International Journal of Biochemistry Research & Review
14(4): 1-12, 2016; Article no.IJBCRR.29575
ISSN: 2231-086X, NLM ID: 101654445

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Linking the Role of Biomarkers Network in Type 2 Diabetes Mellitus with Cardiovascular Risk Development

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Authors' contributions

This work was carried out in collaboration between all authors. Authors NAS and DHZ reached literature and conceived the study, contributed to biochemical/ molecular assays, performed data analysis and wrote the first draft of the paper. Author NSE was involved in protocol development, patient recruitment and data analysis. All authors reviewed, edited the manuscript and approved the final manuscript.

Article Information

DOI: 10.9734/IJBCRR/2016/29575
Editor(s):
(1) Yi-Ren Hong, College of Medicine, Kaohsiung Medical University, Taiwan.
Reviewers:
(1) Atanas Baltadjiev, Medical University, Bulgaria.
(2) Elvira Bormusov, Technion Institute of Technology, Israel.
Complete Peer review History: http://www.sciencedomain.org/review-history/16882

Original Research Article

Received 18th September 2016
Accepted 24th October 2016
Published 11th November 2016

ABSTRACT

Background: Cardiovascular diseases (CVD) mortality risk in type 2 diabetes mellitus (T2DM) is a major problem. This study was undertaken to highlight the role of biomarkers network in cardiovascular diabetic complications.
Methods: 45 sex and age-matched subjects were included; 15 healthy controls, 15 T2DM patients without history of CVD and 15 T2DM patients with CVD. Plasminogen activator inhibitor-1 (PAI-1), fetuin A, interleukin-4 (IL-4), nitric oxide (NO), advanced oxidation protein products (AOPPs) and growth differentiation factor 15 (GDF-15) were analyzed.
Results: Upregulated expression of GDF-15 and increased levels of PAI-1, IL-4 and AOPPs were observed in T2DM patients when compared with normal control group with higher values were detected in T2DM patients with CVD. Meanwhile, fetuin A and NO levels were statistically lowered

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among diabetic patients.
Conclusions: PAI-1, IL-4, fetuin A, GDF-15, NO and AOPPs play potential roles in the development of CV complications in diabetic subjects; which may represent promising prognostic biomarkers and therapeutic targets.

Keywords: Diabetes mellitus; cardivascular disease; fetuin A; plasminogen activator inhibitor-1; growth differentiation factor 15; interleukin-4; nitric oxide.

1. INTRODUCTION

Diabetes Mellitus (DM) has become a major public health problem with its long term sequalae of macrovascular and microvascular complications, currently, the prevalence of type 2 diabetes (T2D) in Egypt is around 15.6% of all adults aged 20 to 79 with an annual death of 86,478 related to diabetes [1].

It is estimated that by the year 2030 Egypt will have at least 8.6 million adults with diabetes and will be the tenth largest population of diabetics worldwide [2]. Type 2 diabetes mellitus (T2DM) is a major risk factor for cardiovascular diseases (CVD) which is the most common cause of morbidity and mortality among diabetic patients [3], about 30% of diabetic patients complicated with CVD, being the leading cause for about 70% of deaths in people with T2DM [1]. Diabetes and CVD, places a huge burden on Egypt's healthcare resources, therefore, better understanding of the pathogenesis of CVD and T2DM is of great interest for early prediction and identification of at-risk patients and for better clinical management. Modifiable factors such as dyslipidemia, obesity, oxidative stress, smoking, exercise and alcohol intake, as well as non-modifiable factors: age, sex, positive family history and genetic predisposition, have been identified as risk factors for both T2DM and CVD [4].

Oxidative stress plays an important role in the pathogenesis of CVD associated diabetes. Oxidative stress is defined as an imbalance between an excessive generation of oxidant compounds and reduced antioxidant defense mechanisms. Plasma proteins are prone to be oxidized by reactive oxygen species (ROS) with production of products, termed advanced oxidation protein products (AOPPs) which are family of oxidized, dityrosine-containing protein compounds generated during oxidative stress and could serve as an early marker of endothelial dysfunction in T2DM [5].

