

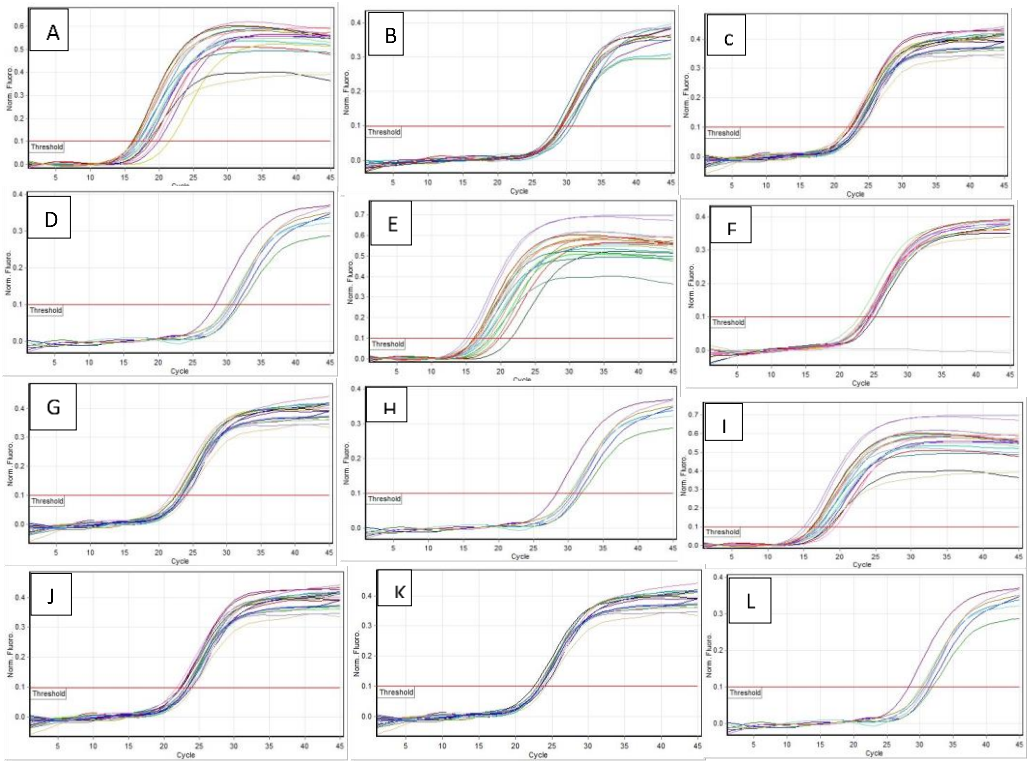

Figure 2. Real time PCR quantitation data analysis for cycling A. green. (A) β-actin in the control group, (B) SESN1 in the control group, (C) SESN2 in the control group, (D) SESN3 in control group, (E), β-actin in the DM group, (F) SESN1 in the DM group, (G) SESN2 in the DM group, (H) SESN3 in the DM group (1) β-actin in DMDL group, (J) SESN1 in DMDL group, (K) SESN2 in DMDL group, (L) SESN3 in DMDL group.

Table 7. Fold change expression of SESN1 gene normalized to β -Actin gene in control, DM, and DMDL calculated by 2^-ΔΔCT method

Groups	Ct Actin β Mean±SD	Ct SESN1 gene Mean±SD	ΔCT	ΔCT Calibrator	ΔΔCT	2^-ΔΔCT	Experimental group/Control group	Fold Change
Control	17.7±1.18	23.94±1.63	6.24	9.97	-3.73	13.27	13.26/13.26	1
DM	17.5±0.29	24.06±1.17	6.56	9.97	-3.41	10.63	10.62/13.26	0.801
DMDL	17.4±0.81	24.6±2.4	7.2	9.97	-2.77	6.821	6.82/13.26	0.514

Table 8. Fold change expression of SESN2 gene normalized to β- Actin gene in control, DM, and DMDL calculated by 2^ΔΔCT method

