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The relationships between 7-kchol, 7β-ohchol, chol-triol, Lp (A) and PON1 with coronary heart disease in patients with diabetes mellitus T1DM and T2DM

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Abstract: Oxysterols (OXY) are oxidized derivatives of cholesterol associated with oxidation and can increase the risk of cardiovascular diseases. The aim of the current study is to examine the relationships between OXY profile, lipids, lipoprotein(a) [Lp(a)] and paraoxonase1 (PON1) with coronary heart disease (CHD) in patients with diabetes mellitus type1 (T1DM) and type2 (T2DM). 120 diabetic patients (T1DM=40, T2DM=80) and 60 healthy subjects were recruited in the study. OXY profile (7-KChol, 7β-OHChol and Chol-triol) was measured using liquid chromatography-mass spectrometry. The clinical profile of the study participants was also collected. 7-KChol, 7β-OHChol and Chol-triol and Lp(a), FBG and glycation parameters were higher in diabetic patients compared to controls ($p<0.01$), whereas PON1 was lower in patients compared to controls ($p<0.01$). Within the T2DM group, 7-KChol and 7β-OHChol levels were associated with CHD, obesity, and smoking ($p<0.05$). In addition, KChol, 7β-OHChol and Chol-triol levels were associated with smoking in T1DM ($p<0.05$). In both diabetic types, 7-KChol, 7β-OHChol and Chol-triol were significantly correlated with TC, LDL, ApoB and Lp(a), glycation parameters and inversely with PON1 ($p<0.05$). OXY profile in diabetic patients can be used as a reliable biomarker of CHD, particularly in T2DM.

Keywords: Oxysterols, 7-ketocholesterol, 7β-hydroxycholesterol, triol, Type 1 diabetes mellitus, type 2 diabetes mellitus.

INTRODUCTION

Oxysterol (OXY) is a form of oxidized cholesterol (Griffiths and Wang, 2019), which is formed after the oxidation of cholesterol by free radicals (Chatuphonprasert *et al.*, 2018; Cilla *et al.*, 2017; Russo *et al.*, 2020). OXY formation is implicated in vascular and neurological complications of diabetes (Czuba *et al.*, 2017) via increasing the synthesis of many pro-inflammatory cytokines, adhesion molecules and growth factors (Koh *et al.*, 2021). OXY includes 7-ketocholesterol (7-KChol), 7β-hydroxycholesterol (7β-OHChol), and cholesterol triol (Chol-triol) (Lefort and Cani, 2021; Nury *et al.*, 2020; Wang *et al.*, 2021). Both OXY and oxidative stress were also implicated in several chronic pathological states such as atherosclerosis and cardiovascular diseases (Poli *et al.*, 2013; Vejux *et al.*, 2020). For example, OXY was elevated in human atherosclerotic lesions and plays an important role in the development of atherosclerotic plaques (Brzeska *et al.*, 2016).

Diabetes mellitus (DM) is a group of chronic disorders characterized by persistent hyperglycemia (Gómez-Zorita *et al.*, 2021; Scott and O'Connor, 2021; Steinke *et al.*, 2020). The prevalence of DM is increasing with years in developing countries due to inactivity and unhealthy lifestyles (Cole and Florez, 2020). DM can be divided into type1 (T1DM) and type2 (T2DM) depending on insulin dependency. Oxidative stress is usually elevated in both types of DM and is considered the main etiological factor for the disease complications (Luc *et al.*, 2019). In the current study, the relationships between OXY profile and CHD were investigated among patients with diabetes. In addition, the relationships between OXY profile and lipid and glycation parameters were also examined.

MATERIALS AND METHODS

Subjects

The study is cross-sectional in design and involved T1DM (n=40) and T2DM (n=80) patients. Patients were recruited from different Hospitals, Madinah, Saudi Arabia. For comparison, 60 healthy subjects were recruited as a control group. The inclusion criteria for the patients were: HbA1C above 6.5%, fasting blood glucose ≥ 7.7 mmol/l (to exclude any borderline one). Exclusion criteria were the presence of acute conditions, cancer, anemia, hepatobiliary diseases, acute or chronic renal failure and thyroid abnormalities. In addition, subjects with vitamin or mineral supplement use were also excluded (Ferderbar *et al.*, 2007b).

