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Diagnostic value of micro-RNAs for coronary artery disease in Egyptian type 2 diabetic patients

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Abstract

With an increasing global burden of coronary artery disease (CAD), early detection and timely management of risk factors are crucial to reduce morbidity and mortality in such patients. Diabetes mellitus (DM) is considered an independent risk factor for the development of CAD. Circulating microRNAs have been recognized as promising biomarkers for various diseases. The present study aimed to explore the potential role of circulating miRNA-149, mi-RNA424 and mi-RNA765 as non-invasive biomarkers for the diagnosis of coronary artery disease in middle-aged (40–60-years old) type 2 diabetes mellitus patients. This study included 120 volunteers of both sexes classified as group I: 30 normal subjects; group II: 30 type 2 diabetic patients (T2D), group III: 30 coronary artery disease patients (CAD) and group IV 30 type 2 diabetic patients with coronary artery disease (T2D&CAD). Plasma mi-RNAs, diabetic biomarkers, lipid profile, inflammatory biomarkers and troponin I were determined. Circulating mi-RNA765 levels were elevated in T2DM with CAD group (7.04 ±0.36), compared with T2DM group (2.01 ±0.27). In contrast, circulating mi-RNA149 and mi-RNA424 levels were decreased in T2DM with CAD group (0.9 ±0.09 &0.84±0.08), compared with T2DM group (4.28 ±0.32 & 4.52 ±0.31) respectively. These results suggest that circulating mi-RNA149, mi-RNA 424 and mi-RNA765 might be non-invasive biomarkers for the diagnosis of coronary artery disease in middle-aged diabetic patients.

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Introduction

Microvascular disease is a growing public health problem, accounting for approximately half of hospital admissions of individuals with heart failure (*Shu, et al., 2019,* and *Schiattarella, et al., 2019*).

People with DM have a higher-than-average risk of having a heart attack or stroke (*Balakumar et al., 2016,* and *Huo et al., 2016*). In fact, DM represents a crucial risk factor for cardiovascular disease (*Shu et al., 2019,* and *Shu et al., 2018*). In 2019, a total of 463 million people were estimated to be living with diabetes, representing 9.3% of the global adult population (20–79 years). This number is expected to increase to 578 million (10.2%) in 2030 and 700 million (10.9%) in 2045.

Atherosclerosis, a progressive inflammatory disease, is the main underlying mechanism of CAD. Endothelial cell (EC) initiate the development of atherosclerosis *(Matsuzawa and Lerman, 2014)*, where, the permeability of ECs changes and macrophages accumulate and release inflammatory mediators. Smooth muscle cells (SMCs) are then activated and begin proliferating and migrating *(Ding et al., 2017)*. Therefore, the pathogenesis of CAD results from numerous changes and interactions between multiple cell types in the artery walls; these changes mainly include lipid deposition, EC dysfunction, macrophage activation, and SMC alteration *(Nurnberg et al., 2015)*.

CAD is hard to diagnose without the help of the well-established invasive coronary angiogram (CAG) technique. Nowadays, electrocardiogram (ECG) and exercise tolerance test (ETT) have been widely used *(Gupta et al., 2010)*.

Micro RNAs regulate EC, SMC, and macrophage function; vascular inflammation; and metabolism, suggesting the possibility that micro RNAs influence the progression of CAD. The levels of circulating mi-RNAs reflect pathological conditions, and some mi-RNAs have been identified as biomarkers with the potential to detect atherosclerosis or CAD at its earliest stages in clinical practice *(Jansen, 2014)*.

Circulating miR-149 and miR-424 were down regulated, whereas mi-RNA-765 was up regulated, in middle-aged diabetic patients, indicating that these mi-RNAs might be noninvasive biomarkers for the diagnosis of CAD *(Ali Sheikh, 2015)*. Mi-RNA-424 has been found to inhibit inflammatory-induced angiogenesis through the direct targeting of CD40 *(Lee, 2017)*. Mi-RNA-765 influences arterial stiffness through modulating apelin expression.

