Rare genetic variants associated with early onset CVD
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Citation for published version (APA):
Maiwald, S. (2015). Rare genetic variants associated with early onset CVD

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Chapter 2

Monocyte Gene Expression and Coronary Artery Disease


Abstract

**Purpose of review:**
Despite current therapy, coronary artery disease (CAD) remains the major cause of morbidity and mortality worldwide. CAD is the consequence of a complex array of deranged metabolic processes including the immune system. In this context monocytes and macrophages are indisputable players. Thus, monocyte gene expression analysis could be a powerful tool to provide new insights in the pathophysiology of CAD and improve identification of individuals at risk. We discuss current literature assessing monocyte gene expression and its association to CAD.

**Recent findings:**
Monocyte surface markers CD14\(^{++}\) and CD16\(^+\) have been established as biomarkers for increased CVD risk in a large number of studies. More in depth gene expression analysis identified several interesting genes, such as *ABCA1*, *CD36* and *MSR1* with an increased expression in circulating monocytes from patients with CAD. The results for *CD36* were replicated in one other study. For *ABCA1* and *MSR1* conflicting data are published.

**Summary:**
Recent findings indicate that genetic differences exist in circulating monocytes of patients suffering from CAD, giving us more insights in the underlying mechanisms. However, larger studies are required to proof that monocytes expression signature could serve as a marker for diagnostic purposes in the future.

**Key points:**
- Small studies and criteria for patient collection have hampered the identification of new genes in circulating monocytes predictive for the increased risk on CVD.
- Circulating monocyte gene expression profiles do not serve as a biomarker for CVD yet.
- The CD16\(^+\) surface marker does seem to be a useful biomarker for CVD prediction so far.
Introduction
Cardiovascular disease (CVD) is the major cause of morbidity and mortality worldwide, and is the consequence of a complex array of processes, including adaptive and innate immunity [1], as a response towards local injury. Vessel wall damage occurs predominately at sites of disturbed laminar flow or low shear stress area such as observed at branch points of arteries [2] and involves infiltration of circulating monocytes [3]. In the last years the interesting concept was proposed that the transcriptome signature of the circulating monocytes in patients with CAD may already be adapted to the altered environment and associates with increased risk.

The aim of this review is to (I) discuss the role of monocytes in atherosclerosis (II) summarize the current literature on relevant gene expression in monocytes in relation to coronary artery disease, and (III) explore, if expression profiles could be applied as diagnostic tool to identify apparently healthy individuals at risk for coronary atherosclerosis.

I. The role of monocytes in atherosclerosis
Atherosclerosis develops in the presence of dyslipidemia, hypertension and/or proinflammatory stimuli. In this environment endothelial cells secrete adhesion molecules enabling leucocytes to adhere to the cell surface [4]. Due to aberrations in the endothelial cell layer local trapping of mainly low density lipoprotein (LDL) particles, the most abundant circulating class of cholesterol-containing lipoprotein particles in the blood compartment, into the intima region occur [5]. In the hypoxic environment lipids are oxidized and taken up by local macrophages via scavenger receptors (SR-A and CD36), which then become foam cells and turn into a pro-inflammatory cell, when rescue mechanisms to remove the foam cells fail and apoptosis occurs. Endothelial cells (ECs) secrete more chemo-attractants, such as tumor necrosis factor α (TNF-α), monocyte chemotactic protein 1 (MCP-1), adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1) and p-selectin glycoprotein ligand 1 (PSGL-1), which leads to more attraction of leucocytes, primarily monocytes and T lymphocytes toward the vessel wall [1,6]. Monocytes adhere and roll on the activated ECs [1], through the interaction of monocyte PSGL-1 with endothelial selectins [7]. Adhesion is followed by entry into the subendothelial space (diapedesis) [8]. An unstable plaque is characterized by an increased number of activated inflammatory cells and an increased release of numerous inflammatory mediators and proteolytic enzymes characterizes [9]. The inflammatory response can lead to the disruption of plaque and subsequent events [9]. Since monocytes are pivotal for the initiation of the process it is an evolving question whether the gene expression in circulating monocytes renders the cells more quickly to be taken up into
the intima region. Circulating monocytes represent a heterogeneous type of white blood cells in blood [10,11]. In addition to the classical monocytes, at least two additional subpopulations of monocytes have been detected [12]: (i) classical monocytes (65–85% of all monocytes), characterized by high surface lipopolysaccharide receptor CD14++ expression, and low FcγIII receptor CD16 expression, express CCR2, the receptor for MCP-1, and are believed to be actively recruited to sites of inflammation [3]; (ii) non-classical monocytes with low CD14+ expression at the cell surface but high CD16++ expression [11] and (iii) intermediate monocytes with high CD14++ expression and low CD16+ levels [13]. An accumulation of CD16+ monocytes leads to progression of atherosclerosis [14]. Inline, increased levels of circulating CD16+ monocytes are associated with progression of atherosclerosis and other inflammatory diseases [14]. It has been documented, in 3 large studies, that a high plasma CD14++ CD16+ monocyte count in patients with CVD was associated with increased prevalence of CAD [15,16]. Interestingly, in patients with familial hypercholesterolemia (FH), who are at increased risk to develop CVD, plasma circulating CD16+ monocyte population was 4 times higher as compared to non-FH individuals. [17]. We could find one study that shows a different result. Herein an increased number of CD14++ CD16+ monocytes predict future cardiovascular events [3]. Altogether, most studies report a positive association between elevated levels of circulating CD14+ CD16+ monocytes with active CVD. These results underline our hypothesis that defining the nature of circulating monocytes could be a useful biomarker for CVD.