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Ethical approval

The study was approved by the Research Ethics Committee of Applied Medical Sciences at Taibah University, Madinah, Saudi Arabia (No. CLS 2019198) that follows the ethical standards of the Declaration of Helsinki 1964 and its amendments. Written informed consent was obtained from participants after a full description of study objectives and protocols.

Anthropometric parameters

Anthropometric parameters including body mass index (BMI) and waist circumference were measured as previously described (Khabour *et al.*, 2018).

Biochemical parameters

Fasting blood samples were collected from each participant for the analysis of biochemical parameters after overnight fasting (12 hours) in plain sterile vacuum test tubes and the serum was separated with the standard centrifugation method. Total cholesterol (TC), triglyceride (TG), HDL, LDL, apolipoprotein A1 (Apo A1), apolipoprotein B (Apo B), Lp(a) and fructosamine were measured using a full auto-analyzer (Cobas C501, Roche Diagnostics). HbA1C was measured using D-10™ Hemoglobin Analyzer Bio-Rad (Nyocard). PON1 level was determined using Elabscience's ELISA kit (Sandwich-ELISA) and fresh serum samples. The concentration of PON1 was determined by plotting the optical density from designed standard curve (Ahmed, 2019). GA was measured with Cobas 6000, (Roche Diagnostics) using commercial kits from Lucica Glycated Albumin-L enzymatic assay (Asahi Kasei Pharma, Tokyo, Japan). OXY profile was measured using liquid chromatography-mass spectrometry (Shimadzu 8040, Shimadzu® Japan) as previously described (Jiang *et al.*, 2011) and with a modification (no need for saponification of serum) protocol (Samadi *et al.*, 2019) using C18 column (100mm × 2.1mm, 5µm) (Thermo Fisher Scientific, USA).

Table 1: Basic characteristics of study groups

Variables	T1DM (n=40)	T2DM (n=80)	Controls (n=60)	P value
Age	36.7±14.1	41.1±9.7	38.3±8.8	0.07
Duration (years)	19.5±8.2	4.7±1.1	-	<0.001**[a]
Gender (male, female)	26, 14	46, 34	33, 27	0.59
BMI	22.9±4.2	24.2±5.8	22.4±2.9	0.23
Normal	32 (80%)	45 (56.3%)	53 (88.3%)	
Over	8 (20%)	26 (32.5%)	7 (11.7%)	-
Obese	-	9 (11.2%)	-	
WC (cm)	80.2±18.4	84.6±28.4	80.7±8.6	0.44
≤ 90	29 (72.5%)	49 (61.3%)	51 (85%)	
≥ 90	11 (27.5%)	31 (38.7%)	9 (15%)	
Blood pressure				0.12
Systolic (mm/Hg)	122.5±22.7	123.4±18.8	121.3±16.1	0.71
Diastolic (mm/Hg)	82.5±12.5	82.2±8.4	81.8±5.1	0.87
Hypertensive	24 (60%)	51 (63.7%)	-	0.68
Non-hypertensive	16 (40%)	29 (36.3%)	-	
Smoking				-
Smoker's	14 (35%)	32 (40%)	12 (20%)	0.03*[b]
Non-smoker's	26 (65%)	48 (60%)	48(80%)	
Hypercholesterolemia				-
≥ 220 mg/dL	12 (30%)	26 (32.5%)	2 (3.3%)	<0.001**[b]
≤ 200 mg/dL	28 (70%)	54 (67.5%)	58 (96.7%)	
Diagnose with history of CHD				-
Yes	6 (15%)	11 (13.7%)	-	0.22
No	34 (85%)	69 (86.3%)	-	
Different medications			-	-
Insulin	40 (100%)	5 (6.3%)	-	-
Oral hypoglycemic drugs	2 (5%)	78 (97.5%)	-	-
Antihypertensive	18 (45%)	33 (41.3%)	-	-
Aspirin	34 (85%)	67 (83.7%)	-	-
Lipids lowering medications	20 (50%)	44 (55%)	-	-