Nitric Oxide (NO) is synthesized from L-arginine by the nitric oxide synthase (NOS) family of enzymes. NO is an important factor in maintaining normal CV function, inhibits thrombosis and coagulation, potent vasodilator, regulator of cell proliferation and protects the endothelium and the underlying intima from inflammatory processes [6].

Growth-differentiation factor-15 (GDF-15), a member of transforming growth factor beta (TGF-β)/bone morphogenetic protein (BMP) super family, is a stress-responsive cytokine, expressed in the CV system and is emerging as a biomarker of cardiometabolic risk and disease burden. It plays an important role in the regulation of the inflammatory response, growth and cell differentiation [7].

It was reported that GDF-15 expression is highly induced in cardiomyocytes after ischemia/ reperfusion, also it is not expressed in heart under normal physiological conditions but increases rapidly in response to CV injury the same as cardiac troponin and natriuretic peptides levels making it specific for cardiometabolic diseases [8].

Cytokines and the cytokine-receptor axis are the subjects of several recent studies for their crucial roles in DM and its complications. Interleukin-4 (IL-4) is Th2 cytokine which mediates Th1/Th2 balance and immune responses by regulating the production of pro-inflammatory mediators from macrophages [9]. No previous reports studied its role in diabetes with CV complication.

Pro-coagulant and fibrinolytic markers have been proposed as risk factors for the development of T2DM [10]. Plasminogen activator inhibitor-1 (PAI-1), a serine-protease inhibitor secreted primarily by adipocytes, endothelial cells, and hepatocytes, acts as a key negative regulator of fibrinolysis through its role as the primary inhibitor of tissue plasminogen activator (tPA), it increases CV risk by favoring clot stability, interfering with vascular remodeling, or both [11].

Fetuin A is an anti-inflammatory mediator that is produced by the liver and exerts a protective effect against ischemia in the cardiomyocyte [12].

Fetuin-A inhibits the pro-inflammatory cytokine synthesis and thus prevents the self-amplification of inflammatory response; it also inhibits insulin receptor autophosphorylation and tyrosine kinase activity that improve insulin sensitivity [13].

It is associated with T2DM, but its association with CVD is uncertain also few previous studies have evaluated the association of fetuin-A with CVD associated diabetes.

The present work aimed to dissect the role played by redox status, IL-4, PAI-1, GDF-15 and fetuin A as potential prognostic biomarkers that could pave the way for better understanding of the pathogenesis of T2DM complicated with CVD. Moreover, to throw light on GDF-15, IL-4 and fetuin A as newly investigated biomarkers for T2DM complicated with CVD with evaluation of their diagnostic values.

2. SUBJECTS AND METHODS

2.1 Study Design

All participants have given their written informed consent, and the study was institutionally approved by the Research Ethical Committee of Faculty of Medicine, Tanta University, Egypt. A prospective study on a total of 45 Egyptian subjects were included in this study.

Fifteen of which served as healthy control group (group I) without any systemic diseases as diabetes, hypertension, CVD or renal insufficiency and they will be served as apparently healthy controls. Patients enrolled in the study were classified into the following groups: 15 T2DM subjects without CVD (group II), and 15 T2DM subjects with CVD (group III) who had one or more CVD manifestations in the form of history of myocardial infarction, stenting operations and/or unstable angina. Patients enrolled in this study were selected from the out-patient and in-patient divisions of the Internal Medicine Department, Tanta University Hospitals, Egypt. Participants were classified as having T2DM if they had one or more components of the American Diabetes Association criteria [14]:- fasting blood sugar (FBG) ≥126 mg/dl (7.0 mmol/l) or 2-h post prandial blood glucose ≥200 mg/dl (11.1 mmol/l) during an oral glucose tolerance test (OGTT) and random blood glucose ≥200 mg/dl (11.1 mmol/l).

Exclusion criteria were defined as: Having the history of thyroid diseases, malignancies, current smoking, acute or chronic infections, acute or chronic inflammatory disease, sever hepatic or renal impairments, immunological diseases, hemolytic disorders and alcohol or drug abuse.

