

Inflammation and peripheral arterial disease: The value of circulating biomarkers (Review)

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Abstract. Peripheral arterial disease (PAD) is a manifestation of atherosclerotic vascular disease and is often associated with other comorbidities, such as hypertension, diabetes and dyslipidemia. An increasing body of evidence supports the notion that inflammation plays an important role in the development and progression of PAD. A number of studies have investigated the association of various acute phase proteins, particularly C-reactive protein (CRP), with PAD. Apart from CRP, other circulating biomarkers, such as matrix metalloproteinases (MMPs), selectins and interleukin (IL)-1, IL-2, IL-6, IL-8 and IL-10 have been considered to play a role in the development of PAD. In this review, the role of these circulating biomarkers in PAD is discussed. Current data indicate that the appropriate use of biomarkers in patients with PAD may contribute to an early diagnosis, an enhanced knowledge of the developmental process of the disease, as well as to the subsequent improvement of current therapies and to the development of new ones.

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1. Introduction

Peripheral arterial disease (PAD), is one of the most common manifestations of atherosclerosis, affecting 27 million individuals in Europe and North America (1).

In 1858 Charcot (3) clearly defined and described this syndrome (and he used the term ‘intermittent claudication’) (2,3). Intermittent claudication is reproducibly elicited by walking-induced muscle ischemia and is consistently relieved by rest that allows reperfusion of the affected limb; thus, it may be considered as ‘a leg effort angina.’ Since the 1950s, Stammers (4) and Allen *et al* (5) independently observed that patients with intermittent claudication have a high risk of mortality due to cardiovascular events (CVs). Subsequent prospective studies on the clinical outcome of patients with PAD have yielded divergent findings; in fact, they rarely progress to limb loss; however, PAD is a powerful and independent predictor of CVs, both cardiac and cerebral (6-10). Similar to all atherosclerotic diseases (myocardial infarction, unstable angina, abdominal aortic dilatation and carotid artery disease), arterial hypertension, smoking, diabetes mellitus and hypercholesterolemia are widely considered as crucial risk factors for the development of PAD. Formerly, it was considered that arterial narrowing (stenosis), more or less critical in terms of haemodynamic efficiency, tended to lead to arterial thrombosis and as a consequence, to tissue ischemia. It is now known that the degree of arterial stenosis due to atheromatous plaque plays a significant role; however, other factors must also be considered in relation to their likelihood of causing thrombosis. A large number of cases of myocardial infarction are caused by stenosis of the coronary arteries with >50% narrowing of the lumen (11); biomechanical stresses experienced by non-obstructing atheroma may be greater than those caused by stenoses, which yield a smaller residual lumen (14). Instead, the thrombus usually occurs due to a disruption of atherosclerotic plaque (12,13). Different models of physical plaque disruption are known. In fact, a superficial erosion may occur, leading to the contact of platelets with pro-aggregatory collagen in the intima membrane (15,16), whilst the majority of thromboses result from a rupture of the protective fibrous cap of the plaque, leading to the contact between blood and the highly thrombogenic material located in the lipidic core (e.g., tissue factor). The rupture of plaque plays a critical role in arterial thrombosis, considering the biomechanical ability

of the plaque fibrous cap; the fibrous cap determines the stability of plaque. Thus, this critical issue must be analysed in molecular terms in order to provide effective means of determining the clinical outcome of patients with PAD and to obtain positive results which may improve prognosis and to reduce the extent of arterial damage and comorbidities in patients with PAD.

Data presented since 1815, the year in which cholesterol was discovered, although not yet correlated with atherosclerosis, have cited inflammation as the underlying cause of atherosclerosis (17,18). In 1858, Virchow (19) found inflammatory cells in vascular plaques, and Osler (20) in 1908 suggested that inflammation and infection play a role in the pathogenesis of atherosclerosis. However, for almost a century, the inflammation hypothesis was ignored, whilst greater emphasis was placed on the putative role of cholesterol in atherosclerosis. Finally, at the end of the last millennium, many lines of evidence suggested alternative mechanisms to the cholesterol theory. Ross (21) branded atherosclerosis an inflammatory disease; to date, atherosclerosis is not only considered a disorder of pathological lipid deposition, but also as a dynamic and progressive pathophysiological process arising from a combination of endothelial dysfunction and inflammation interacting with standard risk factors. All these factors contribute to the initiation and progression of arterial damage, consequently causing clinical manifestations, as well as cardiovascular and atherosclerotic diseases, including PAD (22,23).

