



## Pathogenic gene expression of epicardial adipose tissue in patients with coronary artery disease

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**Background & objectives:** Coronary artery disease (CAD), a leading cause of mortality and morbidity worldwide has multifactorial origin. Epicardial adipose tissue (EAT) has complex mechanical and thermogenic functions and paracrine actions *via* various cytokines released by it, which can have both pro- and anti-inflammatory actions on myocardium and adjacent coronaries. The alteration of EAT gene expression in CAD is speculated, but poorly understood. This study was undertaken to find out the difference in gene expression of epicardial fat in CAD and non-CAD patients.

**Methods:** Twenty seven patients undergoing coronary artery bypass graft (CABG) and 16 controls (non-CAD patients undergoing valvular heart surgeries) were included in the study and their EAT samples were obtained. Gene expressions of uncoupling protein-1, monocyte chemoattractant protein-1 (*MCP-1*), adiponectin, adenosine A1 receptor (*ADORA-1*), vascular cell adhesion molecule-1 (*VCAM-1*) and tumour necrosis factor-alpha (*TNF-α*) were studied by real-time reverse transcription-polymerase chain reaction. Glucose, insulin, lipid profile, high-sensitivity C-reactive protein, homocysteine, vitamin D, *TNF-α* and leptin levels were estimated in fasting blood samples and analyzed.

**Results:** Leptin levels were significantly higher in CABG group as compared to controls ( $P<0.05$ ), whereas other metabolic parameters were not significantly different between the two groups. *MCP-1*, *VCAM-1* and *TNF-α* were upregulated in the CABG group as compared to controls. Further, multivariate analysis showed significantly reduced adjusted odds ratio for *MCP-1* [0.27; 95% confidence interval: 0.08-0.91] in the CABG group as compared to controls ( $P<0.05$ ).

**Interpretation & conclusions:** Our findings showed an alteration in EAT gene expression in CAD patients with significant upregulation of *MCP-1*. Further studies with a large sample need to be done to confirm these findings.

**Key words** Adiponectin - CAD - EAT - inflammatory biomarkers - MCP-1 - paracrine

Epicardial adipose tissue (EAT) is a visceral fat depot located around heart with no fascia separating it from underlying myocardium<sup>1</sup>. EAT is vascularized by the branches of the coronary arteries. The complex physiological functioning of human epicardial fat is not completely elucidated, but is distinguished by mechanical, metabolic and endocrine/paracrine and thermogenic functions<sup>2</sup>.

Mechanically, it protects coronaries against distortion and releases free fatty acids, which are utilized by myocardium as an energy source. It acts as a sink for fatty acids in circulation, protecting heart from lipotoxicity. The expression of uncoupling protein-1 (UCP-1) and related proteins is high in EAT, indicating its role in thermogenesis in humans<sup>1-4</sup>. It is a source of multiple bioactive cytokines such as leptin, adiponectin, resistin, plasminogen activator inhibitor-1, apelin, tumour necrosis factor-alpha (TNF- $\alpha$ ), interleukin-6 and monocyte chemoattractant protein-1 (MCP-1), which are involved in the regulation of endothelial function, coagulation and inflammation through paracrine and endocrine actions<sup>5</sup>. Growing evidence points to the role of epicardial fat involvement in the development of coronary artery disease (CAD)<sup>6</sup>. Owing to its anatomical and functional contiguity with myocardium and coronaries, epicardial fat can secrete a large number of pro-inflammatory and suppressed anti-inflammatory cytokines under pathological conditions<sup>7</sup>. The relative expression (RE) of pro- and anti-inflammatory genes by EAT may be a determinant in the development of CAD<sup>8</sup>.

CAD risk in Indians is reported to be significantly higher in the general population than in Caucasians<sup>9</sup>. Asian Indians have more total, subcutaneous and visceral fat for similar body mass index (BMI) and age, compared with Caucasians<sup>10</sup>. The amount of visceral fat in Indians is more for each BMI level when compared with Caucasians and they fit into a term described as 'metabolically obese' normal-weight individuals<sup>11</sup>.