A detailed medical history and drug treatment (s) were collected for all subjects including disease duration since diagnosed as diabetes mellitus patients. Body Mass Index (BMI) of all subjects was calculated as weight (kg)/height (m2). The mean of two blood pressure (BP) readings, measured on the right arm after participants had been seated for 5 min.

2.2 Sampling

After 12 hours of overnight fasting, 7ml of venous blood samples were taken from every investigated subject on two sets of sterile EDTA treated and one set of plain tubes for serum and plasma separation at 3000 rpm for 10 minutes. The extracted serum, plasma and EDTA treated blood were aliquoted and stored at -80℃ till the assay time. For fetuin A and IL-4 levels assay random urine samples were collected in sterile containers, centrifuged 20 minutes at 3000 rpm, the resultant supernatant was removed and stored at-80℃ for further analysis.

2.3 Chemicals

All chemicals and solvents used, unless otherwise described, were purchased from Sigma (Sigma, St Louis, USA).

2.4 Biochemical Analysis

Serum fasting blood sugar (FBS) was measured by the oxidase method (Biodiagnostic., Egypt), glycated haemoglobin (HbA1c) was measured in whole EDTA blood using quantitative colorimetric measurement of glycohemoglobin as percent of total hemoglobin using commercial kits (Biosystem, Spain). Serum total Lipid profile including total cholesterol (TC), triglycerides (TAG), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein (LDL-C) were measured by enzymatic-colorimetric methods (Biodiagnostic., Egypt). Serum nitric oxide (NO) was calorimetrically measured using commercial kit supplied by (Biodiagnostic., Egypt). Plasma

AOPPs was assayed by calorimetric assay method according to Witko-Sarsat et al. [15].

2.5 Determination of Atherogenic Index (AI) and Coronary Risk Index (CRI)

AI and CRI were calculated as:

AI = (TC (mg/dl)-HDL-C (mg/dl))/HDL-C (mg/dl) [16] and CRI = TC (mg/dl)/HDL-C (mg/dl) [17].

2.6 Immunoassays by Enzyme-linked Immunosorbent Technique (ELISA)

Serum levels of PAI-1 were assessed using sandwich ELISA commercial kit (Assay Max Human), urinary fetuin A level using sandwich ELISA commercial kit (OmniKine™, Assay Biotech. Company, Inc., CA, USA) and IL-4 level using commercial sandwich ELISA supplied kit (Chongqing Piospes Co., Ltd-China). All ELISA techniques were done according to the manufacturer's protocol and read on microplate reader (Stat Fax®2100, Fisher Bioblock Scientific, France), at 450 nm with correction wavelength set at 570 nm. Unknown sample concentrations were calculated from the standard curve.

2.7 RNA Extraction, cDNA Synthesis and Real-time PCR for GDF-15 Gene Expression

Total RNA was extracted from EDTA peripheral blood using QIAamp RNA mini kit (Qiagen, Hiden, Germany) according to manufacturer's instructions. Total RNA was treated with DNase I to eliminate genomic DNA contamination, followed by synthesis of the first strand cDNA using SuperScript® III First-Strand Synthesis System for RT-PCR kit (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. Real-time PCR was carried out with cDNAs. PCR reactions were performed using Power SYBR Green PCR Master Mix (Life Technologies) following manufacturer's instructions. GDF-15 mRNA transcripts were quantified, relative to the housekeeping gene, glyceraldehyde-3- phosphatedehydrogenase (GAPDH) which was used as an internal control. Sequence specific primers were designed by Primer3 software: (http://bioinfo.ut.ee/primer3/) as follows: GDF-15 with the accession number (NM_004864.2) forward primer; (5'-CGAAGACTCCAGATTCCGAGAG-3'), reverse primer; (5'-CCAGCCGCACTTCTGGC-3'). GAPDH with the accession number (NM_001289746.1): forward primer; (5'-AGTGCCAGCCTCGTCTCATAG-3'), and reverse primer (5'-CGTTGAACTTGCCGTGGGTAG-3'). PCR cycling was set as follows: a single cycle of DNA polymerase activation for 5 min hold at 95℃ followed by 40 cycles of 94℃ for 45s for denaturation, 59℃ for 45 s for annealing and 72℃ for 1 min for extension. Amplification and data analysis were conducted on a Rotor-Gene Q 6plex and its specific software for calculation of the threshold cycle (Ct) values (Qiagen, Valencia, CA, USA). Relative gene expression was automatically calculated from the delta cycle threshold values of the target and the reference gene.

