Chemokines in cardiovascular risk prediction

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Summary
In consideration of the important role of inflammation in plaque progression and stability, recent work has focused on whether plasma markers of inflammation can non-invasively diagnose and predict coronary artery disease (CAD) and other forms of atherosclerotic disorders. Although several studies support an important pathogenic role of chemokines in atherogenesis and plaque destabilization, potentially representing attractive therapeutic targets in atherosclerotic disorders, this does not necessarily mean that chemokines are suitable parameters for risk prediction. In fact, the ability to reflect up-stream inflammatory activity, stable levels in individuals and high stability of the actual protein (e.g. long half-life and negligible circadian variation), are additional important criteria for an ideal biomarker in cardiovascular disease. Although plasma/serum levels of certain chemokines (e.g. interleukin 8 and monocyte chemoattractant protein-1) have been shown to be predictive for future cardiac events in some studies, independent of traditional cardiovascular risk factors and C-reactive protein, and although certain gene polymorphisms of chemokines/chemokine receptors (e.g. fractalkine receptor) have been shown to be predictive of future atherosclerotic disease, further prospective studies, including a larger number patients, are needed to make any firm conclusion. While the demonstrations of an association between chemokines and CAD are a necessary first step, such studies do not establish the full clinical utility of a biomarker, which is a demanding process that requires validation in multiple cohorts, and clear demonstration of incremental prognostic value over traditional risk models. If successful, such new biomarker will be a useful indicator for better risk assessment, diagnosis, and prognosis, as well as monitoring pharmacological treatments for atherosclerosis.

Keywords
Chemokines, atherosclerosis, gene polymorphism, biomarkers, inflammation

Introduction
Cardiovascular disease remains a leading cause of death throughout the world despite advances in its detection and treatment. Commonly used risk algorithms, such as the Framingham Risk Score and lipid parameters fail to identify all affected individuals. Novel cardiovascular risk factors that identify these missed individuals would greatly improve overall care of patients. These risk markers should also give prognostic information in patients with established cardiovascular disease as well as being predictors of efficacy for various therapeutic interventions (e.g. statins) in these patients.

Inflammatory mediators as biomarkers in cardiovascular disease
In consideration of the important role of inflammation in plaque progression and stability, recent work has focused on whether plasma markers of inflammation can non-invasively diagnose and predict coronary artery disease (CAD) and other forms of atherosclerotic disorders (1). Numerous studies have shown that inflammatory markers can help in identifying patients with stable CAD and acute coronary syndromes (ACS), as well as being predictive for development of CAD in high-risk patients (2). Moreover, several inflammatory markers improve risk stratification in CAD patients, being independent prognostic markers for cardiovascular events (2, 3). C-reactive protein (CRP), the prototype inflammatory marker, has certainly been the best...
Chemokines in vascular biology

Chemokines – major pathogenic role in atherogenesis and plaque destabilization

Chemokines are a family of chemotactic cytokines characterized by their ability to cause directed migration of leukocytes into inflamed tissue, and raised levels are found in several inflammatory disorders including CAD (12). Moreover, these chemotactic cytokines seem not only to be raised in circulation, but also within the atherosclerotic lesions (13). Hence, there are several reports of enhanced expression of CXC-chemokines (e.g. IL-8, neutrophil-activating peptide [NAP]-2, CXCL16, and interferon-γ-inducible 10 [IP-10], CC-chemokines (e.g. monocyte chemoattractant protein [MCP]-1, leukotactin-1 [Lkn-1], and regulated upon activation, normal T-cell expressed and secreted [RANTES]) as well as some of their corresponding chemokine receptors within human atherosclerotic lesions (12–15). In addition to being potent chemotactants, several other leukocyte responses such as cell proliferation, enzyme secretion and induction of reactive oxygen species, have been observed in vitro after chemokine stimulation (13). Moreover, beyond their effects on leukocytes, chemokines may also interfere with smooth muscle cell (SMC) migration and growth, as well as platelet activation (16, 17). Some of these responses may clearly be relevant to atherogenesis and plaque destabilization, and indeed, the co-expression of chemokines and their receptor within atherosclerotic lesions, involving various cell types such as T cells, macrophages, and vascular SMC, suggests their involvement not only in the regulation of lymphocyte recruitment into atherosclerotic lesions, but also in other processes with relevance to atherogenesis such as regulation of vascular SMC phenotype (13). Moreover, recent studies in vivo have shown that targeted disruption of the genes for MCP-1, CCR2 (i.e. MCP-1 receptor) and CXCR2 (i.e. IL-8 receptor) significantly decreases atherosclerotic lesion formation and lipid deposition in mice prone to develop atherosclerotic lesions (18–20). Also, two independent reports recently indicated the involvement of the CX3C chemokine fractalkine (also known as CX3CL1) in atherogenesis, showing that CX3CR1 deficiency (i.e. the fractalkine receptor) decreased atherosclerosis in animal models (21, 22). These and other studies in gene-modified mice strongly suggest an important pathogenic role of chemokines in atherogenesis.