Plasma inflammatory biomarkers may not adequately reflect this local tissue inflammation<sup>12</sup>. It is postulated that in the adipose tissue, hypoxia leads to increased expression of inflammatory genes and decreased expression of adiponectin<sup>13</sup>. Treatment with beta-blockers, aspirin, angiotensin converting enzyme (ACE) inhibitors and Ca<sup>2+</sup> channel blockers does not affect the adipocytokine gene expression in the EAT<sup>13</sup>. This study was thus undertaken with the aim to find out pathological functioning of epicardial fat in patients

with CAD and the difference in gene expression of EAT in CAD and non-CAD patients.

### Material & Methods

Twenty seven consecutive patients meeting inclusion-exclusion criteria undergoing elective coronary artery bypass graft (CABG), at Dr. K.G. Deshpande Memorial Centre, Nagpur, Maharashtra, India, and willing to participate were recruited in the study during February 2016 to October 2017). Written informed consent was obtained from all participants (both cases and controls) before carrying out any study-related procedure. Patients with renal and hepatic insufficiency and those with psychiatric disorders and pregnancy were excluded. Proper history was taken and thorough physical examination was done. The data of echocardiography and angiography were recorded for each patient. Complete drug history and family history were also noted. Their fasting mixed venous blood sample (10 ml) was collected. The investigations performed were fasting glucose (FBS), fasting insulin (in non-diabetic patients), lipid profile, leptin, TNF- $\alpha$ , haemoglobin A<sub>1c</sub> (HbA<sub>1c</sub>), high-sensitivity C-reactive protein (hsCRP), homocysteine and vitamin D levels. To compare the epicardial fat expression of CAD patients with those without CAD, EAT and fasting blood samples (10 ml) were collected from non-CAD patients (confirmed by angiography) undergoing valvular surgeries (n=16). The study protocol was approved by the Institutional Ethics Committee.

*RNA extraction and gene expression:* The human epicardial fat tissue (0.5-1 g) biopsy was obtained during CABG. The sample was immediately frozen at -70°C till RNA was extracted. Total RNA was isolated using RNeasy Lipid Tissue Mini Kit (Qiagen, Germany) as per the manufacturer's instructions; 1  $\mu$ g of total RNA was first converted to cDNA (complementary DNA) using High-Capacity Reverse Transcription kit (Applied Biosystems, USA). Resulting cDNA samples were analyzed for the expression of pro-inflammatory and anti-inflammatory chemokine genes by real-time reverse transcription-polymerase chain reaction (qRT-PCR). Relative quantification of these genes was performed on Applied Biosystems 7300 real-time PCR using EXPRESS SYBR GreenER qPCR SuperMix.

The threshold cycle (C<sub>t</sub>) values were obtained for target as well as reference genes from both CABG and control samples. Hypoxanthine

**Table I.** Forward and reverse primer sequences for selected genes

Genes	Primer	Sequence	Reference
<i>ADIN</i>	Forward	TATGATGGCTCCACTGGTA	14
	Reverse	GAGCATAGCCTTGTCTTCT	
<i>UCP-1</i>	Forward	TGCCCAACTGTGCAATGAA	15
	Reverse	TCGCAAGAAGGAAGGTACCAA	
<i>MCP-1</i>	Forward	TCGCGAGCTATAGAAGAATCA	16
	Reverse	TGTTCAAGTCTTCGGAGTTTG	
<i>VCAM-1</i>	Forward	CCCTTGACCGGCTGGAGATT	17
	Reverse	CTGGGGGCAACATTGACATAAAGTG	
<i>TNF-<math>\alpha</math></i>	Forward	CCTCTCTCTAATCAGCCCTCTG	18
	Reverse	GAGGACCTGGGAGTAGATGAG	
<i>ADORA-1</i>	Forward	GTCCTCATCCTCACCCAGAG	19
	Reverse	CAGATTGTTCCAGCCAAACA	
<i>HPRT</i>	Forward	ACGAAGTGTGGATATAAGC	20
	Reverse	ATAATTTTACTGGCGATGTC	