2.8 Statistical Analysis

Statistical analysis was conducted as mean and standard deviation using Statistical Package for Social Sciences (SPSS), version 16.0 for Windows (SPSS, Chicago, IL). One-way analysis of variance (ANOVA) was used for multiple comparisons to evaluate the statistical significance between experimental groups followed by post hoc test. Unpaired student t-test was used to evaluate the statistical significance between two groups. The correlation study was calculated using Pearson's correlation. P^* value < 0.05 was considered significant. Receiver operating characteristics (ROC) analysis was used to identify the optimal threshold values of the studied parameters.

3. RESULTS

3.1 Descriptive and Routine Biochemical Findings among All Studied Groups

Table 1 summarizes the main demographic and laboratory data of the studied groups.

There were no significant differences in terms of age and BMI among the studied groups ($p>0.05$). Meanwhile, there were statistically significant increases in disease duration, TAG, TC and LDL-C levels in patient groups when compared to their allied control group with significant higher values were for T2DM with CVD group, meanwhile, HDL-C showed the reverse. There were statistically significant increase in the AI and CRI in diabetic patient groups when compared to control group with higher values were noted among T2DM patients with CVD.

Table 1. Descriptive statistics and routine biochemical findings in studied groups

Parameter/group	Group I (n=15)	Group II (n=15)	Group III (n=15)	P-value
Age (years)	45.4±6.58	45.00±6.61	44.66±6.95	0.956
Sex Female (n, %)	10(66.7%)	9(60%)	10(66.7%)	-
Male (n, %)	5(33.3%)	6(40%)	5(33.3%)	-
Duration (years)	-	6.90±0.6	9.72±0.40	0.001*
BMI (kg/m^2)	24.13±0.92	24.34± 0.99	24.21±1.12	0.860
FBS level (mg/dl)	85.93±3.77	139. 60±7.41 [c]	169. 93±11.68 [ab]	0.001*
Systolic blood pressure (mmHg)	103.33±7.72	133.00±9.78 [c]	162.33±10.67 [ab]	0. 01*
Diastolic blood pressure (mmHg)	67.33±6.11	87.33±8.21 [c]	104.33±9.04 [ab]	0. 03*
Hb A1c%	3.89±0.59	6.37± 0.36 [c]	7.19±0.56 [ab]	0. 01*
TC (mg/dl)	131. 73±12.93	217.53±13.66 [c]	246.73±7.88 [ab]	0.003*
TAG (mg/dl)	114.87±12.32	161.33±13.48 [c]	185.53±10.12 [ab]	0.001*
HDL-C (mg/dl)	60.13± 5.46	43.93±4.94 [c]	36.33±2.32 [ab]	0.00*
LDL-C (mg/dl)	56.73±3.86	78.07±4.11 [c]	136.87±4.1 [ab]	0.001*
AI	1.41±0.33	3.99±0.51 [c]	5.82± 0.47 [ab]	0.003*
CRI	2.41±0.33	4.99±0.51 [c]	6.82±0.47 [ab]	0.002*

*Data presented as mean ± SD; n number of cases; BMI Body mass index; FBS fasting blood sugar; HbA1c: Glycated hemoglobin;TC total cholesterol; TAG triacylglycerol; LDL-C low-density lipoprotein cholesterol; HDL-C high-density lipoprotein cholesterol; AI atherogenic index; CRI coronary risk index. * Significant difference. P Value was calculated by one-way ANOVA test followed by Tukey's post hoc test. To compare significance between the two groups student t- test was used. Identical P Value was considered significant at P<0.05.* [a] *Significant difference between groups III & I;* [b] *Significant difference between groups III & II;* [c] *Significant difference between groups II & I*