n: number. BMI: Body mass index. WC: Waist circumference. CHD: Coronary heart disease. *: Significant ($p<0.05$). **: Significant ($p<0.01$). [a:] unpaired t-test. [b:] chi-square test (fisher exact test for samples less than 5)

Table 2: Biochemical parameters for participants

Variables	T1DM n=40	T2DM n=80	Controls n=60	P value
FPG (mmol/l)	8.1±1.6	8.3±1.8	5.3±0.3	<0.001**
HbA1c (%)	8.3±1.2	8.1±1.8	5.1±0.4	<0.001**
Glycated Albumin (%)	7.1±1.8	6.8±1.5	1.1±0.1	<0.001**
Fructosamine (mmol/l)	393.5±46.3	386.7±53.5	205.3±32.7	<0.001**
Lipids				
TC (mmol/l)	4.3±1.8	4.5±1.9[a]	4.0±1.1	0.21
TG (mmol/l)	1.53±0.3	1.62±0.9	1.42±0.4	0.21
LDL (mmol/l)	3.4±0.9	3.55±1.3	3.2±0.7	0.15
HDL (mmol/l)	1.27±0.8	1.24±0.7	1.4±0.8	0.45
Apolipoprotein A1 (g/l)	1.46±0.4	1.41±0.5	1.59±0.7	0.16
Apolipoprotein B (g/l)	10.1±4.4	10.7±5.1	9.3±3.2	0.18

FBG: Fasting blood glucose. TC: Total cholesterol. TG: Triglyceride. LDL: Low density lipoprotein. HDL: High density lipoprotein. [a]: significantly higher in T2DM compared to controls ($p<0.05$) by unpaired t-test. **: significant at ($p<0.001$) when compared with control.

Table 3: biochemical data in CHD patients

	T1DM With CHD (n=6)	T2DM With CHD (n=11)	P value
FPG (mmol/l)	8.6±1.6	8.7±1.8	0.91
HbA1c (%)	8.7±1.1	8.6±1.4	0.88
Glycated Albumin (%)	8.3±1.5	8.0±1.2	0.65
Fructosamines (mmol/l)	399.5±62.3	394.7±72.5	0.89
Lipids			
TC (mmol/l)	6.4±1.8	6.6±1.9	0.08
TG (mmol/l)	1.82±0.3	1.9±0.6	0.12
LDL (mmol/l)	4.7±1.1	5.1±1.3=1	0.26
HDL (mmol/l)	1.1±0.7	1.06±0.8	0.45
Apolipoprotein A1 (g/l)	1.37±0.6	1.32±0.6	0.08
Apolipoprotein B (g/l)	12.1±5.2	13.4±3.9	0.56
Lp(a) (mg/dL)	46.7±12.9	54.9±16.1	0.30
Paraoxonase (ng/ml)	61.8±9.7	56.1±12.8	0.35
7-KChol (ng/ml)	49.9±22.5	84.2±26.1	0.016*
7β-OHChol	43.8±19.2	70.8±24.7	0.03*
Chol-triol (ng/ml)	37.8±18.2	65.2±20.8	0.016*

*: significant ($p<0.05$), unpaired t-test.

