

## Potential Molecular Biomarkers for Prediction of Coronary Artery Disease, its Severity and Extent

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**Received:** 13 April 2017

**Accepted:** 15 May 2017

**Available online:** 1 June 2017

### Keywords

- molecular Biomarkers
- coronary artery disease (CAD)
- plaque burden

### Abstract

Coronary artery disease (CAD) has high morbidity and mortality, therefore accurate detection of CAD and atherosclerotic plaque burden is essential. Invasive coronary angiography (ICA) can confirm the presence of luminal stenosis caused by atherosclerotic plaque and is an important tool for the quantification of CAD plaque burden. However, it has many limitations. Therefore, new methods for accurate noninvasive diagnosis of CAD using genetic markers were exerted. This study aimed at evaluation of new genetic biomarkers in diagnosing CAD and determining its severity and extent, using Gensini score as a scoring model. Sixty two subjects who had undergone coronary angiography and analyzed for the presence, severity and extent of CAD using Gensini score were selected for this study. Twenty subjects had normal Gensini score, 24 subjects were with mild to moderate CAD and 18 subjects were with severe CAD. Extraction of total RNA from whole blood was done together with PCR amplification of PNPLA2, TUBA3, FTL and AHR genes and the house keeping gene GAPDH. It was found that PNPLA2 and TUBA3 were significantly lower in CAD patients and mild/moderate CAD than in controls. However, PNPLA2 was significantly higher in severe CAD than in mild/moderate CAD. Moreover, PNPLA2 gene had The highest specificity for CAD prediction of (100%), followed by TUBA3 (60%) then FTL and AHR (each 40%), and both PNPLA2 and FTL gene had the highest sensitivity for CAD diagnosis (80.95%), followed by TUBA 3 (76.19%), then AHR (71.43%). On the other hand, PNPLA2 showed the highest specificity in CAD severity prediction (83.33%), followed by TUBA3, FTL and AHR with equal specificity (each 50%), and TUBA 3 showed the highest sensitivity (66.67%) followed by PNPLA2., FTL and AHR with equal sensitivity (each 55.56%). In conclusion, these genes are useful in prediction of CAD and CAD severity especially emphasizing PNPLA2 and TUBA3 and further studies on larger population number and other genes are recommended.

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## Introduction

Coronary artery disease (CAD) is a highly important cause of mortality worldwide. WHO reported that deaths from cardiovascular disease (CVD) represented 31% of all global deaths in 2012. Of these deaths, 42.3% were due to CAD [1]. The evaluation and management of patients with suspected CAD has been based on the noninvasive detection of ischemia followed by the use of invasive coronary angiography (ICA) to confirm the presence of luminal stenosis caused by atherosclerotic plaque [2]. Coronary angiography is an important tool for the quantification of CAD plaque burden in clinical practice [3] and it remains the gold standard for the diagnosis of CAD, despite its many limitations. There are multiple angiographic scoring systems used which are strongly correlated with each other and with atherosclerotic plaque burden. One of the most commonly used scoring system is the Gensini score [4]

Efforts for determining blood genetic markers for accurate noninvasive diagnosis of CAD were exerted [5,6,7]. Of the identified genes that their expression in the peripheral blood correlated with the Duke's CAD index [6], ferritin, light polypeptide (FTL), Tubulin $\alpha$  3 (TUBA3), patatin-like phospholipase domain containing 2 (PNPLA2) genes. Their expression changes in peripheral blood not only correlated with the extent of coronary atherosclerosis but also with the pathophysiological changes in atherosclerotic arteries; Aorta [6]. Also, aryl hydrocarbon receptor (AHR) gene expression that was found to correlate with atherosclerosis [8].

PNPLA2, also known as adipose triglyceride lipase (ATGL), calcium-independent phospholipase A2 (iPLA2) and desnutrin, is expressed in most body tissues with the highest mRNA levels and enzyme activity in the white and brown adipose tissues [9]. PNPLA2 selectively performs the initial step in TGs hydrolysis in the cytosolic lipid droplets that are present in almost any type of cell and in various types of inflammatory cells as neutrophils, macrophages, macrophage derived foam cells and mast cells. It is the rate-limiting enzyme for this step, resulting in the formation of diacylglycerol (DG) and free fatty acids (FFAs) [9,10,11]. For maximal lipolytic activity, PNPLA2 requires comparative gene identification-58 (CGI-58) protein as a coactivator [9].

Tubulin  $\alpha$ -1A chain is a subtype of  $\alpha$  tubulins that in humans is encoded by Tubulin  $\alpha$ -1A gene (TUBA1A); also known as TUBA3. This gene is one of three  $\alpha$ -tubulin genes in a cluster on chromosome 12q [12]. In eukaryotes, tubulin proteins superfamily (globular proteins) contain six families of tubulins. Of them are tubulin  $\alpha$  and  $\beta$  families.  $\alpha$ - and  $\beta$ -tubulins polymerize into microtubules. They are the major components of microtubules. Microtubules are filamentous structures present in virtually all eukaryotic cells. They are principal components of the cytoskeleton, of the mitotic spindle, of cilia and flagella, and of neuronal processes. They are involved in numerous cellular functions including maintenance of cell shape, mitosis, cell movement, and intracellular transport [13].

In humans, the FTL gene in chromosome 19 encodes the light subunit of the ferritin protein. Ferritin is the major intracellular iron storage protein. It is composed of 24 protein subunits of ferritin chains. In humans, there are 3 types of ferritin subunits; Heavy (H), Light (L) and glycosylated (G) chains, where tissue ferritin is composed of variable proportions of H and L subunits types and serum ferritin is composed of almost only L and G subunits. The G subunit appears to be derived from posttranslational modification of the L chain. Ferritin synthesis is regulated by cellular iron at a translational, posttranslational [14,15,16] and transcriptional levels (L chain (FTL)). Ferritin synthesis increases in response to iron increase [17]. Also, ferritin gene expression was found to be increased in other cases, including cell proliferation, differentiation, inflammation, and specific hormonal signals. [18].