II. Literature of gene expression in monocytes in relation to coronary artery disease (table 1)

Monocytes have a library of transcripts of almost 10,000 genes [18], including genes associated with gender, age, lipids, blood pressure, immunity and CAD [18]. The signature of the monocyte transcriptome is sensitive to environmental changes and may therefore provide a specific signature for patients at increased risk for CAD and lead new insight into CAD. To address these question we set out a literature search using the following criteria in pubmed: Human AND (Coronary AND (Arterial Disease OR Arteriosclerosis OR Arterioscleroses OR Atherosclerosis OR Atheroscleroses)) AND (Gene OR Genes OR Genetic OR Genetics OR Genomic OR Genomics) AND (Monocyte OR Monocytes).

First we will discuss articles describing studies in patients with CAD and presenting data on gene expression in monocytes isolated with CD14+ beads representing a pure monocyte fraction (table 1). In total 5 studies met our selection criteria. Two studies presented data on whole genome analyses whereas the other 3 studies only show data from selected gene pathways. In the
second part we discuss larger cohort papers referring to studies investigating gene expression in the PBMC fraction, which represents a heterogeneous pool of circulating cells, consisting of 15% B-cells, 70% lymphocytes, and 15% natural killer cells, which may not all contribute to the pathophysiology of atherosclerosis. However, the isolation of PBMCs from blood is simple and can be applied in larger cohort studies (Table 1).