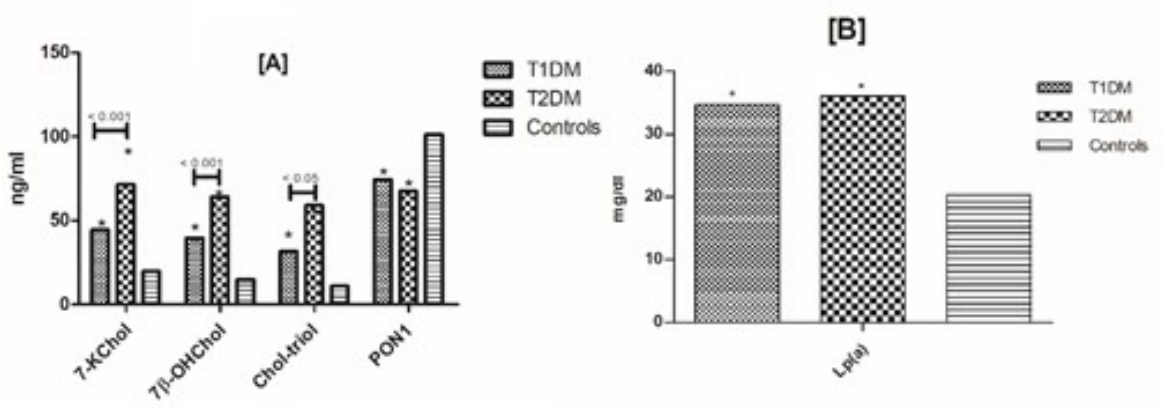

Fig. 1: shows comparison of the following biochemicals across participants: [A] oxysterol profiles (7-Kchol, 7β-OHChol, Chol-triol and PON1); [B] Lp(a) levels. *: significant compared to controls ($p<0.01$).

Table 4: Oxysterol profiles (7-KChol, 7β-OHChol & Chol-triol) levels according to different clinical characteristics in patients with T1DM and T1DM.

T	T1DM			T2DM		
	7-KChol	7β-OHChol	Chol-triol	7-KChol	7β-OHChol	Chol-triol
Total	44.6±19.5	39.5±17.2	31.7±11.2	71.5±22.4	64.2±19.2	59.3±13.1
Male	45.4±15.1	40.2±12.1	32.5±4.1	72.4±28.8	65.5±17.1	60.2±14.1
Female	43.8±11.5	38.7±9.8	30.8±2.8	70.6±20.4	62.9±12.5	58.4±15.3
BMI						
Normal	38.7±19.8	34.2±11.3	33.3±19.3	71.1±12.7	64.1±17.1	53.8±12.1
Over	40.8±19.8	35.1±15.7	34.8±19.3	74.3±18.2	67±12.5	54.7±13.5
Obese	-	-	-	86.8±22.2[a**]	76.1±17.1[a*]	60.8±17.8
WC (cm)						
≤ 90	41.7±12.8	36.1±15.1	34.2±10.2	74.3±22.3	66±22.5	54.9±20.1
≥ 90	43.1±10.3	38.7±20.2	36.4±8.7	78.6±26.6	70±25.1	55.4±16.5
Blood pressure						
Hypertensive	43.9±17.4	38.1±14	35.8±7.7	76.2±23.2	68±19.2	55.2±20.5
Non-hypertensive	40.2±8.4	36.7±12.8	34.8±6.8	72.7±30.2	67±13.5	54±16.2
Smoking						
Smoker's	46.8±10.4[b**]	38.9±6.9 [b*]	36.7±6.2	89.8±13.9 [b*]	69.9±12.7 [b**]	56.1±13.2
Non-smoker's	38.9±7.3	32.1±9.5	34.2±10.2	81.2±19.2	60.8±14.2	54.5±14.9
Hypercholesterolemia						
≥ 220 mg/dL	43.8±11.1	39.5±22.6	36.2±9.8	86.4±23.7[c*]	72.2±20.9[c*]	59.8±11.9
≤ 200 mg/dL	40.6±2=9	35.3±14.2	33.9±7.7	73.1±20.2	62.4±18.2	53.2±16.1
Diagnose with history of CHD						
Yes	46.6±18.1	42.5±6.1	38.8±11.8	91.2±31.4[d*]	82.3±22.7[d*]	61.2±20.4
No	36.1±12.1	34.9±10.4	31.1±9.7	72.4±23.6	69.2±19.2	53.7±17.7
Different medications usage						
Insulin	36.7±16.1	32.6±14.7	28.1±4.9	68.3±27.3	62.2±17.2	56.8±20.2
Oral hypoglycemic drugs	-	-	-	70.2±22.4	61.4±15.5	55.2±18.3
Antihypertensive	34.6±13.2	32.1±11.2	29.1±8.9	69.3±25.1	59.6±17.9	54.2±13.9
Aspirin	30.7±6.1[e*]	29.8±15.9[e*]	26.2±6.1[e*]	61.6±12.2[e**]	53.3±19.1[e**]	49.2±15.5[e**]
Lipids lowering drugs	32.1±6.9[f*]	30.8±11.2[f*]	26.4±5.1[f*]	62.1±13.5[f*]	52.4±13.1[f**]	48.1±14.4[f**]