3.2 Inflammatory Biomarkers

As regards the studied inflammatory markers, GSD-15 gene expression showed significant increase in T2DM patients with CVD group when compared to patients without CVD and control groups with non-significant higher values for T2DM without CVD group when compared to control group. Urinary IL-4 level showed significant increase in patient groups when compared to their allied controls with significant higher values for T2DM with CVD group. On the other hand, fetuin A levels showed evident decrease in diabetic groups when compared to control one with significant decline in diabetic patients with CVD (P<0.05) (Table 2).

3.3 Endothelial and Fibrinolysis Biomarkers

Serum PAI levels showed significant increase in diabetic groups when compared to their allied control with significant higher values for T2DM with CVD, meanwhile NO levels showed significant decrease when compared to normal control group with significant lower values for T2DM with CVD group (P<0.05) (Table 2).

3.4 Oxidative Stress and Glycemic Status Biomarkers

FBS, percentage of HbA1c and AOPPs showed significant increase in diabetic groups when compared with control group with significant higher values for T2DM with CVD group (P<0.05) (Tables 1 & 2).

3.5 Results of Correlation Statistics

Table 3 summarizes correlation statistics in T2DM cases, disease duration, FBS, , HbA1c, AI and CRI showed significant positive correlation with GDF-15, IL-4, PAI 1 and AOPPs, while fetuin A and NO showed the reverse (P<0.05).

Table 4 summarizes correlation in T2DM cases between urinary and blood studied biomarkers, GDF-15, PAI-1, NO and AOPPs showed statistically significant positive and negative with IL-4 and fetuin A respectively (P<0.05).

3.6 Results of ROC Curve

Receiver operating characteristics (ROC) analysis was used to evaluate the diagnostic values of studied parameters and to identify the

optimal cut off values. The area under the curve can range from 0.5 to 1 and diagnostic tests that approach 1 indicate a perfect discriminator. The optimal cut off value of GDF-15 gene expression was 2.6, the sensitivity at this cut off point was 86.7%, the specificity was 100%, the positive predictive value (PPV%) was 100% and the negative predictive value (NPV%) was 88.2% and the area under the curve was 0.92. Furthermore, the optimal cut off value of urinary IL-4 (pg/ml) was 42.2 pg/ml, the sensitivity at this cut off point was 100%, the specificity was 80%, the (PPV%) was 83.3% and the (NPV%) was 100% and the area under the curve was 0.94. The optimal cut off value of urinary fetuin A (ng/ml) was 0.79 ng/ml, the sensitivity at this cut off point was 100%, the specificity was 100%, the (PPV%) was 100% and the (NPV%) was 100% and the area under the curve was 1. Data are depicted in Table 5.

4. DISCUSSION

In fact, the traditional cardiovascular (CV) risk factors do not completely predict the presence of sub-clinical injuries of heart and vessels in diabetic population. A deeper understanding of the complex pathogenic mechanisms of the CV remodeling in diabetes is required for proper management and to improve survival.

The combination of hyperglycemia, insulin resistance, dyslipidemia and chronic inflammation associated diabetes can act both independently and cumulatively over time to significantly increase risk for CVD [18] which is

Table 2. Biochemical and molecular findings of the studied groups

Parameter/group	Group I (n=15)	Group II (n=15)	Group III (n=15)	P-value
GDF-15 gene expression	1.99±0.33	2.12±0.29	3.63±0.40 [ab]	0.002*
IL-4 (pg/ml)	18.07±4.5	33.27±7.29 [c]	50.85±8.03 [ab]	0.013*
PAI-1 (pg/ml)	33.26±2.98	47.83±4.28 [c]	66.55±5.23 [ab]	0.012*
NO (µmol /L)	12.7±5.11	8.93±0.70 [c]	5.71±1.36 [ab]	0.018*
Fetuin A (ng/ml)	1.72±0.67	0.98±0.06 [c]	0.61±0.12 [ab]	0.01*
AOPPs (µmol/ L)	21.37+3.08	42.03+5.81 [c]	54.49+7.75 [ab]	0.013*