AHR is constitutively expressed in many adult, mammalian tissues with the highest levels of mRNA detected in liver, kidney, lung and heart. A substantial levels of expressed AHR protein could be detected in the ex vivo human peripheral blood monocytes [19]. AHR is a cytosolic transcription factor, that upon binding to its ligand, translocates into the nucleus and dimerizes with AHR Nuclear Translocator, AHR/AHR nuclear translocator dimers bind to xenobiotic response elements (XREs) in the promoter and enhancer regions of target genes, enhancing their transcription [19,20]. The genes activated by AHR code for drug and xenobiotics metabolizing enzymes (e.g. cytochrome P450 1A1) as well as for proteins involved in regulation of cell growth,

differentiation, apoptosis [20] and inflammation [21].

The aim of this work was to study the changes of these genes in the peripheral whole blood in CAD, and to evaluate the value of these possible genomic markers in diagnosing CAD and determining its severity and extent, using Gensini score as a scoring model. This is the 1<sup>st</sup> study to evaluate these genes in CAD disease in relation to Gensini score.

## MATERIALS AND METHODS

### *Patient selection and grouping:*

Patients were recruited from those who had undergone coronary angiography in the Cardiac Catheterization Unit, Benha university hospital. Coronary angiography was done for assessment of the presence of CAD and evaluating its severity. A patient was considered to have CAD when a stenosed lesion resulting in reduction in lumen diameter by a 50% or greater existed in one of the coronary arteries at least. The total number of stenosed vessels included the number of major stenoses in epicardial arteries (with at least one stenosed lesion leading to  $\geq 50\%$  reduction of lumen diameter) including left main artery (LMA), right coronary artery (RCA), left circumflex artery (LCA) and the left anterior descending artery (LAD). The severity of CAD was evaluated by Gensini score [22]. Narrowing of the lumen was graded by Gensini score as follows: grade 1 (1%–25% occlusion), grade 2 (26%–50% occlusion), grade 4 (51%–75% occlusion), grade 8 (76%–90% occlusion), grade 16 (91%–99% occlusion) and grade 32 (total occlusion). This score was multiplied by a factor reflecting the importance of

the lesion position in the coronary arterial tree as 5 for LM, 2.5 for proximal LAD, and 1 for the proximal RCA. The severity of the disease was expressed as the sum of the scores for individual lesions. Severe CAD was defined as Gensini scores of 20 or more, which was more or less equal to one stenosed lesion of 70% or more in the proximal LAD [4,22].

Therefore, according to Gensini score results, the subjects included in the study were divided into 3 distinct groups; Control group: including 20 subjects with zero Gensini score, Mild/Moderate CAD group: including 24 subjects with Gensini score of less than 20, Severe CAD group: including 18 subjects with Gensini score  $\geq$  20.

Diabetes, myocardial infarction and congestive heart failure were excluded and a written informed consent was taken from all the subjects included in the study and their clinical and laboratory data were collected.

#### ***Sampling and RNA extraction***

Fasting whole blood sample (2 mL) was collected in sterile EDTA- vacutainer tubes before coronary angiography. Extraction of total RNA from the 100  $\mu$ L whole blood was performed using Direct-zol™ RNA MiniPrepkit-Zymosearch-USA according to the manufacturer instructions. Extracted RNA was evaluated for purity and quantity using nanodrop 2000 (Thermoscientific, USA). Then extracted RNA was preserved at -80°C for further processing.

#### ***Reverse transcription***

Extracted RNA was reverse transcribed using high-capacity cDNA reverse transcription kit, Thermo Fisher Scientific, USA. Two hundred ng

of extracted RNA, completed to 10  $\mu$ L by Nuclease- free H<sub>2</sub>O were added to 10  $\mu$ L of 2X RT master mix. The 2X RT master mix contained 2  $\mu$ L 10X RT Buffer, 0.8  $\mu$ L 25X dNTP Mix (100 mM), 2  $\mu$ L 10X RT Random Primers, 1  $\mu$ L MultiScribe™ Reverse Transcriptase, 1  $\mu$ L RNase Inhibitor 3.2  $\mu$ L Nuclease-free H<sub>2</sub>O. The thermal cycler ; Biorad, USA, program was 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes and 4°C for  $\infty$ . The cDNA product was diluted in 100  $\mu$ L Nuclease-free H<sub>2</sub>O.

#### ***Real time PCR***

Amplification of PNPLA2, TUBA3, FTL and AHR genes and the house keeping gene GAPDH were done in separate PCR tubes using gene specific primers [8,23,24,25] (Table 1). QuantiTect® SYBR® Green PCR kit supplied by Qiagen, Germany was used for amplification. The reaction mix contained 10  $\mu$ L of 2x QuantiTect SYBR Green PCR Master Mix, 5  $\mu$ L of cDNA, 0.5  $\mu$ L of the forward primer, 0.5  $\mu$ L of the reverse primer and 4  $\mu$ L of Nuclease-free H<sub>2</sub>O. The thermal profile of the PCR reaction was 95°C for 15 min., 40 cycles of 94°C for 15 sec, annealing temperature of the gene for 30 sec and 72°C for 30 sec. Melting curve analysis was performed to ensure specificity.

Calculation of the fold changes of gene expression with uncertainty was done using the web-based software of Qiagen Data Analysis Center. This software calculates the average fold change ( $2^{-\Delta\Delta ct}$ ) as the normalized gene expression ( $2^{-\Delta ct}$ ) in the test sample divided the normalized gene expression ( $2^{-\Delta ct}$ ) in the control sample. Fold regulation represents fold-change results in a biologically meaningful way. Fold change values

greater than one indicate a positive- or an up-regulation, and the fold-regulation value is equal to the fold-change. Fold change values less than one indicate a negative or down-regulation, and the fold regulation value is the negative inverse of the fold change.

Fold change uncertainty was calculated by the software of Qiagen Data Analysis Center according to the following equation:

Fold change uncertainty =  $\sigma_{FC} = FC * \ln 2 * \sqrt{(\sigma_x^2/n_x + \sigma_y^2/n_y)}$ . Where,

FC: Fold change.