What is a reliable biomarker in cardiovascular disease?

A biomarker for cardiovascular disease should reflect important pathophysiological processes in atherogenesis and plaque destabilization, and some biomarkers have failed because they are involved in only one pathway in a multiple-pathway disease or they reflect epiphenomena independent of the disease process. However, although chemokines seem to play a central pathogenic role in atherogenesis, this does not necessarily mean that these chemotactic cytokines are suitable parameters for risk prediction. In fact, the leading role of CRP as an inflammatory biomarker in cardiovascular disease is not primarily based on its pathogenic role in these disorders, but rather on its ability to reflect upstream inflammatory activity. Moreover, CRP has a long half-life, exhibits stable levels in individuals, and has negligible circadian variation (32, 33). It is easily measured, and inexpensive standardized assays provide similar results in fresh, stored, or frozen plasma, reflecting the high stability of the protein (32, 33). All these characteristics are important criteria for an ideal biomarker in cardiovascular disease. Unfortunately, measurements of chemokines in plasma/serum are hampered by some
methodological problems which may limit their use as biomarkers in CAD and other cardiovascular disease. First, platelets are an important cellular source for several chemokines such as RANTES, NAP-2, and epithelial neutrophil-activating peptide (ENA)-78 (34), and these parameters should therefore ideally be measured in platelet-poor or platelet-free plasma which is not always available. Thus, measurement in serum may underestimate the concentration in high-risk patients (i.e. less release of chemokines from platelets during coagulation ex vivo due to degranulation and platelet activation in vivo). Moreover, freeze and thaw cycles of ordinary plasma samples may results in ex-vivo release of chemokines from platelets which again may depend on platelet counts as well as the degree of platelet degranulation in vivo. Second, plasma/serum levels of several chemokines are in general low, often just above the detection limit of the various enzyme immunoassays, and these aspects may limit their use as a reliable biomarkers. For example, while serum levels of IL-8 in CAD patients are mostly <10 pg/ml, CRP levels in these individuals will mostly be >1 µg/ml (5, 6, 30, 35). Finally, heparin administration may influence plasma/serum levels of chemokines through enhanced release of heparan sulphate-bound chemokines in the vessel wall, which may limit their use as prognostic markers in ACS (35).

Chemokines as markers for subclinical CAD

Migration of monocytes into the arterial wall is an early event in atheroma formation (1). Based on the suggested important role of chemokines in this process, and in particular IL-8 and MCP-1 (18–20), they could potentially be early markers of CAD, and a few studies have tested this hypothesis (Table 1). In a nested case-control study in the prospective EPIC-Norfolk population study, baseline IL-8 concentrations among 785 apparently healthy individuals, in whom fatal or non-fatal CAD developed during follow-up (average of ~6 years), was significantly higher than in 1,570 matched controls (3.5 pg/ml vs. 3.1 pg/ml, p=0.001) (36). The odds ratio (OR) for future CAD was still significant after adjustment for traditional risk factors and after additional adjustment for CRP and white cell count. Although the authors conclude that IL-8 could represent a novel biomarker for CAD in apparently healthy individuals, there was a considerable overlap between the two study groups and, as discussed above, the levels were mostly just above the detection limit of the assay (i.e. 2.5 pg/ml).

Deo et al. observed strong associations with CAD risk factors such as older age, female sex, hypertension, diabetes, and renal insufficiency after measuring MCP-1 levels in subjects from the Dallas Heart Study (3,499 subjects <65 years old) (37). In this study, MCP-1 was associated with coronary artery calcium score in multivariable analyses adjusting for traditional coronary risk factors. However, when further adjustment was made for age, MCP-1 was no longer independently associated with the presence of subclinical atherosclerosis. The authors conclude that these results suggest that MCP-1 may not be useful as a clinical tool that is additive to the assessment of age, traditional risk factors, and/or CRP for the detection of subclinical atherosclerosis.