*: significant ($p<0.05$). **: significant ($p<0.01$). [a:] significantly higher in T2DM obese than normal subjects. [b:] significantly higher in smokers than non-smokers. [c:] significantly higher in T2DM hypercholesterolemia than normocholesterolemic subjects. [d:] significantly higher in patients with diabetes suffer from CHD than in non CHD. [e:] significantly lower in patients with diabetes who use Aspirin than non. [f:] significantly lower in patients with diabetes who uses Lipids lowering drugs than non-users.

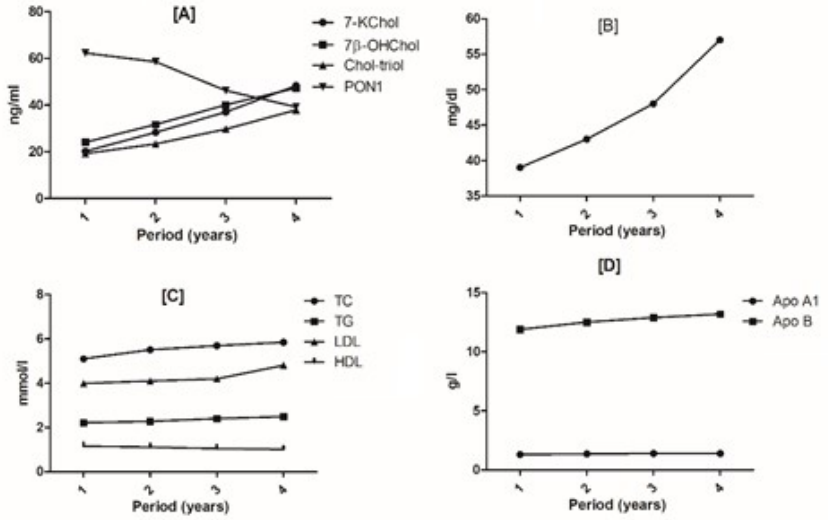

Fig. 2: Relation between oxygenated products of cholesterol (oxysterols) and PON1 with period of previous four years of diabetes in patients whom suffer from CHD (17 patients): [A] Oxysterol profiles (ng/ml), significant direct correlations for oxysterol profiles ($p<0.05$); and significant reverse correlation for PON1 ($p<0.05$); [B] Lp(a) (mg/dl), significant correlation ($p<0.05$); [C] Lipids (mmol/l), not significant; [D] Apo A1&B (g/l), not significant.

Table 5: Correlation coefficient (r^2) between 7-KChol, 7β-OHChol & Chol-triol with biochemical parameters in patients with both diabetes T1DM & T2DM