$\sigma_x$ : standard deviation of Delta (Ct) of test group.

$\sigma_y$ : standard deviation of Delta (Ct) of control group or the calibrator group used.

$n_x$ : number of test group.

$n_y$ : number of control group or the calibrator group.

Relative expression of the target genes included, were calculated as  $2^{-\Delta Ct}$ .  $\Delta Ct$  was calculated by subtracting the Ct of the GAPDH from that of the target gene. Lower  $\Delta Ct$  values and higher  $2^{-\Delta Ct}$  indicated higher expression level of the target gene.

#### **Statistical analysis:**

The study data were analyzed using SPSS version 18. Quantitative variables were expressed as mean  $\pm$ SD or median, IQR and range. Qualitative variables were expressed as number and percentage. Comparison of qualitative data was done using chi-square ( $\chi^2$ ) and Fisher's exact tests. However, comparison of quantitative data was performed using ANOVA with Bonferroni

post hoc test, Mann-Whitney or kruskerwallis tests wherever appropriate. Spearman correlations were done for correlating different variables. ROC curve was used to predict the best cutoff values of  $2^{-\Delta Ct}$  of the different genes with the optimum sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for prediction of CAD and for prediction of the severity of CAD.. P value <0.05 was considered significant.

## **Results**

### **Clinical and demographic data**

Demographic, medical and laboratory data of the study subjects are presented in table 2. Body mass index was significantly higher in the mild/moderate CAD compared to the control and severe CAD. Fasting blood glucose (FBG) was significantly higher in patients with severe CAD compared to the control and mild/moderate CAD. Triglycerides (TGs) level was significantly higher in CAD patients compared to control. LDL-cholesterol levels were significantly lower in the CAD group compared to the control and in the severe CAD compared to mild/moderate CAD, and HDL-cholesterol levels were significantly higher in the CAD group compared to the control, probably reflecting a higher use of statins [26].

### **Gensini score results and correlation analysis of the Gensini score and demographic data**

Gensini score results in the different studied groups are presented in table 3. Gensini score showed positive significant correlation with HDL-cholesterol ( $\rho = 0.46$ ,  $p < 0.001$ ) and negative significant correlation with LDL-cholesterol ( $\rho = -0.37$ ,  $p = 0.003$ ), probably because of the higher use of statins. Moreover, Gensini score was

**Table (1):** Primers and annealing temperature of the genes included in the study

Gene	Forward primer	Reverse primer	Annealing temperature	Reference
PNPLA2	5'-GTGTCAGACGGCGAGAATG-3'	5'-TGGAGGGAGGGAGGGATG-3'	55	[10]
FTL	5'-GCTACGAGCGTCTCCTGAAG-3'	5'-GCTTGAGAGTGAGCCITTCG-3'	53	[11]
TUBA3	5'-TACCGCCAGCTCTCCAC-3'	5'-GAGTGCTCCAGGGTGGTG-3'	56	[11]
AhR	5'-ATTGTGCCGAGTCCCATATC-3'	5'-AAGCAGGCGTGCATTAGACT-3'	53	[8]
GAPDH	5'-TGCACCACCAACTGCTTAGC-3'	5'-GGCATGGACTGTGGTCATGAG-3'	56	[12]

**Table 2:** Demographic and clinical data of the study subjects

Variable	Control N=20	Patients N =42			Significance	
		Mild/moderate CAD N =24	Severe CAD N =18	All patients Number N=42		
<b>Qualitative variables</b>	Number [%]				P value	
<b>Sex</b>					0.7	
Female	4 [20%]	8 [33.3%]	6 [33.3%]	14 [33.3%]		
Male	16 [80%]	16 [66.7%]	12 [66.7%]	28 [66.7%]		
Smoking	7 [35%]	8 [33.3%]	10 [55.6%]	18 [42.9%]	0.5	
Hypertension	8 [40%]	16 [66.7%]	10 [55.6%]	26 [61.9%]	0.3	
Statins use	6 [30%]	13 [54.2%]	16 [88.9%]	29 [69%]	<ul style="list-style-type: none"> <li>• p<sub>1</sub>= 0.003</li> <li>• p<sub>2</sub>= 0.1</li> <li>• p<sub>3</sub>&lt;0.001</li> <li>• p<sub>4</sub>=0.02</li> </ul>	
Fibrate use	1 [5%]	2 [8.3%]	2 [11.1%]	4 [9.5%]	0.9	
<b>Quantitative variables</b>	Mean ± SD				P value	Post Hoc test (Significant relations)
Age [Years]	57.4±9.5	56.7±5.5	57.4±7.5	57.1±6.6	0.98	-
Body mass index [kg/m <sup>2</sup> ]	28.4±2.9	37.3±13.2	27.2±4.3	31.2±10	0.002	p <sub>2</sub> , p <sub>4</sub>
Fasting blood glucose [mg/dl]	95.6±10.6	97.2±8.03	114.1±23.8	104.4±18.5	0.002	p <sub>3</sub> ,p <sub>4</sub>
Triglycerides [mg/dl]	121±42.5	150.3±34	164.3±56.3	158.7±47.7	0.008	p <sub>1</sub> , p <sub>2</sub> , p <sub>3</sub>
Cholesterol [mg/dl]	221.8±20.4	214±40.3	194±33.3	202±36.3	0.06	-
HDL-cholesterol [mg/dl]	35.6±4	36.8±3.6	42.9±6.3	40.5±6.1	< 0.001	p <sub>1</sub> , p <sub>3</sub> ,p <sub>4</sub>
LDL-cholesterol [mg/dl]	148.8±26.8	128.7±32.2	118.2±23.2	122.5±26.6	0.003	p <sub>1</sub> , p <sub>3</sub>

p<sub>1</sub>; CAD patients versus controls, p<sub>2</sub>; Mild/moderate CAD versus controls, p<sub>3</sub>; Severe CAD versus controls, p<sub>4</sub>; Severe CAD versus mild/moderate CAD.