This review focuses on the association between inflammation and PAD. Inflammatory markers along with other circulating biomarkers are discussed in the context of PAD.

2. Inflammation

Inflammation plays a role in the initiation and progression of PAD, and in coronary artery disease (CAD) (23-32). There are several candidate inflammatory markers, including traditional risk factors that play a pro-atherogenic role, at least in part, through an inflammatory mechanism. Cigarette smoking and diabetes mellitus, the strongest risk factors for the development of PAD, promote oxidative stress, which directly and indirectly enhances inflammatory pathways (33-38). Inflammation may also be a factor in arterial hypertension, which affects approximately 80% of patients with PAD (39). Indeed, angiotensin II elicits the production of reactive oxygen species and the expression of vascular cell adhesion molecule-1 from endothelial cells (40,41). Angiotensin II increases the expression of pro-inflammatory cytokines, such as interleukin (IL)-6 and monocyte chemoattractant protein-1 (MCP-1) by arterial smooth muscle cells (SMCs) (41-43). Dyslipidemia can activate inflammatory functions by modifying the oxidation of low-density lipoproteins and of very low-density lipoproteins (44,45). Other conditions that promote a systemic inflammatory response and may predispose the arterial vessels of the lower limbs to atherosclerosis are infections and systemic diseases, such as rheumatoid arthritis and systemic lupus erythematosus. Whatever the inflammatory trigger, a large number of cross-sectional and longitudinal studies have demonstrated a close link between inflammation and PAD (23-32). Notably, each of the inflammatory molecules investigated in these studies is not simply a

marker of inflammation but plays an active role in peripheral atherogenesis (46-53). In 1998, Ridker *et al* (24) in a prospective, nested case control study carried out in apparently healthy men enrolled in the Physician's Health Study, found that the relative risk of developing PAD increased significantly with each increasing quartile of baseline C-reactive protein (CRP) concentration. This result was independent of body mass index, hypercholesterolemia, diabetes mellitus and a family history of premature atherosclerosis. The Edinburgh Artery Study also revealed that elevated levels of soluble intercellular adhesion molecule-1 (ICAM-1), but not of soluble vascular cell adhesion molecule-1, are independently associated with the development of PAD (32).

3. CRP

CRP is a marker of inflammation derived from leukocytes in response to IL-6 stimuli. Several actions are induced by this protein, such as the release of endothelial monocyte chemoattractor protein-1 (MCP-1), which in turn attracts monocytes towards the endothelial barrier. CRP also upregulates the release of the tissue factor and of other pro-inflammatory cytokines. Finally, CRP inhibits the release of nitric oxide (NO). The association between CRP and the development of PAD has been extensively investigated, as demonstrated from the findings of a large cross-sectional study that showed a direct association between higher levels of CRP with a lower ABI value (60). Moreover, the plasma levels of CRP were found to increase progressively as the ABI decreased in patients with PAD. Based on these results, it was postulated that CRP is an independent risk factor for the development of PAD and that subjects with higher levels of CRP have a two-fold increased risk of developing PAD. This inflammatory marker was also considered to predict the clinical outcome of arterial revascularization in patients with PAD, and conclusively a study suggested that a low plasma level of CRP of 50 mg/l is sufficient to predict the success of percutaneous angioplasty in patients with PAD and diabetes. Based on several findings from studies on patients with PAD, the data address the efficacy of a low grade of inflammation in ameliorating the outcome in patients with PAD, and also in reducing the risk of progression towards critical limb ischemia (54-61).

4. Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a family of Zn^{2+} -dependent enzymes that catalyze the proteolysis of many connective extracellular matrix (ECM) tissue proteins, including collagen, fibronectin, gelatin, laminin and proteoglycans (62). MMPs are produced by a number of cells, including leukocytes, macrophages, astrocytes, neurons and microglia. These cells are involved in numerous physiological processes, including tissue remodelling during development and platelet aggregation. The activity of MMPs is negatively regulated by the so-called tissue inhibitors of matrix metalloproteinases (TIMPs) and the level of MMP activity is dependent on the balance between MMPs and TIMPs. Physiologically, the ECM proteins contribute to vessel wall integrity, as demonstrated by the role of MMPs in the development of arterial lesions and also in facilitating monocyte invasion (63). Studies using

the gelatin zymography method have shown that two MMPs (MMP 242 and 943) are involved in arterial remodelling processes associated with atherogenesis. It has been further demonstrated that MMPs are synthesized directly in atherosclerotic plaques and are particularly found in arterial plaques more prone to rupture (64). High plasma levels of MMPs have been found in patients more prone to arterial damage, such as patients with type 2 diabetes and PAD, and the results have demonstrated an increased and chronic or latent level of inflammation in patients with PAD (65).