*ADIN*, adiponectin; *UCP-1*, uncoupling protein-1; *MCP-1*, monocyte chemoattractant protein-1; *VCAM-1*, vascular cell adhesion molecule-1; *TNF- $\alpha$* , tumour necrosis factor-alpha; *ADORA-1*, adenosine A1 receptor; *HPRT*, hypoxanthine phosphoribosyl transferase

phosphoribosyltransferase (*HPRT*) was used as a reference gene. The target gene set was as follows: adiponectin, *UCP-1*, *MCP-1*, vascular cell adhesion molecule-1 (*VCAM-1*), adenosine receptor A1 (*ADORA-1*) and *TNF- $\alpha$*  (Table I). The  $C_t$  values for each gene type in each group were used to determine the relative expression of that gene. The primers were synthesized by Sigma-Aldrich, USA.

The result was expressed as  $\Delta C_t$  i.e., fold change in the expression of the target gene relative to reference gene. The  $\Delta C_t$  values from control and patients' samples were then compared to obtain the  $\Delta\Delta C_t$  value, which is a reliable indicator of the difference in gene expression between control and experimental samples.

**Statistical analysis:** Data on parameters such as age, gender, BMI, behavioural habits and biochemical parameters were obtained for patients undergoing CABG as well as other valvular surgeries, referred to as control group in this study. Descriptive statistics such as mean, standard deviation, frequencies and percentages were obtained. Variables measured on numeric scale were compared between the two groups using *t* test for independent samples, whereas categorical variables were compared using Pearson's Chi-square test.

The  $C_t$  values were obtained for target and reference genes for each patient from CABG and control groups and expressed in terms of mean and

standard deviation. The expression of target gene was normalized to reference gene expression level to obtain  $\Delta C_t$  values:  $\Delta C_t = C_t$  (target gene) -  $C_t$  (reference gene).

Further,  $\Delta\Delta C_t$  value was obtained for each target gene as  $\Delta\Delta C_t = \Delta C_t$  (CABG sample) -  $\Delta C_t$  (control sample). Finally, the fold change, also known as relative expression in qRT-PCR, of target gene expression in CABG sample in comparison with control sample, after normalizing with reference gene was obtained as  $2^{-\Delta\Delta C_t}$ , considering a uniform PCR amplification efficiency of 100 per cent across all samples. The significance of difference in the mean normalized expression levels of target genes between the two groups was determined using *t* test of independent samples and the *P* values were adjusted for multiple testing correction using Benjamin and Hochberg (BH) method<sup>21</sup>. The multivariate logistic regression analysis was performed with groups as dependent variable and demographic and biochemical parameters as independent variables. Hosmer-Lemeshow test<sup>22</sup> was used to decide the goodness of fit of the model. All the analyses were performed in R-3.2.1 programming tool (R Core Team, Vienna, Austria).

## Results

The data on demographic and personal characteristics, metabolic parameters and marker