**Table 3:** Gensini score results in the different studied groups

Gensini score	Control N=20	Patients N =42			P value
		Mild/moderate CAD N =24	Severe CAD N =18	All patients Number N=42	
<b>Gensini score</b>					
• Median	0	8.5	32	12	<0.001
• IQR	0	10	19.9	20.3	
• Range	0	[2-18]	[20-122]	[2-122]	

P<0.05 is significant

significantly higher in males than females ( $p=0.03$ ) and significantly higher in smoker than non-smoker patients ( $P=0.009$ ). On the other hand non-significant associations or correlations with the other demographic data were demonstrated.

#### ***Gene expression analysis results***

The expression of 4 genes was analyzed including, PNPLA2, TUBA3, FTL and AHR genes. The fold change of the studied genes expression in mild/moderate CAD, severe CAD and all CAD patients compared to the control is presented in figure 1. Moreover, the fold change of the studied genes expression in severe CAD patients compared to mild/moderate CAD patients is presented in figure 2.

#### ***Analysis of PNPLA2 gene expression results***

CAD patients showed significant fold down-regulation of 1.68 compared to the controls (0.6 fold change,  $P=0.007$ ). However, its expression in the severe CAD patients showed non-significant fold down-regulation of 1.86 compared to the controls (0.54 fold change,  $P=0.2$ ), which is more than that of mild/moderate CAD patients. Overall, significant 1.76 fold down-regulation in the CAD patients compared to the controls (0.57 fold change,  $P=0.02$ ). Moreover, there was a non-significant 1.13 fold down-regulation of TUBA3 gene expression in severe CAD patients group compared to mild/moderate CAD patients (0.89 fold change,  $P=0.2$ ).

#### ***Analysis of FTL gene expression results***

FTL gene expression in the mild/moderate CAD patients showed non-significant fold down-regulation of 1.17 compared to the controls (0.86

PNPLA2 gene expression in the mild/moderate CAD patients showed significant fold down-regulation of 2.72 compared to the controls (0.37 fold change,  $P<0.001$ ). However, there was non-significant 1.81 fold down-regulation of PNPLA2 gene expression in the severe CAD patients compared to the controls (0.55 fold change,  $P=0.4$ ), which is less than that of mild/moderate CAD patients. Overall, significant 2.28 fold down-regulation in the CAD patients compared to the controls (0.44 fold change,  $P=0.006$ ). In addition, there was a significant 1.52 fold up-regulation of PNPLA2 gene expression in the *severe CAD patients* group compared to mild/moderate CAD patients ( $P=0.02$ ).

#### ***Analysis of TUBA3 gene expression results***

TUBA3 gene expression in the mild/moderate fold change,  $P=0.7$ ) and non-significant 1.3 and 1.02 fold up-regulation in the severe CAD patients and all CAD patients respectively compared to the controls ( $P=0.2$  and  $P=0.5$  respectively). Moreover, FTL gene expression in the severe CAD patients showed non-significant fold up-regulation of 1.53 compared to the mild/moderate CAD patients ( $P=0.2$ ).

#### ***Analysis of AHR gene expression results***

AHR gene expression in the mild/moderate CAD patients, severe CAD patients, all CAD patients showed non-significant fold up-regulation of 1.2, 1.56, 1.35 respectively compared to the controls ( $P=0.1$ ). Moreover, AHR gene expression in the severe CAD patients showed non-significant fold up-regulation of 1.37 compared to the mild/moderate CAD patients ( $P=0.3$ ).

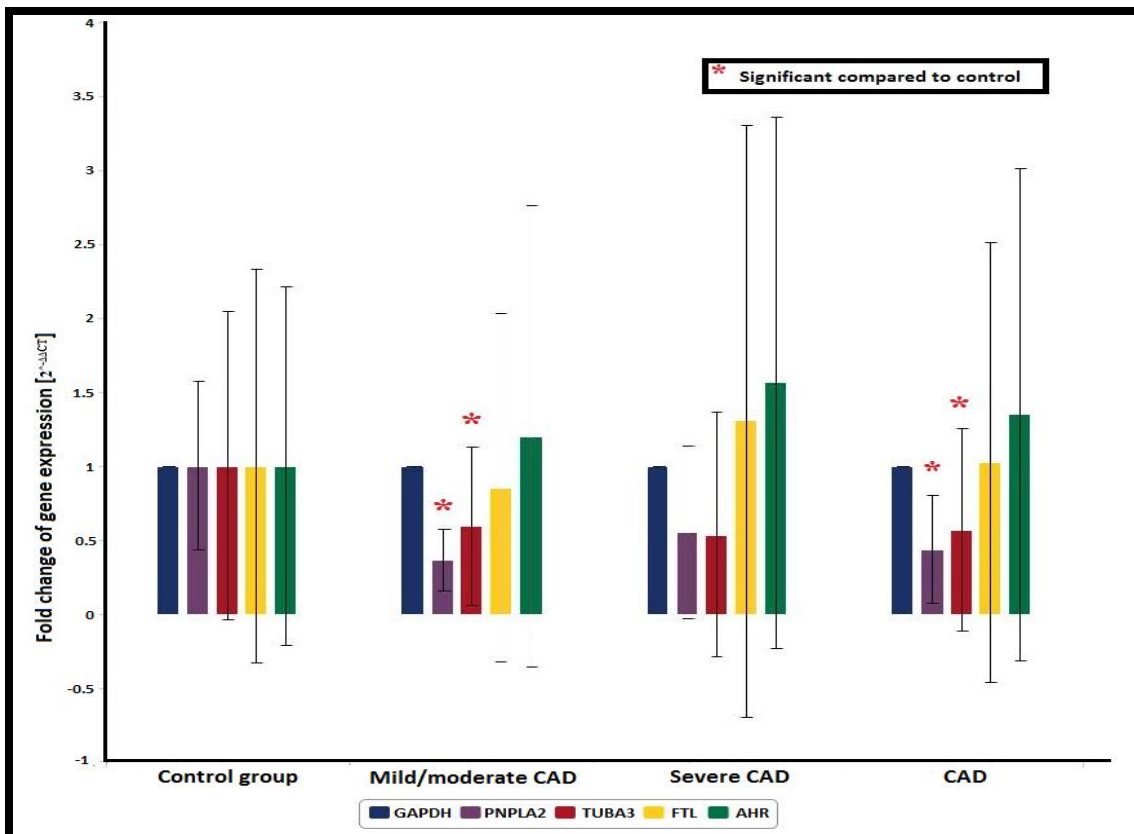


Figure 1: Fold change ( $2^{-\Delta\Delta CT}$ ) with uncertainty of PNPLA2, TUBA3, FTL, and AHR genes expression in mild/moderate CAD, severe CAD and all CAD patients compared to the control.