**Table 1: Differentially expressed genes in atherosclerosis.**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Candidate genes</th>
<th>Disease</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14+ isolated monocytes</td>
<td>$ABCA1(\downarrow)$, $ABCG1(\downarrow)$, $RGSI(\downarrow)$, $ADRB2(\downarrow)$, $FOL3(\downarrow)$</td>
<td>CAD</td>
<td>Sivapalaratnam et al. (2012)</td>
</tr>
<tr>
<td>CD14+ isolated monocytes</td>
<td>$FOS(\uparrow)$, $DUSP1(\downarrow)$</td>
<td>CAD</td>
<td>Patino et al. (2005)</td>
</tr>
<tr>
<td>CD14+ isolated monocytes</td>
<td>$CD36(\downarrow)$</td>
<td>CHD</td>
<td>Teupser et al. (2008)</td>
</tr>
<tr>
<td>CD14+ isolated monocytes</td>
<td>$CD36(\downarrow)$, $MSR1(\uparrow)$</td>
<td>ACS</td>
<td>Piechota et al. (2012)</td>
</tr>
<tr>
<td>CD14+ isolated monocytes</td>
<td>$TLR4(\uparrow)$, $Myd88(\downarrow)$, $B7-1(\downarrow)$, $IL-12(\downarrow)$</td>
<td>AMI, SA, UA</td>
<td>Methe et al. (2005)</td>
</tr>
<tr>
<td>CD14+ isolated monocytes</td>
<td>$p66Shc(\uparrow)$</td>
<td>ACS</td>
<td>Franzeck et al. (2012)</td>
</tr>
<tr>
<td>PBMCs</td>
<td>$TLR4(\uparrow)$</td>
<td>NDHP</td>
<td>Marketou et al. (2012)</td>
</tr>
<tr>
<td>PBMCs</td>
<td>$SR-A(\downarrow)$</td>
<td>ACS, CAD</td>
<td>Nakayama et al. (2007)</td>
</tr>
<tr>
<td>PBMCs</td>
<td>$CXCL16(\uparrow)$</td>
<td>ACS, UA, AMI</td>
<td>Yang et al. (2008)</td>
</tr>
<tr>
<td>PBMCs</td>
<td>$ADAMTS4(\downarrow)$</td>
<td>ACS</td>
<td>Zha et al. (2010)</td>
</tr>
<tr>
<td>PBMCs</td>
<td>$HO-1(\downarrow)$, $Ubiquitin(\downarrow)$</td>
<td>SA, UA, AMI</td>
<td>Chen et al. (2009)</td>
</tr>
<tr>
<td>PBMCs</td>
<td>$CXCR1(\downarrow)$, $CXCR2(\downarrow)$</td>
<td>CAD</td>
<td>Leonard et al (2011)</td>
</tr>
</tbody>
</table>

**Notes:**
- CAD = coronary artery disease
- CHD = coronary heart disease
- ACS = acute coronary syndrome
- AMI = acute myocardial infarction
- SA = stable angina
- UA = unstable angina
- PBMCs = peripheral blood mononuclear cells
- NDHP = Nondiabetic hypertensive patients
Sivapalaratnam et al. [19] performed whole genome expression analysis in circulating monocytes isolated from the blood of male patients with CAD at a young age (n = 22; age 43.4 ± 3.8 years; age of MI 36.3 ± 5.7 years; medication: statin 93%, aspirin 86%, beta-blockers 73%, ACE-inhibitors 41%, nitrates 23%) and lifestyle matched healthy controls (n = 22; age 43.1 ± 3.2 years). Only six genes were significant differentially expressed of which 5 could be validated by RT-PCR. Additionally 12 healthy subjects received statins and aspirin to establish its effect on monocyte gene expression. These findings were replicated in an independent group of 24 cases (age 50.3 ± 5.3 years; CAD 43.8 ± 4.2; MI 54%; similar medication) and 24 controls (age 49.9 ± 5.1 years). The expressions of the beta-2-adrenergic receptor (ADRB2) and the folate receptor 3 (gamma) (FOLR3) were increased, whereas the expressions of ATP-binding cassette sub-family A member 1 (ABCA1), ATP-binding cassette sub-family G member 1 (ABCG1) and regulator of G protein signaling 1 (RSG1) were significantly decreased. Interestingly, they found that the expression of ABCG1 and ADRB2 was highly decreased by statin treatment in the control monocytes. ADRB2 is a cell surface marker receptor and is directly associated with its final effector, the class C L-type calcium channel Ca(V)1.2 [20], which mediates the influx of calcium ions into the cell upon membrane polarization [21]. Previous studies report an association of SNPs in ADRB2, arterial stiffness and acute myocardial infarction (MI) [22]. It has been suggested that ADRB2 mediates SMCs relaxation in small resistant arteries and large conduit arteries [22] resulting in vasodilation of the arteries. FOLR3 is the secreted form of the folate receptor [23] and highly expressed in hematopoietic tissues [24]. So far its role in CVD is unclear. In hyperlipidemic atherosclerotic animal models, increased folate receptor expression was associated with activated macrophages [25]. ABCA1 is an ATP-binding cassette transporter, which facilitates efflux of cholesterol from lipid-laden macrophages to apolipoprotein A1 [26-29]. Rare loss of function mutations in ABCA1 are associated with a low HDL phenotype and an increased risk of CVD [30-32]. ABCG1 participates in the removal of cholesterol from lipid-laden macrophages to high density lipoprotein (HDL) particles [27]. It is an intriguing observation that the expression of both ABCA1 and ABCG1 are decreased implicating a reduced capacity of these monocytes to unload cholesterol [33]. RSG1 is mainly expressed in hematopoietic cells and so far no clear association with atherogenesis or CVD is known.