**Table II.** Descriptive statistics of patient characteristics in the two groups

Characteristics	Levels	CABG (n=27)	Control (n=16)	P
Age (yr)	-	61.30±9.18	52.19±9.93	<0.01 <sup>†</sup>
Gender, n (%)	Male	20 (74.1)	10 (62.5)	0.649*
	Female	7 (25.9)	6 (37.5)	
BMI (kg/m <sup>2</sup> ), mean±SD	-	24.58±4.06	23.02±2.71	0.14 <sup>†</sup>
Smoker, n (%)	Yes	11 (40.7)	5 (31.3)	0.41*
	No	16 (59.26)	11 (68.8)	
HTN, n (%)	Yes	27 (100.0)	6 (37.5)	<0.001*
	No	0	10 (62.5)	
Diabetes, n (%)	Yes	14 (51.9)	6 (37.5)	0.55*
	No	13 (48.2)	10 (62.5)	
Dyslipidaemia, n (%)	Yes	26 (96.3)	7 (43.8)	<0.001*
	No	1 (3.7)	9 (56.3)	
MAP (mm Hg), mean±SD	-	102.03±9.12	86.71±10.76	<0.001 <sup>†</sup>
Platelet count (×10 <sup>5</sup> /μl), mean±SD	-	2.66±0.63	2.45±0.80	0.36 <sup>†</sup>
Homocysteine (μmol/l)	-	21.96±11.83	22.09±8.31	0.96 <sup>†</sup>
hsCRP (nmol/l)	-	50.48±45.90	53.05±33.90	0.83 <sup>†</sup>
Vitamin D (nmol/l)	-	43.32±28.62	59.80±40.27	0.16 <sup>†</sup>
CKMB (μg/l)	-	21.25±14.41	17.64±6.72	0.27 <sup>†</sup>
FBS (mmol/l)	-	6.18±1.59	5.54±1.25	0.15 <sup>†</sup>
Serum insulin (mIU/l), mean±SD <sup>‡</sup>	-	29.36±20.92	20.34±14.95	0.26 <sup>†</sup>
Homostatic model assessment of insulin resistance (HOMA-IR), mean±SD	-	6.77±5.63	4.42±3.23	0.24 <sup>†</sup>
Glycosylated haemoglobin, HbA <sub>1c</sub> (%), mean±SD <sup>‡</sup>	-	7.31±1.76	6.97±2.56	0.64 <sup>†</sup>
TNF-α (pg/ml)	-	17.42±34.12	19.14±20.72	0.84 <sup>†</sup>
Leptin (μg/l)	-	3.88±4.66	1.43±1.16	<0.05 <sup>†</sup>
Non-alcoholic steatohepatitis, n (%)	Yes	13 (48.2)	3 (18.8)	0.10*
	No	14 (51.9)	13 (81.3)	

\*Using Chi-square test; <sup>†</sup>using independent t test; <sup>‡</sup>only of diabetic patients (n=14) in cases and (n=6) in controls  
 BMI, body mass index; SD, standard deviation; hsCRP, high-sensitivity C-reactive protein; MAP, mean arterial pressure;  
 HTN, hypertension; CKMB, creatinine kinase muscle/brain; FBS, fasting blood sugar; CABG, coronary artery bypass graft

expression were obtained on 27 CABG patients and 16 controls. Table II provides the descriptive statistics for parameters and their comparison between the groups. The mean age of patients in CABG group was significantly higher than that of control group ( $P<0.01$ ). Gender bias, mean BMI and smoking habit pattern were not significantly different between the two groups. All the patients from CABG group were hypertensive unlike control group, showing significant difference ( $P<0.001$ ). Dyslipidaemia was also predominantly observed in CABG patients as compared to controls ( $P<0.001$ ). Mean arterial pressure (MAP) was significantly higher in CABG group than control group ( $P<0.001$ ). Other biochemical and haematological

parameters differed insignificantly between the two groups. Among markers of interest, the mean leptin level was significantly higher in the CABG group as compared to the control group ( $P<0.05$ ).

The relative expression or fold change of six target genes was obtained based on  $C_t$  values as given in Table III. It was evident that RE for *MCP-1* was 2.7132 indicating upregulation of this gene in CABG patients as compared to controls. This was followed by upregulation of *VCAM-1*, *TNF-α* and *UCP-1*. Adiponectin showed downregulation in CABG patients. The Figure shows the mean  $\Delta C_t$  values for these genes in CABG group through column chart.