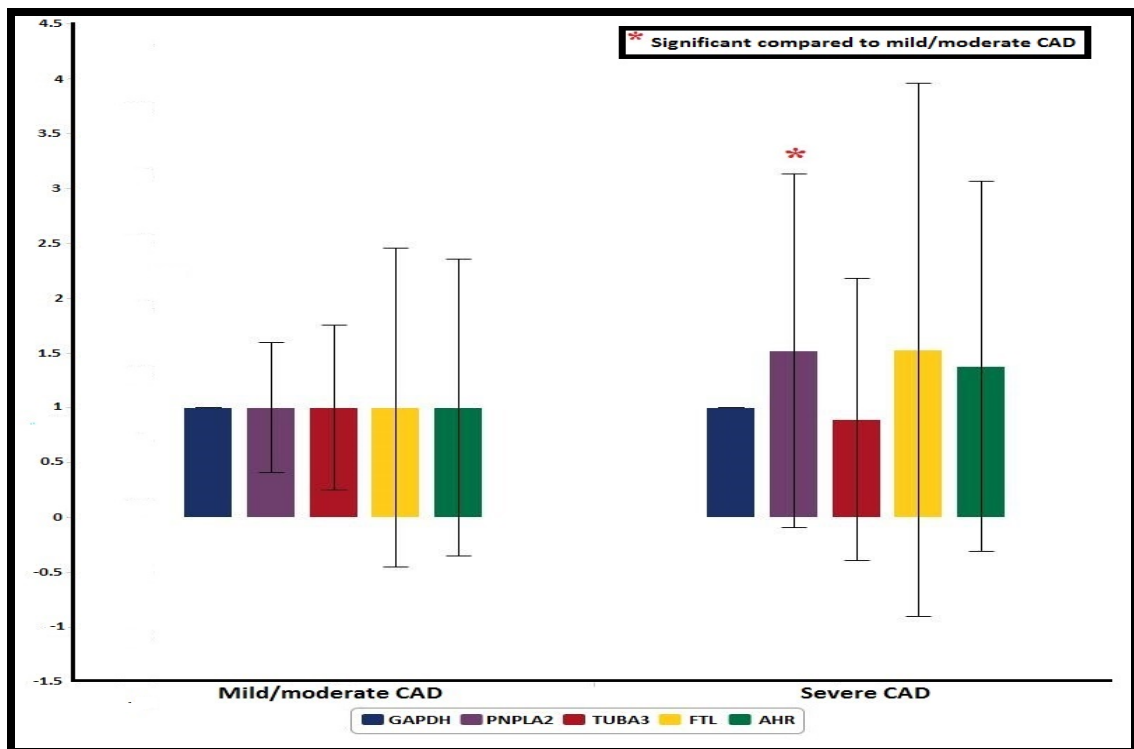


Figure 2: Fold change ( $2^{-\Delta\Delta CT}$ ) with uncertainty of PNPLA2, TUBA3, FTL, and AHR genes expression in mild/moderate CAD compared to severe CAD patients.



**Table 4:** Correlation of expression levels of the studied genes with controls' and patients' quantitative demographic data

Groups	Data	PNPLA2		TUBA3		FTL		AHR	
		rho	P value	rho	P value	rho	P value	rho	P value
Control group	Age	0.3	NS	-0.5	0.025	-0.4	NS	0.7	0.001
	BMI	0.2	NS	0.4	NS	0.1	NS	-0.2	NS
	FBG	-0.6	0.005	0.3	NS	0.2	NS	0.5	0.025
	TGs	-0.8	P<0.001	-0.4	NS	0.1	NS	-0.3	NS
	Cholesterol	-0.3	NS	0.1	NS	0.6	0.005	-0.7	0.001
	HDL- cholesterol	-0.25	NS	-0.2	NS	0.2	NS	-0.4	NS
	LDL cholesterol	0.0	NS	0.3	NS	-0.2	NS	-0.1	NS
Patients group	Age	0.09	NS	-0.42	0.005	0.2	NS	0.2	NS
	BMI	-0.2	NS	-0.2	NS	-0.2	NS	-0.3	NS
	FBG	-0.53	P<0.001	0.2	NS	-0.2	NS	0.68	P<0.001
	TGs	0.3	NS	-0.08	NS	-0.05	NS	-0.2	NS
	Cholesterol	-0.4	NS	-0.52	P<0.001	0.02	NS	-0.25	NS
	HDL- cholesterol	-0.3	NS	0.1	NS	0.43	0.004	0.08	NS
	LDL- cholesterol	-0.3	NS	-0.63	P<0.001	-0.29	NS	-0.04	NS
	Gensini score	0.3	0.05	0.08	NS	0.03	NS	0.09	NS

NS: non-significant, p<0.05 is significant

**Table (5):** Sensitivity, specificity, PPV and NPV of PNPLA2, TUBA3, FTL and AHR gene expression for the prediction of CAD.