Groups	Ct Actin B Mean±SD	Ct SESN2 gene Mean±SD	ΔCT	ΔCT Calibrator	ΔΔCT	2^-ΔΔCT	Experimental group/Control group	Fold Change
Control	17.7±1.18	23.4±1.51	5.7	16.86	-11.16	2288.20	2288.20/2288.20	1
DM	17.5±0.29	23.6±1.03	6.1	16.86	-10.76	1734.13	1734.13/2288.20	0.76
DMDL	17.4±0.81	23.61±2.50	6.21	16.86	-10.65	1606.83	1606.83/2288.20	0.70

Table 9. Fold change expression of SESN3 gene normalized to β-Actin gene in control, DM, and DMDL calculated by 2^- ΔΔCT method

Groups	Ct Actin B Mean±SD	Ct SESN3 gene Mean±SD	ΔCT	ΔCT Calibrator	ΔΔCT	2^-ΔΔCT	Experimental group/Control group	Fold Change
Control	17.7±1.18	30.39±2.54	12.69	17.24	-4.55	23.42	23.42/23.42	1
DM	17.5±0.29	29.81±1.21	12.3	17.24	-4.94	30.69	23.42/30.69	1.31
DMDL	17.4±0.81	30.61±2.16	13.21	17.24	-4.03	16.33	23.42/16.33	0.69

Discussion

All patients were subjected to measure the SESN2 protein in serum and the results indicated reduced concentration when compared with control group. This protein has a cytoprotective effect on physiological and pathological conditions mainly through modulating oxidative stress, endoplasmic reticulum stress, autophagy, metabolism and inflammation; reduction of sestrin levels might impair oxidative defence in diabetic tissues (13). These results agree with Sundararajan et al. who established that the sestrin level decreased with T2DM and dyslipidaemia (14). This protein has a direct influence on lipid and glucose metabolism and liver insulin resistance and modulating mTORC1. Zhang et al. in 2023 found that SESN proteins play a critical role in mTORC signalling pathway. mTORC is strongly associated with regulation of insulin signalling in T2DM and many of its chronic complications. Diabetes disrupts normal metabolic pathways, including those regulated by sestrins. This protein influence metabolism by modulating AMP-activated protein kinase (AMPK) and mammalian target of rapamycin complex 1 (mTORC1) signalling pathways, which play crucial roles in cellular energy homeostasis. This study provides a comprehensive and detailed analysis of T2DM by examining the expression of SESN1, 2, and 3 genes. These results are consistent with the results of Tian et al. indicating that SESN2 expression is downregulated in the skeletal muscle of diabetes humans and SESN2 absence increases insulin resistance and deteriorates glucose

tolerance in mice, implying that SESN2 downregulation contributes to the causes of type 2 diabetes (15). Another study revealed that SESN1 expression is decreased in the adipose tissue of obese and diabetic mice and showed the deficiency exacerbates obesity-induced insulin resistance, indicating that SESN1 downregulation contributes to insulin resistance and metabolic dysfunction in obesity and diabetes (16). This is in agreement with the findings by Nascimento et al. who discovered that the SESN3 gene upregulated in T2D and could influence skeletal muscle differentiation without altering glucose and lipid metabolism (17). However, it disagrees with the results of Chung et al., who demonstrated that Sestrin2 level increased in subjects with metabolic syndrome, particularly in subjects with type 2 diabetes (18).

The prevalence of T2DM increase with age, which recorded 52% of patients over 50 years of age, impaired insulin secretion observed with increasing age and increased insulin requirements due to changes in body composition and muscle mass decline (19, 20). All of the patients have family history of diabetes which recorded 100% positive, that may be due to genetic and environment condition. These results agree with the study of De Pergola et al (21), which showed family history as an important risk factor and there is a significant relation between family history and Diabetes. Patients who have a higher BMI are more likely to develop diabetes, and similar finding was reported by Patel et al. (22). Overall, the reduction in sestrin levels in diabetes is likely due to a combination of insulin resistance, oxidative stress, inflammation, metabolic dysfunction, and genetic/environmental factors. Understanding these pathways may lead to new treatment approaches for controlling diabetes and associated consequences.

It is concluded from this study that the incidence of diabetes in males and females was the same, and increased with age. The level of sestrin2 concentrations in patient sera decreased compared with healthy subjects, while the other factors such as lipid profile, family history, and BMI increased. The expression of SESN gene in isoform 1, 2, and 3 downregulated with diabetes type 2 with and without dyslipidaemia.

Conflict of interest: The authors report no conflict of interest.

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