**Table III.** Descriptive statistics for gene expression and relative expression of target genes

Genes	Control (n=16)	CABG (n=27)	Control-HPRT	CABG patients-HPRT	$\Delta C_t^{(Ref)}$	$\Delta C_t^{(target)}$	$\Delta\Delta C_t$	RE
<i>TNF-<math>\alpha</math></i>	28.70 $\pm$ 1.97	28.25 $\pm$ 1.62	30.11 $\pm$ 0.57	30.02 $\pm$ 0.79	0.09	0.45	-0.36	1.2834
<i>UCP-1</i>	29.40 $\pm$ 1.70	29.00 $\pm$ 1.67	30.11 $\pm$ 0.57	30.02 $\pm$ 0.79	0.09	0.40	-0.31	1.2397
<i>MCP-1</i>	21.82 $\pm$ 1.60	20.29 $\pm$ 1.29	30.11 $\pm$ 0.57	30.02 $\pm$ 0.79	0.09	1.53	-1.44	2.7132
<i>VCAM-1</i>	24.87 $\pm$ 4.94	24.16 $\pm$ 4.66	30.11 $\pm$ 0.57	30.02 $\pm$ 0.79	0.09	0.71	-0.62	1.5369
<i>ADORA 1</i>	30.55 $\pm$ 2.32	30.27 $\pm$ 1.53	30.11 $\pm$ 0.57	30.02 $\pm$ 0.79	0.09	0.28	-0.19	1.1408
<i>ADIN</i>	22.37 $\pm$ 1.36	22.66 $\pm$ 1.15	30.11 $\pm$ 0.57	30.02 $\pm$ 0.79	0.09	-0.29	0.38	0.7684

Values given as mean $\pm$ SD. RE, relative expression

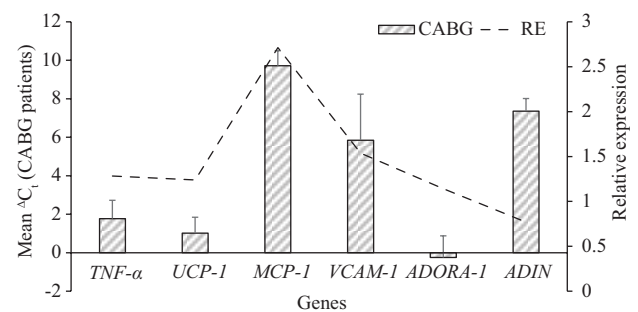
**Table IV.** Risk of coronary artery disease associated with different factors

Factors	OR	95% confidence interval (CI)		<i>P</i> *
		Lower	Upper	
Age (yr)	1.16	0.96	1.41	0.11
Gender (reference:female)	5.71	0.19	173.8	0.32
BMI (kg/m <sup>2</sup> )	1.29	0.86	1.96	0.22
Dyslipidaemia (reference:no)	12.32	0.05	312.2	0.37
hsCRP (nmol/l)	0.94	0.68	1.29	0.70
Glycosylated haemoglobin (%)	1.43	0.68	3.03	0.35
Homocysteine	0.98	0.85	1.14	0.85
CKMB ( $\mu$ g/l)	0.99	0.89	1.10	0.91
TNF- $\alpha$ (pg/ml)	0.98	0.93	1.03	0.42
Leptin ( $\mu$ g/l)	1.92	0.61	6.09	0.27
MCP-1	0.27	0.08	0.91	<0.05

\*Obtained using multivariate logistic regression. OR, odds ratio

The difference between the means of CABG and control groups for *MCP-1* was significant ( $P<0.05$ ) after BH correction. The Figure also shows the relative expression for different genes. *MCP-1* and *VCAM-1* were focused in the downstream functional analysis, although the difference for later was not significant.

Further, the risk of CAD associated with metabolic parameters of interest after adjusting for covariates was obtained (Table IV). The odds ratio associated with homocysteine, creatinine kinase muscle/brain (CKMB) and TNF- $\alpha$  was close to 1.0, indicating marginal effect of unit increase in these parameters on CAD. Increase in the leptin levels increased the risk of CAD, although not significant. The unit increase in  $C_t$  values for *MCP-1* decreased the odds of CAD significantly ( $P<0.05$ ). In other words, decrease in  $C_t$  values of *MCP-1* (upregulation of *MCP-1*) increased the risk of CAD.



**Figure.** Showing mean ( $\pm$ SD) of  $\Delta C_t$  values for target genes in coronary artery bypass graft group (n=27) and the relative expression. *TNF- $\alpha$* , tumour necrosis factor-alpha; *UCP-1*, uncoupling protein-1; *MCP-1*, monocyte chemoattractant protein-1; *VCAM-1*, vascular cell adhesion molecule-1; *ADORA-1*, adenosine A1 receptor; *ADIN*, adiponectin.