Patino et al. [34] performed monocyte expression profile in 2 cases with CAD and three controls. 297 candidate genes were identified with a 1.5-fold increased expression and 267 genes with decreased expression mainly in genes involved in stress response and inflammatory genes. Validation was performed in 25 patients (age 74 ± 8 years) with known atherosclerosis and 19 matched controls (age 70 ± 5 years). Finkel-Biskin-Jinkins osteosarcoma (FOS) and dual
specificity phosphatase 1 (DUSP1) genes were highly expressed in monocytes of patients with atherosclerosis. DUSP1, a stress response phosphatase, is important in the regulation of mitogen-activated protein kinase (MAPK) [34] and therefore is also known as mitogen-activated protein kinase phosphatase 1 (MKP-1) [35]. DUSP1 expression is upregulated by inflammatory stimuli and results in the enhanced expression of cytokines and adhesion molecules [35]. Indeed lack of Dusp1 protects against atherosclerosis in apoE−/− mice [35]. Interestingly, DUSP1 blood levels in coronary artery bypass grafting (CABG) patients were a marker for post-operative recovery [36]. FOS, a pro-inflammatory transcription factor, is highly expressed in macrophages and smooth muscle cells in plaque tissues, but also in circulating monocytes. It is implicated to play a role in the regulation of the cell response towards oxLDL exposure. Of note, FOS expression does seem to be inhibited by statins [37]. Interestingly FOS expression may be modulated by mir-181a which binds to the 3′-UTR of FOS [38]. Furthermore, FOS levels in blood leukocytes are considered a cumulative biomarker for smoking [39].

Teupser et al. [40] assessed monocyte expression of eleven candidate genes, selected on the basis of their known role in atherosclerosis: Macrophage scavenger receptor 1 (MSR1), scavenger receptor class B member 1 (SR-B1), lectin-like oxidized low-density lipoprotein (LDL) receptor 1 (LOX1), CD36, LDL receptor (LDLR) and APOE all involved in lipid uptake and homeostasis, and ABCA1. Additionally expression of inflammatory genes like, TNF-α, macrophage inflammatory protein 1α (MIP-1α), interleukin-6 (IL-6) was analysed. The study comprises 119 subjects undergoing coronary catheterisation. 79 had coronary atherosclerosis (cases, age 64 ± 10 years), and 40 were free of vessel disease (controls, age 57 ± 12 years). Only CD36 expression was significantly increased. A recent study replicates this finding in ACS subjects (n = 100; age 64 years) compared to control subjects (n = 40; age 37 years) [41]. Herein, an increased expression for MSR1 [41] was found. MSR1 and CD36 are scavenger receptors present on the cell surface of monocytes and macrophages [41]. CD36 is involved in the uptake and internalization of oxLDL [42]. In apoE−/− mice the absence of CD36 expression in macrophage was protective against atherosclerosis [43]. Thus, it is speculated that higher expression of CD36 in monocytes from patients with coronary heart disease (CHD) may be an indicator of an increased burden of oxLDL and inflammation in these patients. CD36 has been detected in atherosclerotic plaque and was increased with the progression of atherosclerosis [44]. MSR1, on the other hand, is mainly responsible for the uptake of acetylated lipoproteins [41]. Of note, patients with FH have increased expression of CD36 in circulating monocytes [17,45].