*Data presented as mean ± SD; n number of cases; GDF-15 growth differentiation factor-15; IL-4 interleukin-4; PAI-1 plasminogen activator inhibitor-1; NO nitric oxide AOPPs advanced oxidized protein products . * Significant difference. P Value was calculated by one-way ANOVA test followed by Tukey's post hoc test. Identical P Values was considered significant at P<0.05. [a] Significant difference between groups III & I; [b] Significant difference between groups III & II; [c] Significant difference between groups II & I*

Table 3. Correlations matrix between atherogenic index (AI) and coronary risk index (CRI) and all studied molecular and biochemical studied biomarkers in patient groups

		Disease duration (years)	AI	CRI	FBS (mg/dl)	% HbA1c
GDF-15 gene expression	*r*	0.88	0.76	0.76	0.84	0.50
	P	0.001*	0.001*	0.01*	0.001*	0.005*
IL-4 (pg/ml)	*r*	0.69	0.80	0.80	0.70	0.44
	P	0.003*	0.001*	0.01*	0.016*	0.016*
PAI-1 (pg/ml)	*r*	0.88	0.79	0.79	0.85	0.64
	P	0.001*	0.001*	0.01*	0.001*	0.001*
NO (µmol /L)	*r*	-0.74	-0.71	-0.71	-0.78	-0.65
	P	0.003*	0.001*	0.01*	0.001*	0.009*
Fetuin A (ng/ml)	*r*	-0.87	-0.88	-0.88	-0.79	-0.59
	P	0.001*	0.001*	0.01*	0.001*	0.001*
AOPPs (µmol / L)	*r*	0.63	0.68	0.68	0.72	0.54
	P	0.002*	0.004*	0.01*	0.007*	0.002*

*Correlation study was carried out using Pearson correlation. *Significant at P value <0.05. AI atherogenic index; CRI coronary risk index; FBS fasting blood sugar; HbA1c glycated hemoglobin; GDF-15 growth differentiation factor-15; IL-4 interleukin-4; PAI-1 plasminogen activator inhibitor-1; NO nitric oxide AOPPs advanced oxidized protein products*

clear in this current study as there was a significant increase in disease duration in group III when compared with group II. Inflammatory biomarkers became in use as a diagnostic tool of diabetic patients prone to develop CVD [19].

Table 4. Correlations matrix between urinary and blood studied biomarkers in patient groups

		IL-4 (pg/ml)	Fetuin A (ng/ml)
GDF-15 gene expression	*r*	0.71	-0.84
	P	0.01*	0.001*
PAI-1 (pg/ml)	*r*	0.67	-0.86
	P	0.005*	0.001*
NO (µmol / L)	*r*	-0.65	0.67
	P	0.008*	0.005*
AOPPs (µmol / L)	*r*	0.65	-0.66
	P	0.009*	0.007*

*Correlation study was carried out using Pearson correlation. *Significant at P value < 0.05. GDF-15 growth differentiation factor-15; IL-4 interleukin-4; PAI-1 plasminogen activator inhibitor-1; NO nitric oxide AOPPs advanced oxidized protein products*

GDF-15 is a stress responsive cytokine; it is highly expressed in cardiomyocytes, adipocytes, macrophages, endothelial cells and vascular smooth muscle cells [19].

The present study revealed significant increase in T2DM patients with CVD group when compared to T2DM without CVD and control groups with non-significant higher values for T2DM without CVD group when compared to control group making it a cardiospecific biomarker. Furtherly, GDF-15 correlate positively with disease duration, FBS, percentage of HBA1c, AI and CRI. In harmony with these results Rohatgi et al. [20] reported that GDF-15 is a predictive and prognostic factor for CV disease and diabetes. In addition El-Refaei et al. [21] reported that GDF15 has a potential link to the pro-inflammatory transcription factor, nuclear factor-kappa B (NF-κB) activation, making GDF15 a valuable target for modulating inflammatory conditions.