## Discussion

The key findings of the study were upregulation and higher expression of pro-inflammatory chemokines *MCP-1*, *TNF- $\alpha$*  and *VCAM-1* in EAT of patients



with CAD as compared to controls. The expression of anti-inflammatory chemokines, adiponectin, *ADORA-1* and *UCP-1* which are cardioprotective, was downregulated in CAD patients as compared to controls. However, upregulation of *MCP-1* expression was observed after adjusting for all confounders. The plasma levels of inflammatory biomarkers such as hsCRP, homocysteine, insufficient vitamin D levels and TNF- $\alpha$  were comparable in both groups. This indicates that EAT may have a larger role to play in the development of CAD. Earlier we have reported that epicardial fat mass correlates positively with diastolic dysfunction<sup>1</sup>. Higher levels of *MCP-1*, TNF- $\alpha$  and some other chemokines have been reported in different studies<sup>7,13,23</sup>. Lower levels of adiponectin in the EAT in CABG patients as compared with non-CAD patients have also been shown<sup>24,25</sup>.

*MCP-1* initiates macrophage infiltration of adipose tissue, a hallmark of many studies which have reported presence of inflammatory state of EAT in CAD patients<sup>7,13,22,26-28</sup>. Hirata *et al*<sup>26</sup> have reported the presence of greater number of M1 macrophages (inflammatory) as compared to M2 (inactive) in epicardial fat in CAD patients. The fact that atherosclerotic lesions develop in those parts of coronary arteries, which are surrounded completely by epicardial fat and the amount of fat and macrophage infiltration correlate with atherosclerotic plaque size and composition, further emphasizes the inflammatory role of EAT<sup>29</sup>. Expression of *MCP-1* by EAT is high as compared with subcutaneous adipose tissue (SAT) and omental fat<sup>26,30</sup>. The parts of coronaries, which are free of atherosclerotic lesions are also free of adipose tissue<sup>31</sup>.

The secretion of inflammatory chemokines such as TNF- $\alpha$  and *MCP-1* induces inflammatory cell influx into arterial wall, coronary vasospasm affecting arterial homeostasis, inducing plaque instability and apoptosis<sup>13,32</sup>. Whole genome analysis of EAT from 29 CAD and 15 non-CAD (undergoing valvular surgeries) patients through microarrays has shown complex overactivation of inflammatory cascades in EAT of CAD patients, whereas negative modulators of inflammation were found to be downregulated in them at transcriptional level<sup>33</sup>. Various pro-inflammatory adipokines released from the EAT increase the expression of *VCAM-1*, which mediates vascular endothelial inflammation *via* oxidative stress-dependent NF $\kappa$ B activation<sup>34</sup>. In our

study, we found upregulation of *VCAM-1* gene from EAT in CAD patients but significant upregulation was seen with *MCP-1*.

The finding that inflammatory markers are overexpressed in EAT after adjusting for all confounders including hypertension, diabetes, dyslipidaemia, age, gender and BMI signifies the independent role played by EAT in the development of CAD. The expression of adipokines correlates well with the EAT thickness. EAT can be a potential therapeutic target. Drugs such as sodium glucose transporter 2 (SGLT-2) inhibitors reduce EAT thickness. Epicardial fat decreases after very low calorie diet, aerobic exercise and even after bariatric surgery-induced weight loss<sup>35-37</sup>.

Our study had certain limitations. The sample size for the study was modest. This was a single-centre study and there were constraints in getting age- and sex-matched controls as the mean age of valvular heart disease without CAD was low.

In conclusion, the upregulation of expression of inflammatory markers such as *MCP-1*, TNF- $\alpha$  and *VCAM-1* with downregulation of protective molecules such as adiponectin and *ADORA-1* remained independent after adjusting for various confounders, indicating a pathological functioning of EAT in the development of CAD. *MCP-1* expression was significantly higher in CAD patients as compared with non-CAD patients.

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**Conflicts of Interest:** None.

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