Variables	T1DM			T2DM		
	7-KChol	7β-OHChol	Chol-triol	7-KChol	7β-OHChol	Chol-triol
FPG (mmol/L)	0.29*	0.18	0.1	0.43*	0.39*	0.34*
HbA1c (%)	0.41*	0.41*	0.15	0.44*	0.41*	0.32*
Glycated Albumin (%)	0.43*	0.44*	0.14	0.48*	0.44*	0.4*
Fructosamines (mmol/L)	0.38*	0.43*	0.12	0.42*	0.39*	0.12
TC (mmol/l)	0.82*	0.47*	0.39*	0.9**	0.72**	0.42*
TG (mmol/l)	0.08	0.03	0.02	0.1	0.08	0.04
LDL (mmol/l)	0.57*	0.41*	0.32*	0.66**	0.38*	0.3*
HDL (mmol/l)	-0.18	-0.16	-0.1	-0.32*	-0.39*	-0.11
Apolipoprotein A1 (g/L)	-0.16	-0.11	-0.12	-0.42*	-0.42*	-0.11
Apolipoprotein B (g/L)	0.47*	-0.38*	0.26	0.51*	0.41*	0.24
Lp(a)	0.71**	0.6**	0.6**	0.8**	0.77**	0.66**
PON1	-0.6**	-0.58**	-0.39**	-0.66**	-0.52**	-0.4**

* significant ($p<0.05$). ** significant ($p<0.01$).

STATISTICAL ANALYSIS

The data were analyzed using GraphPad Prism (8.0.1.244) (GraphPad Software, San Diego, CA, USA). Values were expressed as percentages or mean ± SD. Continuous variables were compared using unpaired Student t-test. Categorical variables were compared using chi-squared test. Correlations were computed using Pearson correlation coefficient. Statistical significance was set at $P<0.05$.

RESULTS

Basic demographic and clinical data of participants are presented in table 1. No differences concerning age, gender, BMI and WC between participants of the different groups were detected. The duration of diabetes was higher in T1DM than T2DM ($p<0.001$). The prevalence of smoking was different between the DM groups and the control group ($p<0.05$).

The biochemical parameters of patients and controls were presented in table 2. T1DM and T2DM had higher FPG, HbA1C, GA and fructosamines than controls ($p<0.001$). However, there were no differences with respect to lipids and apolipoproteins between the different groups except TC, which was higher in T2DM than the control group ($p<0.05$). Lp(a), 7-Kchol, 7β-OHChol and Chol-triol were higher in T1DM and T2DM than in the control group ($p<0.001$, fig. 1). On the other hand, PON1 was lower in DM groups than in the control group ($p<0.001$). Moreover, 7-Kchol, 7β-OHChol and Chol-triol were higher in T2DM than T1DM ($p<0.05$, fig. 1).

Biochemical information of patients with T1DM and T2DM with CHD were presented in table 3. No statistically significant differences in FPG, glycation parameters, lipids, apolipoproteins and Lp(a) were detected between the two groups.

Comparison of the mean levels of oxysterol profiles, 7-Kchol, 7β-OHChol and Chol-triol in T1DM and T2DM with different demographic and clinical characteristics were shown in table 4. 7-Kchol and 7β-OHChol were higher in T2DM subjects with CHD, obesity and hypercholesterolemia than the ones without these conditions ($p<0.05$). In addition, 7-Kchol and 7β-OHChol were higher in smokers T1DM & T2DM subjects than non-smokers ($p<0.05$). Moreover, 7-Kchol, 7β-OHChol, and Chol-triol were lower in both patients with diabetes types that use Aspirin and lipid-lowering drugs than none users ($p<0.05$).

Correlations between oxysterol profiles with biochemical parameters in patients with both T1DM and T2DM, were shown in table 5. 7-Kchol, was positively correlated with FBG, glycation parameters, TC, LDL, Apo B and Lp(a) ($p<0.05$), and inversely correlated with PON1 ($p<0.05$). In addition, 7β-OHChol was positively correlated with glycation parameters, TC, LDL, ApoB and Lp(a) and inversely correlated with PON1 ($p<0.05$). Moreover, Chol-triol was positively correlated with TC, LDL, Lp(a) and inversely correlated with PON1 ($p<0.05$).

Fig. 2 shows the relationships between mean levels of oxysterol profiles, PON1, Lp(a), lipids and ApoA1 & ApoB with the previous four years of diabetes; in which, the oxysterol profiles, PON1 and Lp(a) showed significant correlations with the period of diabetes ($p<0.05$).