Methe et al. [46] studied monocyte expression of toll-like receptor 4 (TLR4) and its downstream targets in patients with CAD. TLRs are key recognition proteins of the innate immune system
Co-stimulatory molecules (such as CD80 and CD86; also known as B7-1 and B7-2) and proinflammatory cytokines (such as interleukin IL-1β, IL-6, IL-12 and TNF-α) have been demonstrated as downstream effectors of TLR activation. Blood was collected from 143 patients with the diagnosis of CAD and from 30 healthy controls. The patients were divided in three groups: stable angina (SA), unstable angina (UA) and AMI based on anamnesis and ECG. Transcript levels for TLR4 were significantly higher in the UA and the AMI groups than in controls and SA patients. In a recent study analyzing monocytes isolated from the blood of nondiabetic hypertensive patients \( n = 52; \text{age } 56 \pm 8 \) TLR4 expression was significantly elevated [47]. Several lines of evidence exist for the role of TLR4 in atherosclerosis [48]. Prominent expression of TLR4 in human atherosclerotic tissue was found at the lipid-rich, macrophage-infiltrated shoulder region [46]. Franzeck at al. [49] studied the expression of the adaptor protein p66Sh in 19 CAD patients, 18 acute coronary syndrome (ACS) patients and 16 aged matching controls. In monocytes of ACS but not CAD patients there was higher expression of p66Shc [49]. P66Shc\(^{-/-}\) mice developed less atherosclerotic plaques [49]. P66Shc is a member of the family of ShcA adaptor proteins and contains a unique N-terminal region \( (\text{CH}_2) \), which is crucial for its role as a redox enzyme implicated in mitochondrial reactive oxygen species (ROS) formation an important mediator in cell signaling and vascular cell homeostasis [50,51]. ROS interferes with nitric oxide (NO) causing endothelial dysfunction [52]. Thus, the interplay between oxidative stress and inflammation is crucial in the pathogenesis of atherosclerosis [53]. Nakayama et al. showed a strongly increased expression for SR-\(A\) in PBMCs isolated during the acute phase of ACS [9]. Yang et al. observed an increased expression of Chemokine (C-X-C motif) ligand 16 \( (\text{CXCL16}) \) in patients with ACS [54]. CXCL16, a novel transmembrane chemokine, also functions as scavenger receptor which can bind and uptake phosphatidylserine and oxLDL (named SR-PSOX/CXCL16) [54]. CXCL16 is known to promote foam cell formation through a TNF-\(\alpha\) dependent mechanism [54]. Zha et al. identified an up-regulation of disintegrin and metalloproteinase with thrombospondin motifs 4 \( (\text{ADAMTS4}) \) in subjects with ACS [55]. The ADAMTS family is involved in vascular lesion development by remodeling the endothelial lining through degradation of different proteoglycans at the endothelial cell surface in blood vessels [55]. ADAMTS4 is expressed in macrophage rich areas of human atherosclerotic carotid plaques, suggesting a pathogenic role in the development of ACS [55]. Adamts4 expression is upregulated during the development of atherosclerosis in Ldlr\(^{-/-}\)/ApoB\(^{100/100}\) mice [56].
Chen et al. showed an increased gene expression of heme oxygenase-1 (HO-1) and ubiquitin [57]. The ubiquitin-mediated proteolytic pathway (UPS) is responsible for the non-lysosomal degradation of the majority of intracellular proteins and plays a crucial role in the regulation of many cellular processes [57]. It has been indicated that UPS may be functionally impaired under conditions of increased endogenous oxidative stress. HO-1 is involved in cytoprotective mechanism to prevent tissues from oxidative damage and is removed by UPS [57]. The up-regulated HO-1 expression may be explained by auto protective reaction to injury [57].

Leonard et al. observed increased expression for CXC-motiv-chemokinrezeptor 1 and 2 (CXCR1 and CXCR2) in patients with obstructive coronary disease Leonard [58]. Both are interleukin 8 (IL-8) receptors and play a role in monocyte chemotaxis, adhesion, and accumulation of macrophages in atherosclerotic lesions [58]. In an atherosclerosis-susceptible mouse model, CXCR2 was required for accumulation of macrophages in atherosclerotic lesions [58].