Sayed et al., (2015) Ren et al., (*2013*) and *Hoekstra et al., (2010)* showed an up-regulation of mi-RNA 765 in the middle-aged population of unstable CAD patients.

Aim of the work

The present study was conducted to evaluate the circulating mi-RNAs 149, 424 and 765 in T2DM with CAD patients in an attempt to examine the possibility of considering them as predictors of CAD in Egyptian T2DM patients who are at risk of disease.

Materials and methods

Subjects

This study included 120 volunteers of both sexes classified as group I: 30 normal subjects; group II: 30 type 2 diabetic patients (T2D), group III: 30 coronary artery disease patients (CAD) and group IV: 30 type 2 diabetic patients with coronary artery disease (T2D&CAD). The CAD patients were referred to the outpatient clinics of National Heart Institute, Cairo, Egypt, and T2DM patients were selected from those attending to National Institute of Diabetes and Endocrinology, Cairo, Egypt. The mean ages of volunteers were 53±6 years.

Type 2 DM was diagnosed according to the Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (2006).

A full medical history was taken with special attention to any associated medical problems. Patients with any history of smoking, alcohol habits, liver disease, thyroid dysfunction, chronic inflammation, infectious

disease were excluded. Also pregnant women were also excluded.

The participants in the diabetic groups were uncontrolled type 2 diabetic patients treated with oral hypoglycemic agent with a dose adjusted according to the state of each patient.

The patients were instructed not to engage in any vigorous exercise for at least 3 days before the study. None of the normal subjects were taking any medications known to affect glucose metabolism. The purpose and nature of the study were explained to all subjects and written voluntary consents were obtained before their participation. Approval was taken from the research committee of General Organization of Teaching Hospitals and Institutions.

Methods

Ten ml of venous blood samples were collected from patients and healthy controls in the morning after an overnight fasting. Each blood sample was divided into the following: 2 ml of blood was collected on EDTA coated tube until total RNA isolation, which was, then, performed using a MagNA Pure LC Total (Nucleic Acid Isolation Kit) according to the manufacturer's instructions. cDNA synthesis and real-time polymerase chain reaction amplification from the isolate was performed using an EPIK™ mi-RNA Select Hi/Lo-ROX (Bioline Reagents Ltd). Mi-RNA amplification kit for determination of mi-RNAs (149, 424 and 765) and HbA1c levels on whole blood sample using a commercial assay kit from (BioMed diagnostics, Egypt) according to *Trivelli, (1971)*. 2 ml of blood was collected on fluoride coated tube for determination of plasma fasting glucose (FG) using a commercial assay kit from (Vitro Scient, Egypt) according to *Trinder, (1969)*.The rest of blood sample was kept in a plain vacationer tube without additives to clot at 37 ºC for 20 minutes, and then centrifuged at 3000 rpm for 10 minutes at 4 ºC. The serum was then separated into aliquots and stored at -20 ºC to be thawed only once on demand, except an aliquot was used immediately for the estimation of serum insulin using (BioSource International, Inc, Egypt) according to *Flier et al., (1979)*, Insulin resistance was defined by homeostasis model assessment for insulin resistance (HOMA-IR). HOMA-IR was calculated by dividing the product of plasma glucose (m.mol/ L) and fasting serum insulin (mU/ L) by 22.5 according to *Qu et al., (2011)*. Serum lipid parameters were estimated as follows, total cholesterol, according to *Natio(1984)*, TAG according to *Kaplan et al.,(1984)* using Spin react, Egypt kit and HDL- C, using Spectrum, Egypt kit according to *Lopes-Virella et al.,(1977)*. LDL- C was calculated according to the formula of *Freidwald et al, (1972)*. Inflammation biomarkers: IL-6 using ELISA kit (Quantikine, R&D Systems) according to *Pinto et al.,(2009)* and NO using BioMed diagnostics kit according to *Miranda et al.,(2001)*. Liver enzymes using Spin react kit according to Murray, 1984 and troponin I using ELISA kit purchased from AlpoTM Diagnositic (USA) according to *Takahashi et al., (1996)*.

