

## Original Research

# Early Cardiac Gene Transcript Levels in Peripheral Blood Mononuclear Cells Reflect Severity in Stable Coronary Artery Disease

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**Introduction:** The early cardiac marker genes *myocardin*, *GATA4* and *Nkx2.5*, play a role in both embryonic cardiovascular development and adult cardiovascular disease. We evaluated transcript levels of *myocardin*, *GATA4* and *Nkx2.5* in peripheral blood mononuclear cells (PBMCs) in patients with stable coronary artery disease (CAD) and we examined the relationship between these levels and the severity of the disease, estimated by the number of stenotic vessels involved.

**Methods:** Ninety-eight patients with stable CAD (age  $66 \pm 9$  years) who underwent coronary angiography participated in the study; 66 healthy individuals (age  $58 \pm 13$  years) were also included for comparison. Gene transcript levels were determined by quantitative real-time reverse transcription polymerase chain reaction.

**Results:** Patients with 3-vessel CAD had elevated transcript levels of *myocardin* (median difference 2.7,  $p=0.001$ , 95% confidence interval, CI: 1-5.8), *GATA4* (median difference 0.3,  $p=0.015$ , 95% CI: 0.1-1.9) and *Nkx2.5* (median difference 16.1,  $p<0.001$ , 95% CI: 4.5-23) compared to healthy controls. Patients with 3-vessel CAD also showed elevated transcript levels of *myocardin* (median difference 2.3,  $p=0.001$ , 95% CI: 0.49-5.5) and *Nkx2.5* (median difference 11.8,  $p<0.001$ , 95% CI: 1.5-21.5) compared to patients with 1-vessel CAD.

**Conclusions:** Early cardiac marker gene transcript levels are significantly higher in the PBMCs of patients with severe stable CAD than in those of healthy controls, and show alterations in their expression profile according to the disease severity status. Our results indicate for the first time that changes in the early cardiac gene expression in the peripheral blood of stable CAD patients, possibly as a result of alterations in circulating cardiovascular progenitor cells that express these genes, may reflect the level of disease severity.

**C**oronary artery disease (CAD) is one of the major health issues worldwide and is a leading cause of death in Western countries.<sup>1</sup> Despite considerable advances in prevention and treatment, cardiovascular mortality and morbidity remain high in CAD. The need for the development of new strategies for the accurate non-invasive diagnosis of CAD and the identification of novel treat-

ment targets becomes more urgent as the ageing population, with its greater incidence of cardiovascular disease risk factors, continues to grow.<sup>2</sup> The identification of novel biomarkers and investigation techniques that could lead to more accurate diagnosis and monitoring of the disease process and response to treatment becomes more important in the context of more closely focused intervention thera-

pies.<sup>3,4</sup> However, atherosclerosis is a complex multifactorial disease process involving multiple pathways; thus, deeper insights into the pathophysiological pathways leading to atherosclerotic vascular disease can improve our understanding and help in the development of more individualised risk stratification and targeted therapy.

At the cellular level, vascular progenitor cells (endothelial and smooth muscle progenitor cells) are involved in the complex pathophysiology of atherosclerosis, vascular regeneration, arterial remodelling and angiogenesis.<sup>5-8</sup> It has been shown that the level of circulating progenitor cells predicts the occurrence of cardiovascular events and death from cardiovascular causes.<sup>9</sup> In addition, alterations in the function of vascular smooth muscle cells (VSMCs) seem to play an important role in cardiovascular disease.<sup>10,11</sup> The proliferation and migration of VSMCs are a key component in the stenotic process of an injured artery and play an important role in arterial remodelling processes.<sup>12</sup> Myocardin is a transcription factor that functions as a “master regulator” of SMC differentiation during development and is the primary target of signalling pathways leading to remodelling of VSMCs in cardiovascular diseases.<sup>13-17</sup> *GATA4* has been shown to play a role in stress compensation and cardiomyocyte viability,<sup>18</sup> as well as in angiogenesis, helping to maintain the balance between hypertrophy and capillary density.<sup>19</sup> Circulating very small embryonic-like cells expressing the early cardiogenic markers *GATA4* and *Nkx2.5* have also been implicated in cardiovascular disease.<sup>20</sup> *Myocardin*, *GATA4* and *Nkx2.5* are also part of the “foetal gene program”, which is reactivated in the adult stressed heart as part of an adaptation process.<sup>21-23</sup>

In the present study, we evaluated gene expression levels of selected developmental transcription factors in peripheral blood mononuclear cells of patients with stable CAD in relation to the disease severity. We selected the early myocardial marker genes *myocardin*, *GATA4* and *Nkx2.5*, encoding developmental transcription factors that participate in embryonic heart development and seem to play a role in several cardiovascular conditions in adults.<sup>21,24</sup>

## Methods

### Study population

The study population consisted of 98 patients with stable CAD (mean age  $66 \pm 9$  years, 80 men and 18

women) who underwent coronary angiography for suspected CAD in our department. Sixty-six healthy volunteers (mean age  $58 \pm 13$  years, 44 men and 22 women) were also enrolled in the study.