### Table 1: Studies [references] on plasma/serum levels of chemokines as risk predictors for cardiovascular disease.

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Population</th>
<th>Numbers</th>
<th>Follow-up</th>
<th>Associated disease/event</th>
<th>Study design</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8 (36)</td>
<td>Healthy individuals</td>
<td>785 cases</td>
<td>6 years</td>
<td>Fatal and nonfatal CAD</td>
<td>Nested case-control</td>
<td>Higher levels of IL-8 in cases</td>
</tr>
<tr>
<td>MCP-1 (37)</td>
<td>Healthy individuals</td>
<td>3,499</td>
<td></td>
<td>Subclinical atherosclerosis</td>
<td>Cross sectional</td>
<td>MCP-1 associated with coronary artery calcium score</td>
</tr>
<tr>
<td>MCP-1 (38)</td>
<td>CAD/PAD</td>
<td>412/209 cases</td>
<td></td>
<td>CAD/PAD</td>
<td>Case control</td>
<td>Higher levels of MCP-1 in cases</td>
</tr>
<tr>
<td>IL-8, IP-10, MCP-1, RANTES, MIP-1, Eotaxin (39)</td>
<td>CAD</td>
<td>312 cases</td>
<td></td>
<td>Angiographically confirmed and stable CAD</td>
<td>Case control</td>
<td>Higher levels of IL-8 and IP-10, lower RANTES in cases</td>
</tr>
<tr>
<td>MCP-1 (40)</td>
<td>ACS</td>
<td>2,270</td>
<td>10 months</td>
<td>Death or MI</td>
<td>Prospective</td>
<td>MCP-1 levels above 75th percentile associated with increased event risk</td>
</tr>
<tr>
<td>MCP-1 (41)</td>
<td>ACS</td>
<td>183</td>
<td>13 months</td>
<td>Composite (death, MI, unstable angina, revascularization)</td>
<td>Prospective</td>
<td>MCP-1 predicted a new coronary event</td>
</tr>
<tr>
<td>Eotaxin-3 (42)</td>
<td>CAD</td>
<td>1,026</td>
<td>2.7–4.1 years</td>
<td>Cardiovascular death, MI</td>
<td>Prospective</td>
<td>Lower eotaxin-3 levels predicted future events</td>
</tr>
</tbody>
</table>

IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1; IP-10, interferon (INF)-inducible protein of 10 kDa; MIP-1α, macrophage inflammation protein-1; CAD, coronary artery disease; PAD, peripheral artery disease; ACS, acute coronary syndrome; MI, myocardial infarction.

Chemokines as markers of established cardiovascular disease

A few case-control studies have examined the ability of chemokines to predict established atherosclerotic disease (Table 1). In
the Atherosclerosis Risk in Communities (ARIC) study, 209 cases with lower extremity peripheral artery disease (PAD) and 412 cases with incident CAD were compared with 733 and 709 subjects without PAD and CAD, respectively (38). Participants with incident CAD and in particular those with PAD had significantly higher levels of MCP-1 than controls. MCP-1 levels in the upper versus lower tertile were associated with PAD, independent of traditional CAD risk factors. However, this association seemed not to be independent of CRP, which weakens the usefulness of MCP-1 as a reliable inflammatory biomarker in this population. Moreover, in another case-control study, Rothenbacher et al. included 312 patients, aged 40 to 68 years, with angiographically confirmed and stable CAD and 472 age- and gender-matched controls (39). The authors found no universal upregulation of chemokines in CAD patients as compared to controls, with up-regulation of IP-10 and IL-8 versus down-regulation of RANTES, and no clear disease association for MCP-1, MIP-1α, or eotaxin. Moreover, the association with CAD was attenuated or lost when carrying out multivariate adjustments for a variety of covariates, even if CRP was not included in these models.

Chemokines as predictors of cardiovascular events in patients with overt CAD

In contrast to these somewhat disappointing case-control studies, MCP-1 seems to be of some value in predicting cardiovascular event in patients with ACS (Table 1). In a sub-study of the Oral Glycoprotein IIb/IIIa Inhibition with Orbifiban in Patients with Unstable Coronary Syndromes (OPUS-TIMI) 16 trial, the authors showed that among 2,279 patients with ACS, MCP-1 levels above the 75th percentile (238 pg/ml) were associated with an almost two-fold increased risk of death or MI during follow-up (10 months) after adjustment for standard risk predictors including CRP (40). The authors suggest that MCP-1 could be attractive as a surrogate biomarker in these patients and merits further study as a potential therapeutic target. A similar finding has also been reported by Kervinen et al. showing in a much smaller study (n=183) of unselected ACS patients with a very high rate (64%) of coronary events (i.e. cardiac death, recurrent MI, unstable angina, or revascularization) during follow-up (13 months), that increased plasma levels of MCP-1, as well as the T-cell marker soluble IL-2 receptor, were helpful for predicting new coronary events independent on other inflammatory mediators (i.e. CRP and IL-6) (41).