All chemicals and reagents used were purchased from Sigma Chemical Company (St. Louis MO, USA) & Aldrich Chemical Company. Kits were purchased from bio diagnostic, (Egypt); spectrum, (Egypt) and sigma company, (St. Louis MO, USA).

Statistical analysis

Data are presented as means ±SD. The data were analyzed by one-way analysis of variance (ANOVA). A P value less than 0.05 was considered statistically significant. To compare the difference among the groups, post hoc testing was performed by the Bonferroni test. Pearson's correlation coefficient analysis was used to determine the correlations between studied micro RNAs and the different studied parameters.

The predictive values of the studied parameters for the patients group was compared to control group data by ROC curve analysis, the data were expressed as area under the curve (AUC). If AUC = 1, the index is an ideal predictor, and if AUC = 0.5, the index has no predictive value. Statistical analysis was carried out by the aid of a digital computer, using Excel & SPSS for Windows (version 23, Chicago IL, USA).

Results

Fasting plasma glucose, HbA1c%, insulin, and HOMA-IR levels showed a highly significant increase ($P < 0.001$) in diabetic patients and T2DM patients with CAD compared to control group with percent change (128.9% , 162.1%), (118.8%, 157.2%), (80.5% , 106.6%) and (110.6% , 153.2%) respectively.

Troponin I showed a highly significant increase ($P < 0.001$) in both CAD patients and T2DM patients with CAD compared to control group with percent change (30158.2 %, 31641.9%) respectively. Nitric oxide showed a highly significant decrease ($P < 0.001$) in both CAD patients and T2DM patients with CAD compared to control group with percent change (30.9 %, 52.8 %) respectively.

Interleukin 6 showed a highly significant increase ($P < 0.001$) in T2DM, CAD, and T2DM with CAD patients compared to control group with percent change (146.6%, 58.9% and 120.5%) respectively (Table 1).

Table 1. Levels of diabetic biomarkers, Troponin I and inflammatory biomarkers in all studied groups.

Group	FG (mg/dl)	HbA1c %	Insulin (mU/L)	HOMA-IR	NO (µmol/L)	Trop-I (ng/ml)	IL-6 (Pg/ml)
G: I Normal controls	91.40±1.94	5.21±2.15	6.27±0.015	2.35±0.57	66.66±1.45	0.031±0 .003	0.75±0.02
G:II T2DM	209.30±5.25	11.4±0.09	11.32± 1.01	4.95±1.2	70.33±1.19	0.036±0.006	1.85±0.07
Chang%	128.9	118.8	80.5	110.6	5.50	16.1	146.6
G:III CAD	95.73±2.40	6.41±2.15	6.96± 0.9	2.7±0.57	46.06±1.18	9.38±0.26	2.94±0.15
Chang%	4.73	23.03	11.0	17.39	- 30.9	30158.2	58.9
G:IV CAD with T2DM	239.53±5.04	13.4±0.07	12.96± 2.3	5.95±1.2	31.42±0.86	9.84±0.30	4.08±0.13
Chang%	162.1	157.2	106.6	153.2	- 52.8	31641.9	120.5

Note: values are given as means ±SD for groups (30 individuals).

Plasma mi-RNA765 showed a highly significant increase ($P < 0.001$) in CAD patients and in CAD patients with T2DM compared to normal controls with percent changes 177.6 % and 332.3% respectively. On the other hand, plasma mi-RNAs 424& 149 showed a highly significant decrease ($P < 0.001$) in CAD patients and in CAD patients with T2DM compared to normal controls with percent changes (72.4 %, 82.4 %) and (70.9 % and 80.6%) respectively.

Table 2. Levels of lipid profile in all studied groups.