	PNPLA2	TUBA3	FTL	AHR
Cut off value of ( $2^{-\Delta ct}$ )	0.63	0.38	20.39	0.048
Sensitivity	80.95%	76.19%	80.95%	71.43%
Specificity	100.0%	60.0%	40.0%	40.0%
PPV	71.4%	44.4%	72.7%	70.0%
NPV	100.0%	72.7%	44.4%	36.4%
Correctly classified	87.1	70.97%	67.74%	61.29%
AUC	0.8857	0.6476	0.4667	0.5714

**Table (6):** Sensitivity, specificity, PPV and NPV of PNPLA2, TUBA3, FTL and AHR gene expression for the prediction of CAD severity.

	PNPLA2	TUBA3	FTL	AHR
Cut off value of ( $2^{-\Delta ct}$ )	0.58	0.18	26.17	0.055
Sensitivity	55.56%	66.67%	55.56%	55.56%
Specificity	83.33%	50.0%	50.0%	50.0%
PPV	71.4%	50.0%	40.0%	45.5%
NPV	71.4%	66.7%	54.5%	60.0%
Correctly classified	71.43%	57.14%	52.38%	52.38%
AUC	0.657	0.537	0.491	0.519

### *Correlation of gene expression data with demographic data*

The expression levels of the 4 genes indicated by  $2^{-\Delta Ct}$  were correlated with the quantitative demographic data as demonstrated in table 4.

PNPLA2 gene expression showed a positive significant correlation with Gensini score in the patients group. On the other hand it showed a negative significant correlation with FBG in both the control and CAD groups and TGs in the control group. TUBA3 gene expression showed a negative significant correlation with age in both the patients and control groups, and with cholesterol and LDL-cholesterol in the patients group.

FTL gene expression showed a positive significant correlation with the blood cholesterol level in the control group and with HDL-cholesterol in the patients group.

In addition, AHR gene expression showed a positive significant correlation with age in the control group and with FBG in both the control and patients groups. However, it showed a negative significant correlation with blood cholesterol level in the control group.

Moreover, the study of the association of the expression of the four genes with the qualitative demographic data revealed that AHR was significantly higher in smokers and hypertensive patients in both the patients and control groups, otherwise, all remaining associations were non-significant.

### *Gene expression data as predictor for CAD and severity of CAD*

The best cut off values for  $2^{-\Delta Ct}$  of the expressed genes with the highest specificity, sensitivity, PPV and NPV for diagnosis of CAD ( table 5) and its severity (table 6) were determined and analyzed as shown in the tables.

As regards CAD diagnosis, the study revealed that PNPLA2 gene expression had the highest specificity for diagnosis of CAD (100%), followed by TUBA3 (60%) then FTL and AHR (each 40%). Moreover, both PNPLA2 and FTL gene expression values showed the highest sensitivity for the diagnosis of CAD (80.95%), followed by TUBA 3 (76.19%), then AHR (71.43%). On the other hand, as regards CAD severity prediction, PNPLA2 showed the highest specificity (83.33%), followed by TUBA3, FTL and AHR with equal specificity (each 50%), and TUBA 3 showed the highest sensitivity (66.67%) followed by PNPLA2, FTL and AHR with equal sensitivity (each 55.56%). Moreover, the best PPV and NPP for diagnosis of CAD or its severity were that of PNPLA2.

### **Discussion**

Atherosclerosis is the most frequent underlying cause of CAD. It is a chronic inflammatory disease of the vessel wall which eventually leads to blood vessels obstruction [27,28]. Understanding the process of atherogenesis is crucial for understanding the gene expression changes in CAD disease. In atherosclerosis, Plasma molecules and lipoprotein particles extravasate through the leaky and defective endothelium into the subendothelial space, where potentially atherogenic lipoproteins are retained and modified (e.g., oxidized) and become cytotoxic, proinflammatory, chemotactic, and proatherogenic [27]. This leads to activation of the endothelium with the increase in the expression

of adhesion molecules and chemoattractants that recruit monocytes and T-lymphocytes, and monocytes differentiate into macrophages in the subendothelium [27,28]. These macrophages promote plaque development by secreting many inflammatory mediators as cytokines and chemokines, which sustain the inflammatory milieu, recruit leukocytes to the vessel wall and regulate immune functions [29]. So, plaques are formed, which are characterized by lipid droplets accumulation in foam cells derived from the macrophages and the smooth muscle cells (SMCs), SMCs proliferation and matrix proteins accumulation in the extracellular spaces, leading to intima thickening [28].

In this study we evaluated the expression of 4 genes in the peripheral blood of CAD patients. The first gene evaluated in this study was PNPLA2. Our study demonstrated a significant decline of PNPLA2 gene expression in the CAD patients compared to the control subjects. Many studies are required to evaluate the pathophysiological mechanisms of decrease of PNPLA2 gene expression in the blood of CAD patients, since in the pathogenesis of atherosclerosis, which is a complex disease, many cell types and many cytokines and growth factors are involved [28]. Moreover, this study revealed significant increase in PNPLA2 gene expression in the severe CAD group compared to the mild/moderate CAD group. This coincides with current study' detected positive significant correlation of the PNPLA2 gene expression with Gensini score in the patients group. This means that there are pathological events related to the progression of disease that led to this increase. To our knowledge, no previous

studies reported changes of Blood PNPLA2 in CAD disease, with only Sinnaeve et al. (2009) reporting significant correlation of the whole blood PNPLA2 gene expression with Duke CAD-Index without mentioning the changes in the gene expression [6].

Different studies argued about whether PNPLA2 is atherogenesis promoting or protector. Peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ), a direct transcriptional activator of PNPLA2 expression, was reported to increase in expression in the macrophage foam cells, endothelial cells and SMCs of atherosclerotic lesions. It was found to be atheroprotective by its anti-inflammatory effects [30,31,32,33] suggesting that PNPLA2 is anti-atherogenic.

Decrease of PNPLA2 gene expression leads to reduction of macrophage phagocytic function [9], as it is important for ATP synthesis essential for maximal phagocytic activity of macrophages [9,10].