Eotaxins (eotaxin, eotaxin-2, and eotaxin-3) are members of the CC chemokine branch that mainly act on CCR3-bearing cells like eosinophils, basophils, and lymphocytes of the Th2 helper cell type (42). There are few data on the role of these chemokines in CAD, but recently Falcone et al. reported that lower eotaxin-3 concentrations were predictive of future cardiovascular events, whereas both eotaxin and eotaxin-2 showed no association with risk, in a study population with confirmed CAD (n=1,026; 841 with stable and 185 with unstable angina) and with 105 cardiac events during follow-up (2.7–4.1 years) (42) (Table 1). The highest risk of future cardiovascular events was observed in subjects with combined elevation of CRP and reduction of eotaxin-3, and receiver-operating-characteristic curves analysis suggested a superior prognostic value of eotaxin-3 compared with CRP for predicting cardiac events. However, although there are some reports of potential anti-inflammatory effects of eotaxin-3 (42), the reason for the association between low eotaxin-3 levels and cardiac events remain obscure. The authors have previously reported an association between high eotaxin levels and documented CAD, further underscoring such a notion (43).

Genetic variation in the chemokine genes as a risk factor for atherosclerosis

Epidemiologic genetic studies in genes related to lipid metabolism have been of major importance for our understanding of the role of these factors in atherogenesis. Similarly, polymorphism studies in chemokine or chemokine receptor genes, trying to relate these genetic variations to increased risk for cardiovascular disease (Table 2), are of importance for the study of the pathogenic role of these mediators in the atherosclerotic process (44). Moreover, such analyses could also be of importance for

Table 2: Studies [references] on polymorphisms in chemokine and chemokine receptor genes and risk for cardiovascular disease.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutations</th>
<th>Populations</th>
<th>Associated disease/clinical events</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1</td>
<td>−2518G, G-927C, A-2578G</td>
<td>Healthy individuals, CAD, stroke HIV patients</td>
<td>−2518G, G-927C and A-2578G variants in homozygous form appears as a genetic risk factor for IMT, CAD, occult ischemia and MI</td>
</tr>
<tr>
<td>CCR2</td>
<td>V64I</td>
<td>CAD/MI</td>
<td>V64I is associated with a higher prevalence of MI. However, data is conflicting regarding this rare allele.</td>
</tr>
<tr>
<td>CCR5</td>
<td>CC53A32</td>
<td>CAD/MI</td>
<td>CCR5A32 allele seems to protect against an early episode of MI.</td>
</tr>
<tr>
<td>RANTES</td>
<td>−28G, −403A</td>
<td>CAD, DM type II</td>
<td>−403A allele is associated with CAD, cardiac events and all cause mortality.</td>
</tr>
<tr>
<td>CX3CR1</td>
<td>−1249, CX3CR1-M280</td>
<td>CAD, ACS</td>
<td>CX3CR1−1249 and CX3CR1-M280 alleles are associated with a markedly reduced risk of CAD and ACS and improved endothelium-dependent vasodilation. One study (60) suggests protective effect of the M280 and harmful effect of the I249 polymorphism on the occurrence of ACS.</td>
</tr>
</tbody>
</table>

MCP-1, monocyte chemoattractant protein-1; CCR2, −5, CC-chemokine receptor 2; −5; CX3CR1, CX3C receptor 1; CAD, coronary artery disease; HIV, human immunodeficiency virus; MI, myocardial infarction; DM, diabetes mellitus; ACS, acute coronary syndrome; IMT, intima-media thickness.
risk stratification, in particular in the coming years where such molecular methodology will be more accessible.

**Polymorphisms in the genes for MCP-1 and its receptor CCR2**

A polymorphism in the promoter of MCP-1 (the substitution of G for A at position –2518) has been associated with increased transcription of the MCP-1 gene, and Szalai et al. found that the frequency of the –2518G homozygote variant was significantly higher in 318 CAD patients referred to coronary bypass surgery, than in 320 controls, significantly associated with elevated lipoprotein (a) levels, a known risk factor for CAD (43). This MCP-1 polymorphism was also shown to independently predict occult ischemia in an apparently healthy high risk population (679 apparently healthy 24- to 59-year-old siblings of people with premature CAD) (46). Interestingly, this –2518G polymorphism has also been found to be associated with a five-fold increased risk for atherosclerosis in HIV-infected individuals, as assessed by ultrasonography (47).