Group	TC (mg/dl)	TAG (mg/dl)	LDL- C (mg/dl)	HDL- C (mg/dl)
G: I Normal controls	152.07±6.41	107.07±8.6	93.84±5.57	39.78±2.33
G:II T2DM	165.30±7.58	120.50±13.49	97.35±7.2	37.53±2.54
Chang%	8.69	12.54	3.74	-5.73
G:III CAD	266.47±6.23	194.07±8.58	142.85±6.73	32.24±3.13
Chang%	75.1	81.2	52.2	-18.95
G:IV CAD with T2DM	288.93±5.05	230.6±3.56	159.17±6.39	29.59±2.45
Chang%	89.9	115.3	69.6	-25.6

Note: values are given as means ±SD for groups (30 individuals).

Non- significant changes were observed in plasma mi-RNAs 765, 149 and 424 in T2DM patients compared to controls.

A significant increase in TC, TAG and LDL- C was shown in T2DM patients compared to control group with percent change (8.69%, 12.54%, and 3.74%). In contrast, significant decrease of HDL- C was shown in T2DM patients compared to control group with percent change (-5.73%). This increase was augmented in CAD patients and T2DM patients with CAD compared to control group with percent change (75.1 %, 89.9 %), (81.2 %, 115.3 %), and (52.2 %, 69.6 %) respectively. In contrast, highly significant decrease of HDL-C ($P < 0.001$) was shown in CAD patients and CAD patients with T2DM compared to control group with percent change (18.95 % , 25.6%) respectively.

Table 3. Area under the curve for mi-RNA149, mi-RNA424, mi-RNA765 and Trop-I in all studied groups.

Parameter / Group	Micro RNA149 (ng/ml)	Micro RNA424 (ng/ml)	Micro RNA765 (ng/ml)	Trop-I (ng/ml)
Group II (T2DM)	0.793	0.811	0.153	0.228
Group III (CAD)	0.240	0.243	0.738	0.804
Group IV (T2DM+CAD)	0.124	0.110	0.896	0.863

Evaluating the Accuracy of Parameters of Diagnostic Performance

The Receiver Operating Characteristic (ROC) curve and areas under the curves (AUC). The coordinates of the curve were most helpful because they provided some guidance for determining what should serve as the cut off for determining positive and negative assay results.

Receiver Operating Characteristic curve figure (2) and table (3) showed the area under the curve for mi-RNA 765, 149, 424 and Trop I, it was noted that, mi-RNA 765 has the most dignostic value (AUC: 0.896) followed by Trop I (AUC: 0.863).

Discussion

Coronary artery disease is among the leading cause of morbidity and mortality worldwide and puts an enormous economic burden in the society *(Luo et al., 2019)*. Therefor early diagnosis of CAD has an important role in patient management.

DM can cause serious acute and chronic complications that adversely impact the quality of life and survival of the majority of people with this disease. It is among the top 10 causes of death in adults, and was estimated to have caused four million deaths globally in 2017 *(IDF, 2017)*. DM is considered to be an independent risk factor in the development of CAD *(Lloyd-Jones et al., 2018)*.

Mi-RNAs are reported from whole blood, peripheral blood mononuclear cells, platelets, serum, plasma, and other body fluids. The expression level of mi-RNAs in body fluids have a potential role intended for early detection, diagnosis, severity assessment markers and prognostic indicators *(Jessica et al., 2010)*.

Altered regulation of both mi-RNAs levels and function has been associated with several conditions, including CAD (*Da Costa Martins et al., 2010*).

In the present work, it was found that the plasma level of mi-RNAs149 and 424 were remarkably decreased, whereas mi-RNA765 was increased in CAD patients and T2DM patients with CAD compared with control subjects.