All recruited subjects underwent a full clinical examination and basic laboratory tests. Eligible patients included men and women 18 years or older, with evidence of coronary heart disease in the form of a previous documented myocardial infarction (>3 months before screening), percutaneous or surgical coronary revascularization (>6 months before screening), or angiographic evidence of  $\geq 70\%$  narrowing of at least one major coronary artery (right, left anterior descending, or left circumflex coronary artery). The symptom complex remained stable for at least 3 months before recruitment. The severity of coronary artery stenoses was evaluated by more than 2 experienced interventional cardiologists, who were blinded to the early cardiac gene transcript data, using quantitative coronary angiography. All patients had typical chest pain with ST-segment depression  $\geq 1$  mm during an exercise stress test, or a reversible perfusion defect on exercise thallium<sup>201</sup> myocardial perfusion scintigraphic study. Patients with evidence of an acute coronary syndrome or cardiogenic shock <3 months previously, as well as those with a left ventricular ejection fraction <55% or age >75 years, were excluded. Other exclusion criteria were: significant valvular disease; myocarditis and history or signs of neoplastic or haematological disease; heart, renal or hepatic failure; and any chronic inflammatory or other infectious disease during the last 6 months. Any cardiovascular medication had been unchanged for 3 months prior to the study in all patients.

The study was approved by the hospital’s ethics committee, all institutional guidelines were followed and all participants gave written informed consent.

### RNA isolation and quantitative RT-PCR

Blood samples were collected into EDTA (ethylenediaminetetraacetic acid) collection tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by Histopaque-ficoll (SIGMA) centrifugation, total RNA was isolated using the TRI-Reagent (Ambion) and 1  $\mu$ g RNA was reverse-transcribed with oligo-(dT) using the Reverse Transcription System (Promega) in 20  $\mu$ L reactions. Measurements of mRNA levels were performed by real-time reverse transcription-polymerase chain reaction (RT-PCR) using the STRATAGENE Mx3000P Detection Sys-

tem. PCR assays were performed in 1  $\mu$ L of cDNA template using the SYBR Green PCR Master Mix (Bio-Rad). All sample procedures were performed in triplicate. The standard curve method was used for absolute quantification of the amplification products and specificity was determined by performing a melting curve analysis. Standard curves for the expression of each gene were generated by serial dilution of known quantities of cDNA template. The housekeeping gene *GAPDH* (*glyceraldehyde-3-phosphate-dehydrogenase*) was used as an endogenous reference gene, and relative quantification was done by normalising the signals of the different genes with respect to the *GAPDH* signal. Primer sequences for *myocardin*, *GATA4*, *Nkx2.5* and *GAPDH*, as well as the experimental design strategy to achieve specificity, were as previously described.<sup>25</sup>

### Statistical analysis

For baseline characteristics of patients, continuous variables are summarised as mean values (SD), and discrete variables as counts and proportions. The original distributions of gene expression data were extremely positively skewed. Even the square root and cubic root transformations were still skewed and heteroskedastic (unequal variances), not permitting parametric analysis. Therefore non-parametric tests were performed. We used cubic root transformation of original data for better depiction. The Jonckheere–Terpstra test was used to explore differences among groups. *Post-hoc* Bonferroni-adjusted Mann–Whitney pairwise tests were used for 2-group comparisons.

Data are presented as median differences and 95% Hodges–Lehman confidence intervals (CI) for median differences. All tests were 2-sided and the level of  $p < 0.05$  was considered to indicate statistical significance. Statistical analysis was performed using a commercially available statistical package (IBM SPSS Statistics 19.0; Chicago, IL, USA).

### Results

The expression levels of the early myocardial marker genes *myocardin*, *GATA4* and *Nkx2.5* were studied in PBMCs of patients with stable CAD ( $n=98$ ) and of healthy controls ( $n=66$ ) for comparison. In CAD patients, transcript levels of these genes were examined in relation to the severity of the disease. Baseline data from our patients are presented in Table 1.