Moreover, in a recent study of 470 patients with ischemic stroke, Brenner et al. found an association between the occurrence of two other MCP-1 polymorphisms (i.e. MCP-1 A-927G and MCP-1 A-2578G) and the common carotid artery intima media thickness (48). One of these polymorphisms (i.e. A-2578G), was recently found to be associated with higher serum levels of MCP-1 and higher prevalence of MI in the Framingham heart study, also after adjustment for other CAD risk factors (49). This latter study, trying to relate a gene polymorphism not only to the prevalence of cardiac events, but also to phenotypic MCP-1 alterations (i.e. raised serum levels) in a relatively large study population (n=1,609), may be of particularly importance. In contrast to these associations between MCP-1 polymorphism and atherosclerotic disorders, the relation between the polymorphism of the MCP-1 receptor CCR2 gene, in which the valine at amino acid 64 in the first transmembrane domain is replaced with isoleucine (V64I), and CAD is unclear (44, 50, 51). This may at least partly reflect that the mutant CCR2-V64I protein has not been shown to be functionally defective or to have different levels of expression (44), underscoring the importance of relating gene polymorphisms to functional alterations.

**Polymorphisms in the genes for RANTES and its receptor (CX3CR1) gene**

Two polymorphisms have been identified in CX3CR1; one which causes a codon change from valine to isoleucine at position 249 (CX3CR1-I249) and another that causes a codon change from threonine to methionine at position 280 (CX3CR1-M280) (56). These changes are located in the sixth and seventh transmembrane domains, respectively. At least four important studies on the association between CX3CR1 polymorphisms and atherosclerosis have been published. First, when CX3CR1 genotypes were analyzed in 151 patients with ACS and in 249 healthy controls, Moatti et al. found that CX3CR1 I249 heterozygosity was associated with a markedly reduced risk of acute coronary events, independent of established acquired coronary risk factors (57). In another study, genotyping of the CX3CR1-V249I polymorphism was performed in a cohort of 339 white individuals who underwent cardiac catheterization (197 with and 142 without CAD), showing that the CX3CR1 I249 allele was associated with decreased risk of CAD and improved endothelium-dependent vasodilation (58). Third, McDermott et al. showed that CX3CL1-dependent cell-cell adhesion under conditions of physiologic shear was severely reduced in cells expressing CX3CR1-M280 (59). This was associated with marked reduction in the kinetics of CX3CL1 binding as well as reduced CX3CL1-induced chemotaxis of primary leukocytes from donors homozygous for CX3CR1-M280. Importantly, these authors also showed that CX3CR1-M280 is independently associated with a lower risk of atherosclerotic cardiovascular disease in the Offspring Cohort of the Framingham Heart Study, a long term prospective study of the risks and natural history of this disease (204 cases and 1,655 controls) (59).
This latter study, reporting association between CX3CR1 coding polymorphisms and various atherosclerosis end points in the Framingham Offspring Cohort contrasts with most association studies principally because the authors included functional evaluation of the actual polymorphisms. Finally, a recent cross-sectional study by Niessener et al., analyzing 720 patients with verified CAD, suggests a more complex interaction between the two CX3CR1 polymorphisms showing protective effect of the M280 polymorphism and a harmful influence of the I249 polymorphism on the occurrence of the I249 polymorphism (60).

Limitations of the polymorphism studies

Will the presence of the homozygous state for certain chemokine/chemokine receptor polymorphism come to be viewed as a standard cardiovascular risk factor? The tools and general knowledge that are necessary to approach these issues appear to be available. The results from some of these studies may provide new conceptual, diagnostic, and therapeutic approaches to vascular diseases. However, the single nucleotide polymorphisms (SNPs) studies also have several limitations (61). Although the standards and quality seem to be improving, there is nevertheless a risk that SNP-based association analyses will squander academic trust and scientific resources owing to unsatisfactory analysis. Moreover, some of the chemokine/chemokine receptor genotypes are rare, making epidemiological conclusions difficult except in the largest cohorts. Furthermore, the strength of such studies will greatly improve if the actual polymorphism could be related to phenotypical alterations with relevance to atherogenesis.

Conclusions

Although several studies support an important pathogenic role of chemokines in atherogenesis and plaque destabilization, potentially representing attractive therapeutic targets in atherosclerotic heart disease, their role as clinical biomarkers is unclear, and further prospective studies, including a larger number of patients, are needed to make any firm conclusion. While the demonstration of an association between chemokines and CAD is a necessary first step, such studies do not establish the full clinical utility of a biomarker, which is a more demanding process that requires validation in multiple cohorts, a reliable and cost-effective assay, and clear demonstration of incremental prognostic value over traditional risk models. If successful, however, such new a biomarker will be a useful indicator for better risk assessment, diagnosis, and prognosis, as well as for monitoring pharmacological treatments for atherosclerosis.

References