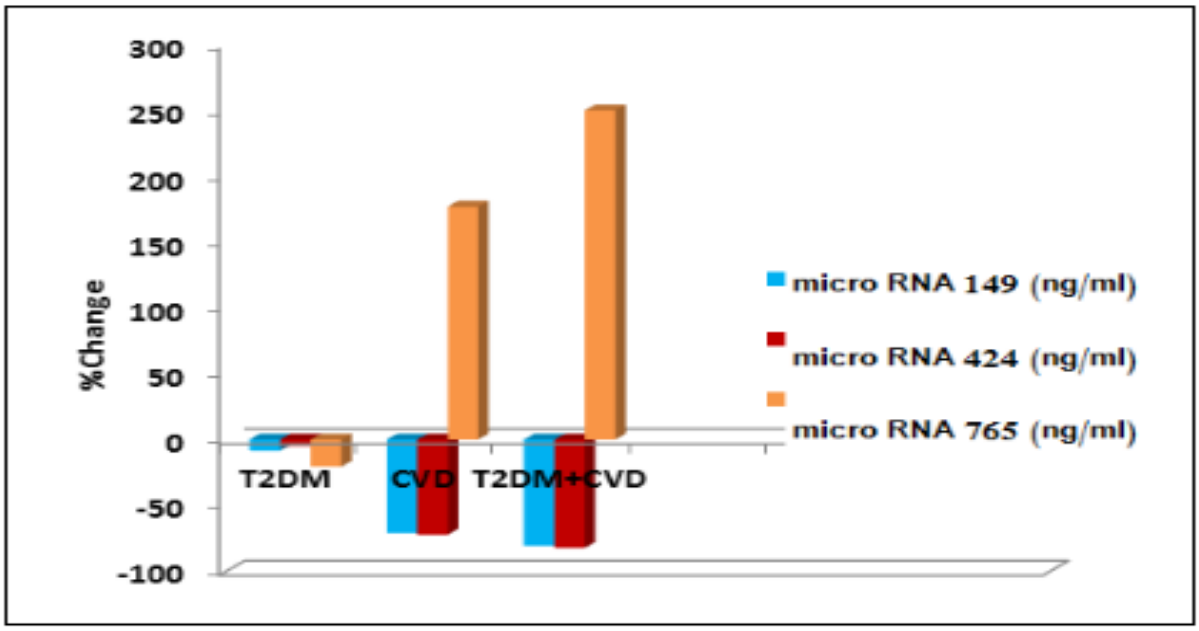

Fig. 1. Percentage change of mi-RNA149, mi-RNA424 and mi-RNA765 between all studied groups.

This could be explained that, mi-RNAs play a role in regulating key signaling and lipid homeostasis pathways that alter the balance between the progression and regression of atherosclerotic plaques *(Wagschal et al., 2015)*. Importantly, mi-RNAs are also implicated in the regulation of endothelial cell inflammation and plaque progression (*Novak et al., 2015)*. In addition, mi-RNAs regulate leukocyte recruitment and activation in atherosclerosis, one of the earliest pathogenic events in atherosclerosis *(Feinberg et al., 2016)*.

Also, micro RNA 424 has been found to inhibit inflammatory-induced angiogenesis through the direct targeting of CD40 *(Lee et al., 2017)*. CD40, a trans membrane receptor of the tumor necrosis factor (TNF) gene superfamily, is largely expressed on antigen presenting cells (APCs), including B cells, macrophages, and monocytes *(Chatzigeorgiou et al., 2009)*. While it has been shown to be expressed at low levels in ECs, pro inflammatory stimuli such as tumor necrosis factor alpha (TNFα) and lipopolysaccharide (LPS) causing rapid induction of CD40 expression in ECs *(Yang et al., 2012, Omari and Dorovini-Zis 2003)*.

Additionally, it was found that mi-RNA424, which has highly conserved seed sequences, was predicted to bind to the 3´ untranslated region (UTR) of CD40. This mi-RNA is highly expressed in ECs and is known to have important roles in maintaining vascular homeostasis *(Kim 2014 and Lee et al., 2014)*.

On the other hand it was found that mi-RNA 149 is a new immune modulator of the innate immune responses. Its overexpression in macrophages has been linked to a significant decrease in MyD88 protein expression (a protein which act as an adaptor, connecting proteins that receive signals from outside the cell to the proteins that relay signals inside the cell), as well as a reduced production of inflammatory mediators such as NF-κB, TNF-α, and IL-6 in response to infection or LPS stimulation *(Xu et al., 2014)*.