5. Selectins

Selectins, include E-selectins, L-selectins and P-selectins. L-selectins are a family of type-1 cell surface glycoproteins, and selectins are expressed on a variety of white blood cells, such as granulocytes, monocytes and lymphocytes. P-selectin is stored in platelet granules and in Weibel-Palade bodies of endothelial cells. These glycoproteins are moved to the cell surface of endothelial cells and platelets when these cells are present in an activated form. E-selectin is not normally expressed, but is upregulated by the presence of inflammatory cytokines. The similar sequence of their homology suggests that they are able to bind similar sugar structures. This part of the molecules is known to be responsible for the different targeting and behavioural abilities of selectins. P-selectin is a target for secretory platelet granules, E-selectin for plasma membranes, and L-selectin for the tips of microfolds in leukocytes. The molecules play a crucial role in immune response, and thus these cells are deeply involved in ischemia, and also in determining the severe pathophysiological situation of ischemia-reperfusion. The effective role played by selectins in thrombus formation has been well documented by the interaction between P-selectin glycoprotein ligand 1 and P-selectin, and this interaction promotes the upregulation of the tissue factor. P-selectin is largely involved in platelet aggregation, which is a crucial key point for arterial thrombosis. These selectins were the focus of a study carried out on patients with PAD without or with type 2 diabetes (66). The results revealed high plasma levels of E-selectin (67), suggesting the involvement of this selectin in the activation process of endothelial cells that is known as crucial in the atherogenic process. In patients with PAD, plasma levels of all selectins were found to be higher compared with other groups and these levels increased following incubation with PAD plasma (68). This finding is significant, demonstrating the adhesion of neutrophils to fibrinogen. Conclusively, P-selectin plays a role in modulating the adhesion of neutrophils and is possibly associated with a lower wall shear stress of the arteries. Consequently, P-selectin is able to enhance the recruitment of activated white blood cells, leading to arterial injury. In patients with severe forms of PAD, such as critical limb ischemia, higher levels of E-selectin have been found. The role of selectins was demonstrated by a study on the polymorphisms of E-selectin. The polymorphisms were found to be independently and significantly associated with the development of PAD. Thus, it can be hypothesized that patients with PAD have a common pro-inflammatory genetic profile (66,69).

In light of this, we have to consider the clinical and beneficial effects of anti-platelet agents (aspirin, clopidogrel as an

antagonist of platelet P2Y₁₂ ADP receptor and cilastazol as a phosphodiesterase inhibitor) on the plasma levels of P-selectin. Thus, this can be considered as another mechanism to explain the efficacy of anti-platelet treatment for patients with PAD beyond the simple inhibition of platelet aggregation (70-73). E-selectin can be also utilized as a medical target to evaluate the efficacy of physical supervised training in patients with PAD. The findings from a study (73) on PAD showed the positive effects of an eight-week period of exercise training on the plasma levels of E-selectin. The levels reduced after the training period; these results are proof of the pivotal role that physical training plays in the treatment of PAD and may aid in elucidating the underlying mechanisms.