DISCUSSION

In the present study, the results showed a strong association between OXY profile and Lp(a) with CHD in patients with DM. In addition, a strong association with the period of DM and levels of OXY were also reported.

This indicates a high level of oxidative stress in patients with DM (Samadi *et al.*, 2019).

Oxidative stress plays a crucial role in the development of both cardiovascular and microvascular complications in patients with DM as a result of bad control of glucose and lipid metabolism (Tan *et al.*, 2020). This leads to the overproduction of endothelial cells' mitochondrial superoxide and the oxidation of cholesterol and the formation of OXY compounds (Ferderbar *et al.*, 2007a).

In agreement with previous studies, OXY profile was found to be elevated in both T1DM and T2DM (Ferderbar *et al.*, 2007a; Samadi *et al.*, 2019; Samadi *et al.*, 2020). In addition, OXY was more profound in T2DM compared to T1DM (Samadi *et al.*, 2019). The observed correlations between OXY in DM with glycation parameters support the association between OXY and the intensity of glycoxidation process, which results from impaired uptake of glucose leading to activation of multiple cascades of tyrosine phosphatases and serine kinases and the subsequent development of insulin resistance (Kakiyama *et al.*, 2020). Moreover, the imbalance between antioxidant and oxidative stress biomarkers (particularly increases of reactive oxygen species concomitant with the decreased antioxidant capability) could be responsible for the impairment of insulin-mediated PI_3 kinase activation of insulin-mediated glucose uptake (Evans *et al.*, 2002). Furthermore, hyperglycemia impairs many cellular events such as NADPH oxidase which inturn increases superoxide anion that reacts with nitric oxide and generates peroxynitrite that induces lipid peroxidation and oxidative DNA damage that contribute to endothelial cell and vascular dysfunctions (Ighodaro, 2018).

7-KChol and 7β-OHChol of OXY profile were higher in T2DM patients with CHD, obesity and hyper-cholesteremia than patients without such conditions. In addition, out of all OXY profiles, 7-KChol consider was the major one and comprised about 57% of the total OXY, followed by 7β-OHChol (21%) and Chol-triol (10%) (Helmschrodt *et al.*, 2013). These findings agree with previous reports (Brown *et al.*, 1996; Endo *et al.*, 2008; Hultén *et al.*, 1996). The association between 7-KChol and 7β-OHChol reported in the present study with obesity is supported by previous studies conducted with other metabolic syndromes (Alkazemi *et al.*, 2008; Tremblay-Franco *et al.*, 2015). Furthermore, 7-KChol and 7β-OHChol were associated with smoking. This finding is in agreement with a study conducted by Samadi *et al* (Samadi *et al.*, 2019). Smoking is well documented to be associated with oxidative stress and is a strong risk factor for several conditions including diabetes and CHD.

Interestingly, 7-KChol, 7β-OHChol and Chol-triol in T1DM and T2DM were decreased significantly in patients with diabetes that used Aspirin and lipid-lowering drugs (statin). These findings indicate a good outcome of these drugs against oxidative stress and atherosclerosis and these are in agreement with previous reports (Franzoni *et al.*, 2003; Guan *et al.*, 2004; Samadi *et al.*, 2019). Aspirin has a beneficial effect on cardiovascular health which diminishes endogenous oxidative stress by up-regulating the expression of antioxidant genes encoding many antioxidants enzymes such as catalase, superoxide dismutase, and glutathione peroxidase (Santilli *et al.*, 2015). In addition, aspirin is widely used as an analgesic medication, an anti-inflammatory agent, inhibits platelet aggregation, and stimulates the expression of some positive signaling molecules (Capodanno and Angiolillo, 2016).

CONCLUSION

OXY profile (particularly 7-KChol and 7β-OHChol) is a reliable biomarker of CHD in DM. Medications such as aspirin and statin, smoking cessation, and body weight control might be useful in the management of OXY in DM.

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