The results of this study were in agreement with other studies where *Van Rooij et al., 2008* found that mi-RNA149 and 424 were significantly down regulated in CAD,*Wu et al., 2013* who established that human mi-RNAs149 and 424 were strongly associated with increased risk of CAD, finally, *Sayed, et al., 2015* and *Zhu, et al., 2014* who documented that mi-RNAs149 and 424 were down-regulated in CAD in elderly and middle-aged patients respectively. Mi-RNA 765 influences arterial stiffness through modulating apelin expression, and enhancing MMP-2 and MMP-

9 *(Lee et al., 2017)*.

These results are in agreement with *Sayed et al., 2015* that showed an up-regulation of micro RNA 765 in the middle-aged population of unstable CAD patients. Furthermore, mi-RNA 765 showed the highest diagnostic value with AUC 0.896 and cut-off value 4.4 ng/ ml in T2DM with CAD patients group.

Hence, the current study showed that mi-RNA 765 is the helpful marker for early diagnosis of CAD in T2DM patients. In the present study, FG and HbA1c showed significant elevations in all diabetic groups of patients compared to normal controls. This could be due to defect in carbohydrate, fat and protein metabolism resulting from deficiencies in insulin secretion, insulin action or both *(Kanwar et al., 2015; Kumar et al., 2010)*. These results are in agreement with *Coimbra et al., (2014)* who reported that the lack of insulin or lack of response to insulin results in glucose elevation.

Insulin resistance is important since it is not only the most powerful predictor of future development of T2DM, but it is also a therapeutic target once hyperglycemia is present *(Ghaffari et al., 2016)*.