When we examined gene transcript levels in healthy controls and CAD patients in relation to the disease severity estimated by the number of stenotic vessels involved, the Jonckheere–Terpstra test showed significantly elevated *myocardin* ( $p=0.001$ ), *GATA4* ( $p=0.005$ ) and *Nkx2.5* ( $p < 0.001$ ) levels as the number of stenotic vessels involved increased (Table 2). *Post-hoc* Bonferroni-adjusted Mann–Whitney pairwise tests revealed significant differences between 3-vessel CAD patients and healthy controls.

More specifically, we found that patients with 3-vessel CAD ( $n=22$ ) had elevated transcript levels of *myocardin* (median difference 2.7,  $p=0.001$ , 95% CI: 1.5–5.8), *GATA4* (median difference 0.3,  $p=0.015$ , 95% CI: 0.1–1.9) and *Nkx2.5* (median difference 16.1,  $p < 0.001$ , 95% CI: 4.5–23) compared to healthy con-

**Table 1.** Baseline characteristics of patients ( $n=98$ ).

Age (y)	66 $\pm$ 9	Creatinine (mg/dL)	1.1 $\pm$ 0.7
Men / women	80 / 18	Total cholesterol (mg/dL)	220.9 $\pm$ 46.1
Smoking	44 (43.1%)	Glucose (mg/dL)	130.5 $\pm$ 55.9
Diabetes mellitus	32 (31.4%)	Haematocrit	40.3 $\pm$ 4.2
Hypertension	55 (53.9%)	Haemoglobin (g/dL)	13.2 $\pm$ 1.4
Hyperlipidaemia	62 (60.8%)	Left ventricular ejection fraction (%)	54.5 $\pm$ 11
Family history	31 (30.4%)	Medication	
Medical history of STEMI	21 (20.6%)	Aspirin	98 (100%)
Medical history of non-STEMI	14 (13.7%)	Clopidogrel	98 (100%)
Coronary artery affected:		Statins	81 (79.4%)
Left anterior descending artery	67 (65.7%)	Beta-blockers	55 (53.9%)
Right coronary artery	49 (50%)	ACE inhibitors	42 (41.2%)
Circumflex	36 (35.3%)	Angiotensin receptor inhibitors	26 (25.5%)
1-vessel disease	44 (43.1%)	Amlodipine	13 (12.7%)
2-vessel disease	32 (31.4%)	Nitrate	14 (13.7%)
3-vessel disease	22 (21.6%)	Diuretics	15 (14.7%)

STEMI, ST elevation myocardial infarction; ACE, angiotensin-converting enzyme.

trols (n=66) (Figure 1). Significantly elevated *Nkx2.5* levels (median difference 7.6, p=0.001, 95% CI: 2.6-11.4) were also observed in patients with 2-vessel CAD compared to healthy controls (Figure 1C).

When we examined gene transcript levels in CAD patients in relation to the disease severity estimated by the number of stenotic vessels involved, the Jonckheere–Terpstra test showed significantly elevated *myocardin* (p=0.022) and *Nkx2.5* (p=0.010) levels as the number of the stenotic vessels involved increased (Table 2). *GATA4* levels also increased, but just failed to reach significance (p=0.068). *Post-hoc* Bonferroni-adjusted Mann–Whitney pairwise tests did not detect any difference between 1-vessel and 2-vessel disease levels in any of the parameters, whereas they revealed significant differences between 1-vessel and 3-vessel disease levels in both *myocardin* and *Nkx2.5*, with *myocardin* also differing between 2- and 3-vessel disease.

More specifically, we found that patients with 3-vessel CAD (n=22) had elevated transcript levels of *myocardin* (median difference 2.3, p=0.001, 95% CI: 0.49-5.5) and *Nkx2.5* (median difference 11.8, p<0.001, 95% CI: 1.5-21.5) compared to patients with 1-vessel CAD (n=44) (Figure 1A, C). *Myocardin* also showed a trend towards a difference (median difference 2.3, p=0.087, 95% CI: 0-5.5) between 2- and 3-vessel patient groups (Figure 1A).