Reduction of the macrophage phagocytic function results in reduction of the scavenging of *modified lipoproteins* by macrophages, leading to the accumulation of extracellular oxidized LDL (oxLDL), that produces apoptotic and /or necrotic cell death by its cytotoxic effect. Also, it competes with apoptotic cells for macrophage binding, leading to defective clearance of apoptotic cells in atherosclerosis [34]. In addition, ox LDL induces the activation of the endothelial cells which express many pro-inflammatory molecules as adhesion molecules, resulting in monocytes recruitment to the vessel wall [34,35].

In addition, reduction of macrophage phagocytic activity results in defective clearance of the

*apoptotic cells* by the macrophages, that induce proinflammatory cytokines production by macrophages and a reduced release of anti-inflammatory proteins, thus contributing to a persistent state of chronic inflammation [35].

All of the above mentioned effects due to decrease of the phagocytic function of macrophages support that decrease PNPLA2 may mediate the initiation and progression of atherosclerosis. However, other cells are involved in the pathogenesis of atherosclerosis as mast cells that are in a continuous, systemic increase in their activation during plaque development leads to increased plaque progression. Upon mast cell activation, gene expression of CGL-85, the co-activating factor of PNPLA2 increases, leading to increase PNPLA2 gene expression, which by hydrolysis of TGs in the mast cell releases the arachidonic acid needed for the synthesis of eicosanoids including PGD2 [11,36,37]. PGD2 may play a role in the evolution of atherosclerosis. [12,38]. This support that PNPLA2 may be atherogenic. However, Different eicosanoids derived from arachidonic acid can either alleviate or worsen the inflammatory responses and influence the magnitude, nature and duration of the immune responses. Even the same eicosanoid produced may in different situations promote or help resolve the inflammation [39,40].

Our study detected a significant negative correlation of PNPLA2 gene expression with FBG in the control and CAD groups. It was previously reported that ATGL mRNA concentrations increase during fasting and decrease during refeeding. Glucocorticoids could be responsible for the increase of ATGL mRNA levels in the fasted state. Insulin, the main inhibitor of lipolysis, reduces

ATGL mRNA. In defective insulin signaling, increase lipolysis and increased ATGL mRNA level occur, thus induction of ATGL gene expression might contribute to elevated FA mobilization, providing fatty acids for oxidation [41].

Our study also reported a significant negative correlation of PNPLA2 gene expression with serum TGs in the control group. Since PNPLA2 performs the first step of TGs hydrolysis, it is logically to find such a correlation. Yang et al. (2014) reported that fasting serum PNPLA2 level was negatively correlated with serum TGs [38].

The 2<sup>nd</sup> gene evaluated in this study was TUBA3. Our study reported a significant decrease of the whole blood TUBA3 gene expression in the CAD group compared to the control and non-significant decrease in the severe CAD compared to the mild/moderate CAD group. Moreover, our study reported non-significant positive correlation of whole blood TUBA3 gene expression with the Gensini score with rho value of 0.08. However, a significant correlation of the whole blood TUBA 3 gene expression with Duke CAD-Index (rho value of 0.28), with similar pathophysiological changes in atherosclerotic aorta were previously reported [6].

It was found that PPAR- $\gamma$  is a transcription factor that can bind TUBA 3 promoter [12]. TUBA 3 gene expression was found to be increased in the absence of PPAR- $\gamma$  in mouse endothelial cells [31] implicating that PPAR- $\gamma$  is an inhibitor of TUBA3 gene expression. Since previous studies documented the expression of PPAR- $\gamma$  in endothelial cells of atherosclerotic lesions and the endothelial PPAR- $\gamma$  was predicted to play a protective role in atherosclerosis [31], this may suggest that the

decrease of whole blood TUBA3 gene expression in the CAD group may reflect its atheroprotective role. However, further studies have to be done before ensuring that.

We revealed that whole blood TUBA3 gene expression decreases significantly with increasing age in both control and patients group. Also, we found that whole blood TUBA3 gene expression was negatively correlated with total cholesterol and LDL-cholesterol levels in the patients group. To our knowledge, there were no studies reporting these correlations.

The 3<sup>rd</sup> gene evaluated in this study was the FTL gene. Studies on humans revealed that increased serum ferritin, which reflects the level of total body iron stores, was found to be associated with increased risk of carotid atherosclerosis [18] and acute myocardial infarction [42]. Pang et al. (1996) reported higher expression of L-ferritin and H-ferritin mRNA (10 folds) and protein in human atherosclerotic aorta. L-ferritin and H-ferritin mRNA were induced mainly in the macrophages and in some endothelial cells of human early atherosclerotic lesions. While their induction occurred mainly in the smooth muscle cells of advanced atherosclerotic lesions. Iron deposits were present only in advanced lesions but not in early lesions indicating that the upregulation by iron was not the primary cause of induction of ferritin synthesis in the early lesions [17].

You et al. (2003) reported that FTL protein expression was significantly increased in the diseased coronary arteries compared to the normal coronaries. However he also reported that expression of FTL mRNA was significantly decreased in diseased coronary arteries, suggesting

that increased expression of FTL protein in the CAD coronary arteries may be related to increased protein stability or upregulation of expression at the posttranscriptional level in the diseased tissues. They speculated that increased expression of the FTL may contribute to pathogenesis of CAD by modulating oxidation of lipids within the vessel wall through the generation of reactive oxygen species. This supports the association between excessive iron storage and a high risk of CAD [43]. It was reported that small amounts of iron are an absolute requirement for LDL oxidation by cultured cells, suggesting iron involvement in LDL oxidation which is a pathological event in atherosclerosis [17].

In contrast, our study demonstrated non significant increase of blood FTL gene expression in the CAD patients and the severe CAD patients compared to the controls. However, non significant decrease was demonstrated in the mild/moderate CAD patients compared to the controls. Also, the FTL gene expression was non significantly higher in the severe CAD patients compared to the mild/moderate CAD patients.

Sinnaeve et al. (2009) reported significant correlation of the whole blood FTL gene expression with Duke CAD-Index with rho value of 0.24 [6]. In contrast, our study reported non significant positive correlation of the whole blood FTL gene expression with the Gensini score with rho value of 0.03.