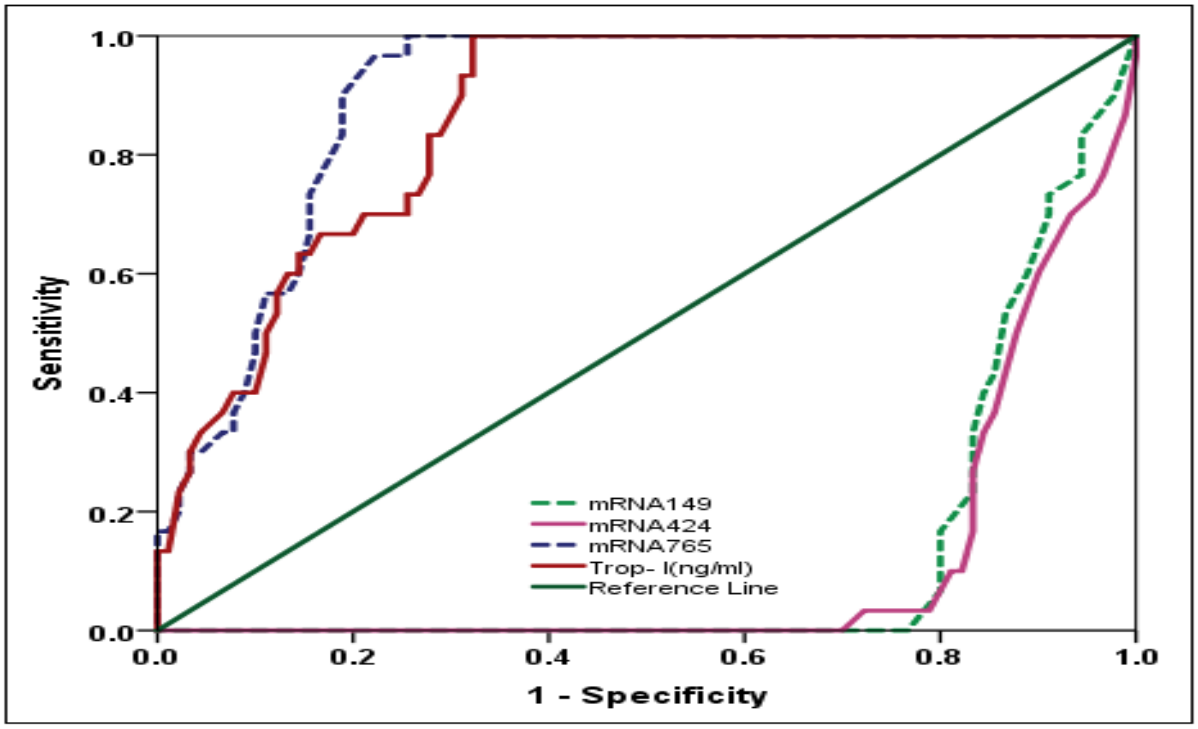

Fig. 2. Receiver operating characteristic (ROC) curves for mi-RNA149, mi-RNA424, mi-RNA765 and Trop-I in T2DM with CAD group.

In this study HOMA-IR levels showed a highly significant elevation in type 2 diabetic patients, while non-significant results were recorded in CAD patients compared to controls. These results confirmed the presence of insulin resistance, and diminished capacity for insulin dependent glucose up take (*Taha et al., 2006)*.

These results are in agreement with *Bonora et al., 2004* whom reported that insulin resistance has been showed to be highly associated with T2DM.

Typical diabetic dyslipidemia which is characterized by low HDL- C, high TAG, TC, LDL- C and VLDL- C was seen in the present study. These results are in agreement with *Al-Jameil et al., 2014*.

The decrease in HDL- C, which normally oppose atherosclerosis directly by inhibiting the oxidation of LDL- C and by limiting the inflammation process, was in parallel with down regulation of mi-RNAs 149 and 424 which inhibiting the inflammatory induced angiogenesis that underlie atherosclerosis.In the

present study, Trop.I levels showed elevated values in CAD patients and T2DM patients with CAD. This finding is consistent with previous study that has also shown elevation of Trop.I in stable coronary artery disease patients (*Eggers et al., 2013*).

Many people with metabolic syndrome have a low-grade inflammation that may place them at risk for the development of cardiovascular diseases (*Wang et al, 2004)*.

A meta-analysis by *Hou et al., 2015* and other studies found associations between IL-6 levels and CAD severity, coronary events, mortality and progression to heart failure *(Su et al., 2013)*.

In this study, IL-6 showed a highly significant increase in all patients groups compared with control group.

These results are in agreement with *Shirai et al., 2016* who showed that patients with pre-existing CAD had higher levels of IL-6 compared to patients without CAD.

This could be explained that endothelial dysfunction, a common finding in CAD, occurs in response to numerous cardiovascular risk factors and precedes the development of atherosclerosis. One of the main regulators of vascular endothelial cell function is nitric oxide (NO), which acts as an important relaxing factor and atheroprotective agent (*Tousoulis et al., 2012*) and inhibits several key steps in atherosclerosis including platelet activation, platelet and leukocyte adhesion, adhesion molecule and chemokine expression, inflammatory cell infiltration, and smooth muscle cell migration and proliferation (*Badimon et al., 2012*).

Therefore, impaired endothelial function caused by reduced NO levels in CAD patients may play a role in the pathogenesis of cardiovascular events.

The results of the present study indicated a reduced level of NO in CAD patients with and without T2DM which is in line with decrease of mi-RNAs 149 and 424 in both CAD patients and CAD patients with T2DM.

Decreased NO levels in atherosclerotic vessels may be caused by oxidative stress in CAD *(Naseem. 2005)*. NO scavenge reactive oxygen species which were generated by oxidative stress, thereby reducing NO bioavailability (*Pacher et al., 2007)*.

Conclusion

In conclusion, our findings suggest that circulating mi-RNA 149, mi-RNA 424 and mi-RNA 765 might be used as non-invasive biomarkers for the diagnosis of CAD in middle-aged Egyptian diabetic patients. Mi-RNA 765 was the most sensitive and specific biomarker.

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