Peroxisome proliferator-activated receptors (PPARs) play an important role in the regulation of genes involved in CVD [59]. PPARγ is a key regulator of monocyte/macrophage differentiation [60]. In both stable plaques (n = 24) and unstable plaques (n = 24) statin treatment significantly increased PPARγ expression, which was also observed in human monocytes in-vitro [61]. In line, Sueyoshi et al. [62] examined human atherosclerotic lesions on the expression of PPARγ and PPARα. The authors showed an upregulation of both PPARγ and PPARα in monocytes and macrophages in atherosclerotic plaques, which correlate with the severity of the lesions [62]. These results link the anti-inflammatory responses of PPARγ-agonists through monocytes by decreasing cytokines in-vitro [63]. Yet it has not been evaluated whether PPARγ expression in circulating monocytes predispose to increased disease prevalence.

III. Can the gene expression profile of monocytes be used as diagnostic tool?

To date, based on the reported literature, there is insufficient evidence that monocyte gene expression can be applied as a diagnostic tool. First, there is no major replication of genes differentially expressed in monocytes of diseased patients in the different studies. This inconsistency in gene expression could be due to the relative small sample sizes, variation in isolation methods and in clinical phenotype of the included patients as well as the influence of medication use. Second, the process of harvesting pure monocytes is still time consuming and complicated. The use of CD14+ microbead selection makes it an expensive tool. Direct facsing
of cell surface proteins in whole blood may provide an alternative as implicated by a study performed in FH patients [17].

So far only monocyte CR36 expression was confirmed by two studies [40,41] and thus could be a potential biomarker. But replication has to occur in larger prospective cohort studies. The complexity of gene expression analysis is illustrated by a recent study reporting on the analysis of the transcriptome of circulating monocytes in 3,336 individuals from the Gutenberg Heart Study [18]. Herein the authors conclude that despite the large number of identified expression quantitative trait loci (eQTLs), the transcriptome of circulating monocytes appear of modest help to dissect the relationship between genome variability and complex diseases such as cardiovascular risk.

**Conclusion**

In conclusion, the transcriptome of circulating monocytes may be useful in analyzing CAD related pathways and gain more insights into the pathophysiology of CAD but do not serve as a potential biomarker yet.
Acknowledgements:
SM is supported by a grant from NETSIM. SS is supported by a grant from ATHEROS.

Conflicts of interest:
None
Research into atherosclerosis led to many compelling hypotheses about the pathophysiology of atherosclerotic lesion formation and of complications such as myocardial infarction and stroke. But we still lack definitive evidence to show that processes such as lipoprotein oxidation, inflammation and immunity have a crucial involvement in human atherosclerosis. Experimental atherosclerosis in animals furnishes an important research tool, but extrapolation to humans requires care. Understanding how to combine experimental and clinical science will provide further insight into atherosclerosis and could lead to new clinical applications.


Variability of gene expression in human may link gene sequence variability and phenotypes; however, non-genetic variations, alone or in combination with genetics, may also influence expression traits and have a critical role in physiological and disease processes. This study
Monocyte Gene Expression

demonstrates that the monocyte transcriptome is a potent integrator of genetic and non-genetic influences of relevance for disease pathophysiology and risk assessment.


By whole genome expression arrays six genes were identified to have differential expression in the monocytes of patients versus controls; ABCA1, ABCG1 and RGS1 were downregulated in patients, whereas ADRB2, FOLR3 and GSTM1 were upregulated. Aspirin and statins altered gene expression of ABCG1 and ADBR2.


Chapter 2


This article was the first to link the expression profile of monocytes with atherosclerosis using transcriptome profiling


The scavenger receptor CD36 was found to be upregulated in the monocytes of patients with CAD. This is the only replicated result so far.


Acute Coronary Syndromes (ACS) are a group of disorders caused by the significant reduction of circulation in coronary arteries. This study describe that CD36 and MSR1 mRNA in circulating monocytes was significantly higher in ACS subjects; for CD36 this is a replication of ref 40.


TLRs are known for their important role in the immune system. This was the first study to show that in monocytes of ACS patients the expression of TLR4 was elevated.


The adaptor protein p66Shc is implicated in atherogenesis and oxidative stress related responses in animal models of diseases. This study suggests an involvement of p66Shc in the transition of a stable CAD to an ACS patient. p66Shc was associated with states of increased oxidative stress.