Our study demonstrated a positive significant correlation of FTL gene expression with blood cholesterol level in the control group. However, such significant correlation was not demonstrated in the CAD patients group, probably because of the significantly higher use of cholesterol-lowering

agents, statins, in the CAD group, which may also explain the positive significant correlation of FTL gene expression with the HDL-cholesterol in the patients group. Pang et al. (1996) demonstrated that both L- and H-ferritin mRNAs were markedly increased in aortas of rabbits after feeding with a high cholesterol diet for 6 wk, which was also the time period after which the formation of atherosclerotic lesions became evident [39]. A Korean study demonstrated a positive association between total cholesterol level and serum ferritin levels in male adolescents and a significant negative correlation between HDL-cholesterol and serum ferritin levels in both genders [44].

The 4<sup>th</sup> gene evaluated in the current study was AHR. AHR mediates mainly the toxic effects of many environmental pollutants, including halogenated aromatic hydrocarbons (HAHs), such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polycyclic aromatic hydrocarbons (PAHs), such as benzo(*a*)pyrene (BaP) [45,46]. Activation of AHR by its ligands dioxin or benzo[*a*]pyrene (BaP), a constituent of cigarette smoke, has been demonstrated to promote atherosclerosis via induction of vascular inflammation via activation of proinflammatory genes in the macrophages and release of inflammatory mediators [29,47]

Inflammation is known to accelerate vessel dysfunction and aging [20]. Vascular inflammation leads to upregulation of pro-inflammatory genes in macrophages and blood vessels. Thus, high level AHR expression or its activation in the vessels could contribute to endothelial dysfunction. In addition, activation of AHR or increase its expression were reported to decrease the activity of endothelial nitric oxide synthase (eNOS)

, by increasing its phosphorylation, and decrease nitric oxide (NO) produced by this enzyme [47]. Endothelium-derived NO regulates vessel tone and produces vasodilatation and anti-atherosclerosis effects, which counteract the vascular actions of endogenous vasoconstrictor substances [48]. Therefore, decreased NO production can contribute to vascular stiffness and atherosclerosis. Several *ex vivo* and *in vivo* studies reported that expression and/or activity of the eNOS are reduced with increasing age and in disease and that decreased phosphorylation of eNOS contributes to age-related vascular stiffness. Furthermore, AHR activation was found to result in suppression of human endothelial cell migration, similar to what is observed in the aged vasculature

[29]. Also, there was positive correlation between AHR expression in blood cells and vessel stiffness in healthy human subjects indicative of vascular aging [29]. All these coincide with our study detected significant increase of AHR expression with age as was also reported by Kim et al. (2005) [49]. Also, they coincide with the revealed increase of AHR gene expression in mild/moderate CAD and further increase in severe CAD characterized by more atherosclerotic changes and also with the positive correlation of AHR gene expression with Gensini score, although being non-significant as these non-significant results may be attributed to low number of cases. Huang et al. (2015) reported that peripheral blood AHR mRNA level was increased significantly by 2.197-folds in CAD patients compared with the controls [8].

It is worth noting that in the absence of exogenous ligands, AHR signaling is subject to regulation by either endogenous ligands or nonligand activators as

modified LDL [46]. OxLDL have been identified as AHR agonists. OxLDL and saturated fatty acids contained in a Western diet activate AHR and contribute to obesity and inflammation in C57Bl/6 J mice [50]. Thus activation of AHR gene occur by OxLDL in the atherosclerosis lesion, leading to vascular inflammation and promoting atherosclerosis.

Our study demonstrated that whole blood AHR gene expression significantly negatively correlated with serum cholesterol level. It was reported that AHR activation results in repression of genes involved in cholesterol biosynthesis in the liver [21].

Our results revealed a positive correlation between AHR expression levels and FBG. In support to this finding, Korecka et al. (2016) observed that AHR<sup>-/-</sup> mice exhibited lower fasting glucose levels and improved glucose tolerance, compared with AHR<sup>+/+</sup> mice indicating partial protection against diet-induced glucose intolerance in AHR<sup>-/-</sup> mice [51]. In addition, serum TCDD level was found to be positively associated ( $P < 0.01$ ) with FBG [52].

This study demonstrated that AHR gene expression was significantly higher in smokers than nonsmokers. Tobacco smoke is a remarkable source of polycyclic aromatic hydrocarbons that trigger the AHR signaling pathway, leading to several pathological effects in humans through AHR-dependent changes in gene expression [53].

Moreover, AHR gene expression was significantly higher in hypertensives than in non-hypertensives in this study. The AHR agonist, TCDD, has been demonstrated to induce high blood pressure in both epidemiology studies and research using mouse

models, in which AHR mediated cytochrome P450 overexpression, may be involved [46]. Moreover, as already said, that activation of AHR by its ligands dioxin or BaP, a constituent of cigarette smoke, has been demonstrated to promote atherosclerosis [29,47]. However, in the absence of exogenous ligands, AHR deficient mice were demonstrated to develop hypertension, most probably due to decreasing cardiac function [54] and elevated circulating Angiotensin II and plasma endothelin-1 (ET-1) levels [55].

To evaluate the 4 studied genes as possible biomarkers for prediction of CAD and its severity, the best cut off values for  $2^{-\Delta\Delta Ct}$  of the expressed genes with the highest specificity, PPV and NPV for diagnosis of CAD and its severity were determined. As regards CAD diagnosis or CAD severity prediction, PNPLA2 gene expression had the best PPV and NPV suggesting its value as a potential biomarker for prediction of CAD and its severity. However, TUBA 3 showed a less but important value in prediction of CAD severity. To our knowledge, there was no previous study that reported the performance characteristics of the studied genes in the prediction of CAD &/or its severity.

In conclusion, whole blood PNPLA2 showed the best value in prediction of CAD and/or its severity. However, the value of TUBA3, FTL and AHR in CAD prediction and CAD severity prediction can not be ignored, and further study on larger population number is recommended. In addition, a larger genetic studies involving evaluation of more genes should be done, in order to avoid exposing the possible CAD patients to invasive coronary angiography.

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