Additionally, GDF-15 is expressed through p53 activation, a transcriptional factor that links GDF-15 with increased production of pro-inflammatory cytokines and insulin resistance, then GDF-15 expression is rapidly induced by pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukins as IL-4, PAI-1 and TGF-β and thus serves as a potential marker of inflammation, moreover, insulin resistance and increased GDF-15 both are associated with endothelial dysfunction that lead to metabolic derangement, inflammation and vascular injuries as reported by Kempf et al. [22].

IL-4 plays an important role in inflammatory reactions via modulating growth, differentiation and cytokines production. Shanmugam et al. [23] reported that NF-κB increased the expression of IL-4 in monocytes subjected to high glucose levels.

The current study revealed increase of urinary IL-4 in T2DM patients with and without CVD when compared with control group. Moreover, there was statistically significant difference between patient groups with and without CVD. Also IL-4 correlates positively with disease duration, FBS, percentage of HBA1c, AI and CRI. In accordance with our current findings. Semenchenko et al. [24] reported that cytokine profiles including IL-4 played a critical part in T2DM pathogenesis. IL-4 was traditionally considered as an anti-inflammatory cytokine however, it can acts as a pro-inflammatory cytokine and plays a critical role in the progression of CV events, hence it induces pro-inflammatory environments by over-expressing inflammatory mediators in vascular endothelial cells as IL-1β, TNF-α and/or lipopolysaccharide (LPS)- induced vascular cell adhesion molecule-1 (VCAM-1) [25].

Table 5. The performance characteristics for GDF-15 gene expression, IL-4 and fetuin A in diabetic patients with and without CVD

	Cut off	Sensitivity	Specificity	PPV	NPV	Area under the curve
GDF-15 gene expression	>2.6	86.7	100.0	100.0	88.2	0.92
IL-4 (pg/ml)	>42.2	100.0	80.0	83.3	100.0	0.949
Fetuin A (ng/ml)	> 0.79	100.0	100.0	100.0	100.0	1

PPV: positive predictive value, NPV: negative predictive value, GDF-15 growth differentiation factor-15; IL-4 interleukin-4

Interetingly, IL-4 increases the endothelial cell turnover by accelerating apoptosis, the event which may alter the function of the vascular endothelium and thereby promote CVD incidence and progression [25].

Dyslipidemia is a mechanism by which diabetes promotes diabetic complications specially CVD. The current study revealed distorted lipid profiles of diabetic patients with statistically evident dyslipedemia in patients wih CVD as previously reported [26].

The total cholesterol (TC)/HDL-C and the LDL-C /HDL-C molar ratios have a good predictive value for future CV events. The present study revealed increase in the atherogenic indices in diabetic groups when compared to normal control. However, there was statistically significant increase in T2DM with CVD group when compared with T2DM without CVD as supported by previous studies of [27].

Insulin resistance and diabetes are associated with prothrombotic risk (coagulation factors VII, XII, and fibrinogen) and with suppression of fibrinolysis due to elevated concentrations of the fibrinolytic inhibitor PAI-1 [28].

PAI-1 favors intravascular fibrin deposition and promotes clot stability by inhibiting plasmin production from its inactive precursor, plasminogen. Moreover, it inhibits fibrinolysis in the vessel wall, interfering with vascular remodeling and promoting the development of an unstable plaque [28]. In addition, PAI-1 is considered an acute phase protein and could act as an inflammatory mediator, increase CVD risk [29]. Given the association of PAI-1 with CVD, intervention that reduces PAI-1 could yield a benefit through one or several of these pathways mainly though suppression of NF-κB pathway as previously reported [30].

The current study revealed increased PAI-1 and decreased NO levels in T2DM with CVD when compared to other groups and showed positive and negative correlation with disease duration, FBS, percentage of HbA1c, AI and CRI respectively that unravel their roles as biomarkers of disturbed endothelial dysfunction in T2DM with CVD [31].

Barrett et al. [32], reported that insulin has important vascular actions to stimulate production of NO from endothelium, this leads to capillary recruitment, vasodilation, increased blood flow which is impaired in insulin resistance associated diabetes.