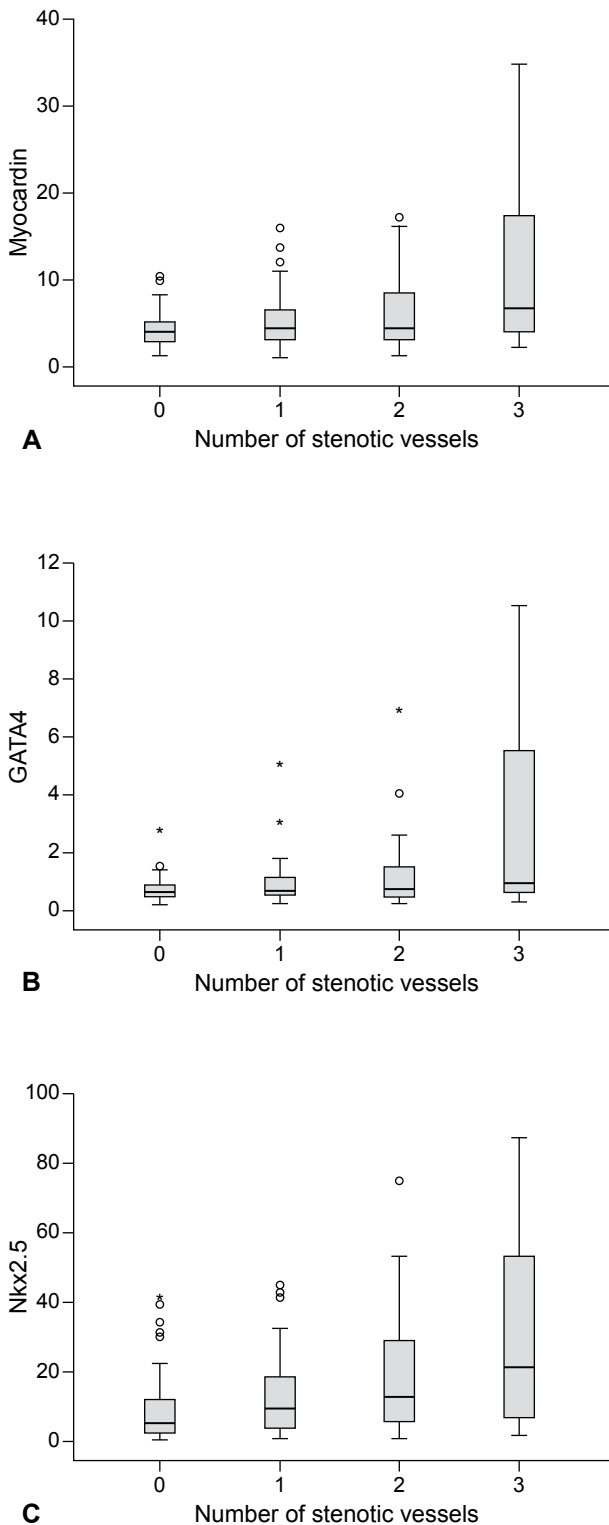
## Discussion

Our study provides data that demonstrate for the first time a relationship between early cardiac gene expression levels in the PBMCs of patients with stable CAD and their disease status. This is the first study to show, even indirectly, a relationship between circulating bone marrow-derived cardiovascular progenitor cells expressing early cardiac marker genes and disease severity in stable CAD. We have demonstrated that early cardiac marker genes show altered expression patterns in the PBMCs in CAD patients, and that there is an association between this altered pattern of expression and the severity of the disease.

Specifically, the expression levels of the early cardiac marker genes *myocardin*, *GATA4* and *Nkx2.5* were examined in the PBMCs of stable CAD patients and healthy controls. When we examined early cardiac gene transcript levels according to the disease severity status, estimated by the number of stenotic vessels involved, we found higher transcript levels in patients with more severe disease. Patients with multi-vessel disease showed higher *myocardin*, *GATA4* and *Nkx2.5* transcript levels than healthy controls. Patients with multi-vessel disease also showed higher *myocardin* and *Nkx2.5* transcript levels than patients with 1-vessel disease. Our data indicate that early cardiac genes may possibly be implicated in the cardiovascular changes that occur in CAD patients.

**Table 2.** Gene expression levels in healthy controls and patients according to the disease severity, estimated by the numbers of stenotic vessels involved. Levels are presented as cubic root-transformed arbitrary units. Jonckheere–Terpstra test p values for differences among groups and median and interquartile ranges are presented.