6. IL-1

IL-1 is a typical pro-inflammatory cytokine and is a glycoprotein shown in two isoforms (IL-1 α and IL-1 β) both encoded by separate genes on chromosome 2. These two glycoproteins act through sharing their receptor, IL-1R, present in a number of cell types. They act as endogenous pyrogens that are specifically synthesized by mononuclear cells. IL-1 stimulates both B and T lymphocyte proliferation and IL-2 receptor expression and the expression of neutrophil adhesion factors on the surface of endothelial cells. It also increases the proliferation of neutrophils, macrophages, lymphocytes, as well as chemotaxis activity. Furthermore, IL-1 increases collagen synthesis in fibroblasts and in epithelial cells, and increases the hepatic synthesis of acute phase proteins. Based on the listed biological activities, it seems to be easy to explain the potential pro-atherogenic role played by IL-1 (29,74-76). IL-1 is now considered a pivotal cytokine involved in vessel wall abnormalities that occur during high or low levels of inflammation and the start of atherosclerotic plaque formation. The data presented in several studies demonstrate that the increased plasma levels of IL-1 correlate with a higher risk of plaque instability (77). However, high levels of IL-1 have not been reported in structured studies focused on patients with PAD. The pro-atherogenic role of IL-1 may be attributed to the increased production of IL-6, as was found in the Edinburgh Artery Study, that showed IL-1 as an important predictive marker of progressive outcome in patients with PAD (32). The putative role of IL-1 in PAD can also be demonstrated through the anti-inflammatory activity of the receptor antagonist of IL-1 (IL-1ra). It is another member of the IL-1 super family that seems to have a paradoxical anti-inflammatory property by regulating IL-1 activity. It has been demonstrated that higher IL-1ra levels are often associated with prothrombotic situations. On the other hand, the exogenous administration of IL-1ra proved useful in many inflammatory diseases by improving clinical conditions and reducing organ damage. Similarly, IL-1ra has been shown to be upregulated in atherosclerosis and to correlate with the clinical progression of PAD (79).

7. IL-2

The IL-2 family consists of four members: IL-2, IL-4, IL-5 and granulocyte-monocyte colony-stimulating factor (GM-CSF). IL-2 is a glycoprotein with a molecular weight of 15,500 Da encoded by a gene found on chromosome 4. It is produced by

activated Th1 cells and antigens. IL-2 is a T and B lymphocyte growth factor, and indeed a key activator of cytotoxic lymphocytes and nuclear killer (NK) cells. In turn, lymphocytes also stimulate the production of other cytokines, such as IL-4 and interferon γ (IFN- γ). In atherosclerotic diseases, high levels of IL-2 have been detected in atheromatous plaques and also in the plasma and in the vascular tissue of patients with abdominal aortic aneurysms (80-82). Despite this evidence, the role of IL-2 in the pathogenesis and/or in the progression of various atherosclerotic diseases has not yet been determined. In light of this, the results of two studies, the Veterans Affairs (VA) Cooperative Study #410 and the Iron (Fe) and Atherosclerosis Study (FeAST) did not show an association between IL-2 plasma levels and the mortality risk of patients with PAD. However, a direct association was found between the ferritin plasma level and IL-6 levels (83,84).

8. IL-6

IL-6 is a glycoprotein whose gene is located on chromosome 6 and is synthesized by several cells, including Th2 lymphocytes, B lymphocytes, macrophages, endothelial cells, fibroblasts, mast cells and some tumour cells (67). Several studies carried out in recent years have established a wide range of functions of IL-6 in many physiological and pathophysiological processes. Moreover, the pivotal role played by IL-6 in many pathological conditions is well known (85). The involvement of IL-6 in the pathophysiology of atherosclerosis has been well studied; it was specifically considered in studies focused on the association between the inflammatory process and PAD. It is known that IL-6 plays a role in stimulating hepatic production in a wide range of inflammatory molecules, so-called acute-phase proteins, such as CRP, fibrinogen production, the release of complement factors and the production of serum amyloid A (86). IL-6 has several activities in the formation and maintenance of atherosclerotic plaque and it stimulates the production of MCP-1 and IL-8 by endothelial cells and macrophages. It also increases the release of chemokines by intimal cells of the arterial wall and it seems to be able to increase the production of ICAM-1 by SMCs, and it promotes the homing of leukocytes into atherosclerotic plaque. Finally, IL-6 promotes the transformation of SMCs into foam cells (32). The findings from studies on healthy individuals have shown a direct and significant association between the plasma levels of IL-6 with certain inflammatory biomarkers involved in the progression of atherosclerosis (i.e., TNF- α and CRP), and these markers are associated with an increased risk of mortality in patients with cardiovascular diseases. It has been shown that IL-6 is an innovative predictor of PAD and a reliable marker in predicting disease clinical progression over a 12-year period. In fact, its role in PAD has been clearly defined and data from community studies have shown that it must be considered as a predictive and independent marker in the development of PAD (87). Furthermore, we have previously demonstrated that the IL-6 GG genotype promotes the development of PAD among individuals with type 2 diabetes by inducing the increased release of IL-6. Higher concentrations of IL-6 among those patients with the GG genotype is associated with increased plasma concentrations of fibrinogen and CRP (88).