Fetuin A, a hepatic secretory protein that simultaneously inhibits arterial calcification and insulin action, is associated with T2DM, but its association with CVD is uncertain. The present study revealed statistically significant decrease in urinary fetuin A levels in diabetic patients with CVD when compared with other groups. Also it negatively correlated with disease duration, FBS, percentage of HBA1c, AI and CRI as previously supported [33].

Data obtained from ROC curve analysis showed that urinary fetuin A showed the best accuracy which makes it an accurate diagnostic biomarker for T2DM complicated with CVD.

Physiologically, during inflammation, pro-inflammatory cytokines, whose production is usually inhibited by fetuin A, will decrease fetuin A liver synthesis which will facilitate the ongoing inflammatory process and overproduction of cardiotoxic cytokines such as TNF-α so fetuin-A concentration indicates the state of inflammatory imbalance, because it is the result of pro- and anti-inflammatory cytokine interaction [34].

Collectively, fetuin A is a pleotropic molecule with diverse, sometimes even contradictory effects in different systems, and its role in T2DM with CVD is unclear so it is an interesting candidate to be studied as a novel, cheap and non-invasive biomarker.

Chronic oxidative stress in diabetic humans and animals, purportedly related to the metabolism of excess substrates (glucose and fatty acids) present in the hyperglycemic state, as well as to the mitochondrial dysfunction associated with insulin resistance [35].

Diabetic hyperglycemia, by the process of free radical production, causes protein glycation and oxidative degeneration. The degree of such protein glycation is estimated by using some biomarkers such as HbA1c and fructosamine levels.

This current study revealed increased level of both FBS and percentage of HbA1c in group of T2DM with CVD when compared with T2DM without CVD and control groups reflecting the glycemic status role as diagnostic and prognostic biomarker for diabetes with and without CV risk as previously reported by Juutilainen et al. [36]

who reported that, an increment of 1 unit (%) of HbA1c increased CVD mortality by 52.5% in T1DM subjects and by 7.5% in T2DM subjects.

AOPPs might be formed during oxidative stress by reaction of plasma proteins with chlorinated oxidants, and have been considered as novel markers of oxidant-mediated protein damage their roles in the development of cardiovascular disease might be of great importance [37].

The present study revealed increased level of AOPPs in group of T2DM with CVD when compared with T2DM without CVD and control groups, moreover AOPPs showed positive correlation with FBS, percentage of HBA1c, AI and CRI, those results are in line with Brzović-Šarić et al. [38] who reported that AOPPs are moderately elevated in adult patients with T1DM and more markedly elevated in those with T2DM as a result of oxidative stress associated diabetes.

The present study showed significant correlation between urinary IL-4 and fetuin A levels with the other studied biomarkers so they act as novel non invasive, easy and cheap mirror images to invasive costly GDF-15 gene expression levels and the invasive traditionally used biomarkers which are PAI 1, NO and AOPPs making together a metabolic map for diabetes associated CVD which can be attacked as a therapeutic approach for better management.

To our knowledge there were no studies showed an evaluation of GDF-15 gene expression in association with the beforementioned studied biomarkers in T2DM patients that gives a new linkage between inflammation, immune response, fibrinolytic activity and oxidative stress in the pathophysiology of diabetic complication. Limitations of the current study were; poor patients cooperation and small number of patients enrolled.

5. CONCLUSION

CVD accounts for the majority of morbidity and mortality associated with diabetes mellitus. As we continue to learn more about the complex pathophysiology underlying this crucial health problem, more effective therapies for prevention and treatment will emerge. This study revealed the role played by GDF-15, IL-4, PAI-1, NO, fetuin A and AOPPs in CV risk associated T2DM through different mechanisms which may be useful as potential biomarkers for effective assessment of disease incidence and progression and may help in the clinical management of the disease and in the measurement of response to therapy. In addition, measurement of urinary IL-4 and fetuin A may emerge as a novel, easy-to-obtain, non-invasive and cheap method for better assessment with reducing suffering of patients. Although, further researches with larger sample size are required for confirmation of our current study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/16882