	No of stenotic vessels		Percentiles			Jonckheere–Terpstra p values	
		n	25th	50th	75th	0-3	1-3
<i>myocardin</i>	0	66	2.98	4.08	5.18	0.001	0.022
	1	44	3.13	4.48	6.77		
	2	32	3.21	4.46	8.73		
	3	22	4.09	6.79	17.47		
<i>GATA4</i>	0	66	0.44	0.61	0.84	0.005	0.068
	1	44	0.51	0.66	1.10		
	2	32	0.41	0.71	1.50		
	3	22	0.57	0.91	5.72		
<i>Nkx2.5</i>	0	66	2.67	5.60	12.67	<0.001	0.010
	1	44	4.13	9.87	19.32		
	2	32	5.97	13.22	30.15		
	3	22	7.21	21.70	55.42		



**Figure 1.** Box-plots of *myocardin* (A), *GATA4* (B) and *Nkx2.5* (C) levels in healthy controls and CAD patients according to the disease severity estimated by the number of stenotic vessels involved. Levels are presented as cubic root transformed arbitrary units.

Early cardiac genes in peripheral blood are expressed in circulating stem-progenitor cells that reside in the bone marrow; thus, alterations in the expression levels of such genes may reflect alterations in these cell populations. Cardiac lineage commitment occurs upon the expression of early cardiac transcription factors such as *Nkx2.5* and *GATA4*, which play a role in both embryonic cardiovascular development and stem-cell cardiomyogenesis.<sup>26-29</sup> The early cardiac marker genes we examined in this study have previously been shown to be expressed in PBMCs,<sup>25,30</sup> and they have also been shown to be expressed in human bone marrow mesenchymal stem cells,<sup>31</sup> which have the capacity of cardiomyogenesis and vasculogenesis and can integrate into a chronically injured heart.<sup>32,33</sup> Mobilisation in peripheral blood of bone marrow-derived very small embryonic-like cells expressing endothelial, early cardiac (*Nkx2.5* and *GATA4*), and pluripotent stem cell markers after myocardial infarction has also been reported.<sup>34,35</sup> Circulating progenitor cell mobilisation and recruitment is a physiological response to ischaemia and exerts a protective role against adverse pathology progression. Great efforts have been made in recent years to find ways to enhance this physiological phenomenon.<sup>36</sup>

Güven et al<sup>37</sup> showed that the number of circulating endothelial progenitor and angiogenic cells is directly related to the extent of coronary atherosclerosis in patients with CAD. We found increased early cardiac gene transcript levels in patients with more severe CAD. While examining the expression of early cardiogenic marker genes, we target subpopulations of the cells that studies using surface markers detect as circulating progenitor cells. We target circulating bone marrow-derived cardiovascular progenitor cells expressing these early cardiogenic marker genes.<sup>29,38</sup> *Myocardin* is also expressed in vascular smooth muscle progenitor cells, which play an important role in the building and repair of blood vessels.<sup>39</sup> To the best of our knowledge, there is a lack of data regarding circulating bone marrow-derived cardiovascular progenitor cells in patients with stable CAD, although there are conflicting data concerning circulating endothelial progenitor cell mobilisation in CAD.<sup>40</sup> Nevertheless, it is now well established that high levels of circulating progenitor cells decrease the likelihood of severe coronary disease and increase event-free survival following cardiovascular events.<sup>6</sup> In that respect, the elevated early cardiac gene transcript levels we found, which probably reflect changes in the circulating cardiovascular progenitor cells in more severe dis-

case, could represent a physiological response to ischaemia in its more severe stages.

The early cardiac genes we examined are also part of the “foetal gene program”, which is re-activated in adults under stress conditions as a survival adaptation mechanism.<sup>21,22</sup> We recently showed that increased *myocardin* and *GATA4* transcript levels in PBMCs of patients with idiopathic dilated cardiomyopathy and heart failure might reflect better left ventricular function in those patients, supporting the hypothesis that a return to the foetal gene profile may be an adaptive process associated with survival.<sup>30</sup> We also suggested a possible reactivation of the foetal gene profile in hypertensive heart disease, which might reflect the severity of the disease, as part of this survival adaptation process.<sup>41</sup> Coronary artery disease is a stress condition of the heart and a possible reactivation of the foetal gene program in CAD, too, could be part of this survival adaptation process.

### Conclusions

The data of this study indicate that alterations in the early cardiac gene transcript levels in peripheral blood of patients with stable CAD, possibly reflecting alterations in circulating cardiovascular progenitor cells as a survival adaptation mechanism, might reflect the level of disease severity. Further studies in larger populations are needed in order to clarify this and reinforce or refine the clinical and physiological importance of our indicative observations. The nature of the circulating cells that express early cardiac markers, their role in the progression or stabilisation of vascular disease, and their impact on clinical outcome remain important topics for further study.

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