9. IL-8

IL-8 was originally known as neutrophil activating protein-1 (NAP-1) and is a small protein consisting of only 79 amino acids. It is mainly produced by monocytes, macrophages and endothelial cells in response to various inflammatory stimuli. It acts by a specific receptor found only on neutrophils and it also induces the expression of β -2 integrins on neutrophil surfaces. This last activity plays a fundamental role in trans-endothelial migration (78). It has been widely demonstrated that IL-8 is closely related to the progression of atherosclerotic lesions and in fact, high levels of IL-8 have been found in atherosclerotic plaques, rich in macrophages. In particular, IL-8 has been found in patients with abdominal aortic aneurysms and carotid artery disease (67). In addition, mice negative for the IL-8 receptor (-/-) have shown limited atherosclerotic plaques (79,80). IL-8 is also able indeed to reduce the stability of atherosclerotic plaque by leading to an increased activity of MMPs through the blockade of TIMP-1 (81). To date, there are conflicting data on the role played by IL-8 in patients with PAD. However, the results from a study performed on patients with PAD that were submitted to vascular surgical procedure revealed a higher production of IL-8 in polymorphonuclear leukocytes [Marino *et al* (89)]. The plasma levels of IL-8 were lower in patients with PAD compared to healthy individuals and to subjects with cardiovascular risk, both under resting conditions and following stimulated production.

10. IL-10

IL-10 is a pure protein and is mainly produced by Th2 lymphocytes, and, to a lesser extent, by monocytes, macrophages and activated T lymphocytes. It inhibits the production of cytokines by Th1 lymphocytes (IFN- γ , TNF- α , IL-2 and IL-3) and it also inhibits the production of interleukins derived from macrophages (IL-1, IL-6, IL-8, GM-CSF, TNF- α). IL-10 gene transfer can significantly reduce the atherosclerotic plaque area and the macrophage infiltrated area (90). Other relevant proof of the anti-inflammatory and immunosuppressive activity of IL-10 has been demonstrated by Mallat *et al*. They showed that the lack of the IL-10 (IL-10-deficient C57BL/6J mice) determines a significant 3-fold increase in lipid accumulation in atherosclerotic plaque compared with wild-type mice, and also showed an increased T-cell infiltration and an abundant IFN- γ expression, and decreased collagen content in the atherosclerotic lesions of deficient mice (91). In advanced atherosclerotic plaques, IL-10 was found and this finding was associated with the reduced apoptosis of cells of the lipid core and thereby in the reduced risk of the plaque rupture (92). To date, few studies have focused on the role of IL-10 in PAD. In particular, it is difficult to demonstrate the correlation between the levels of IL-10 with the progression of atherosclerotic lesions and clinical outcome. In fact, it is still unclear whether high plasma levels of IL-10 are to be considered as a marker of anti-inflammatory patterns (with a reduced likelihood of developing atherosclerosis), or conversely whether they are as counter regulatory consequences related to primitive pro-inflammatory patterns. Data from studies on CAD have linked the high plasma levels of IL-10 with a lower pro-inflammatory profile and with a better prognosis in

patients with acute coronary syndrome (93,94). By contrast, few studies suggest a correlation between PAD and reduced plasma levels of IL-10 and this emphasizes the uncertain role played by this cytokine in the pathogenesis of atherosclerotic processes. Of note, such polymorphisms of the IL-10 gene are related to altered transcriptional activity and protein levels. A study performed by Blanco *et al* (95) showed that the IL-10 proximal promoter haplotype IL-10G*11/-1082G/-819C/-592C is more frequent in patients with aortic severe occlusive disease than in the control subjects. In addition, it was found that the IL-10 ATA haplotype seemed to correlate with a short-term risk of acute post-operative cardiovascular events and in PAD patients with reduced endothelial function (96).

11. Conclusion

It is clear from this review that recent progress in the pathophysiology of PAD have implicated inflammation as a key contributor to the initiation of vascular damage. Circulating biomarkers, apart from CRP, such as MMPs, selectins, IL-1, IL-2, IL-6, IL-8 and IL-10, are useful for the clinical characterization of PAD. In fact, an increasing body of evidence supports the notion that several circulating biomarkers are associated with the main aspects of PAD. The demonstration that cytokines and MMPs play a pathogenic role in PAD may encourage the development of novel therapeutic approaches for the prevention and management of PAD. However, additional studies on this matter are required to develop clinically useful markers of PAD by using novel approaches, such as proteomics.

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