


Inflammatory Markers Related to Innate and Adaptive Immunity in Atherosclerosis: Implications for Disease Prediction and Prospective Therapeutics

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Abstract: Several lines of evidence have linked a dysregulated inflammatory setting to the pathogenesis of atherosclerosis, which is a form of chronic vascular inflammation. Various inflammatory biomarkers have been associated with inflammation and are recognized as potential tools to monitor the progression of atherosclerosis. A well-studied inflammatory marker in the context of cardiovascular diseases is C-reactive protein (CRP) or, more accurately, highly sensitive-CRP (hs-CRP), which has been established as an inflammatory biomarker for atherosclerotic events. In addition, a growing body of investigations has attempted to disclose the potential of inflammatory cytokines, enzymes, and genetic polymorphisms related to innate and adaptive immunity as biomarkers for predicting the development of atherosclerosis. In this review article, we clarify both traditional and novel inflammatory biomarkers related to components of the innate and adaptive immune system that may mirror the progression or phases of atherosclerotic inflammation/lesions. Furthermore, the contribution of the inflammatory biomarkers in developing potential therapeutics against atherosclerotic treatment will be discussed.

Keywords: atherosclerosis, inflammation, immune response, biomarker, hs-CRP

Introduction

Since the introduction of the “response to injury” hypothesis, atherosclerosis has been considered as chronic inflammation in the vascular system.¹ This hypothesis holds that endothelial injury stimulates a complex of responses, particularly inflammatory reactions, that finally manifests as atherosclerosis. The accumulation of low-density lipoprotein (LDL) in the arterial intima of vessels and its subsequent oxidation (ox-LDL) are the initial steps involved in the initiation of atherosclerosis. Then, the inflammatory state in the endothelium develops as a series of events that lead to the infiltration of monocytes and lymphocytes into the vessel wall. These events promote an inflammatory condition in the vessel wall.² Monocytes recruited to the inflammation site differentiate into macrophages, which engulf the ox-LDL and then develop into foam cells. Consequently, these occurrences increase the size and volume of atherosclerotic plaques. The inflammatory immune cells present in the plaques release several factors, such as matrix metalloproteinases (MMPs), that contribute toward the degradation of the extracellular matrix (ECM).

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MMPs and other enzymes promote the loosening of the fibrous cap and plaque instability/rupture. Ultimately, atherosclerotic plaque rupture culminates in the development of a thrombus (Figure 1). Acute coronary syndrome occurs in the case of the vessel being occluded by the rupture of the plaque.³

The bulk of evidence suggests that various inflammatory factors may be increased in different phases of atherosclerosis initiation and perpetuation. These factors have been studied concerning the development of inflammatory biomarkers related to innate and adaptive immunity to identify specific and sensitive elements in the prognosis of cardiovascular risk.⁴ In this study, we review the research on the development of inflammatory biomarkers in the context of optimized monitoring of atherosclerosis.

Endothelial Function in Atherosclerosis

Over the past few decades, endothelial dysfunction has been suggested to be linked to cardiovascular events.⁵ The vessel endothelium plays a critical function in establishing homeostasis of the vessel wall. In normal physiology, the vessel endothelium modulates the relaxation of the vascular tone

and establishes a lower state of oxidative stress by regulating the function of angiotensin as well as producing mediators such as endothelin, prostacyclin, and nitric oxide (NO). The vessel endothelium is also involved in controlling the permeability of the vessel wall to blood serum, adhesion and accumulation of circulating immune cells and platelets, and coagulation events such as thrombosis.⁶

However, various settings can cause an imbalance in the physiological state of the vessel endothelium.⁷ A number of stimulating factors might alter the phenotypical characteristics of the endothelium toward a condition called endothelial dysfunction, in which the mechanisms regulating homeostasis of the endothelium are abrogated. During pathological endothelial dysfunction, the vascular tone is dysregulated, the release of inflammatory and thrombosis-stimulating mediators is promoted, expression of adhesion molecules is upregulated, and generation of oxidative stress components is enhanced.⁸

Several lines of evidence endorse the involvement of endothelial dysfunction in the initial phases of atherosclerosis development. The formation and progression of atherosclerotic plaques have also been associated with endothelial dysfunction. People with cardiovascular risk

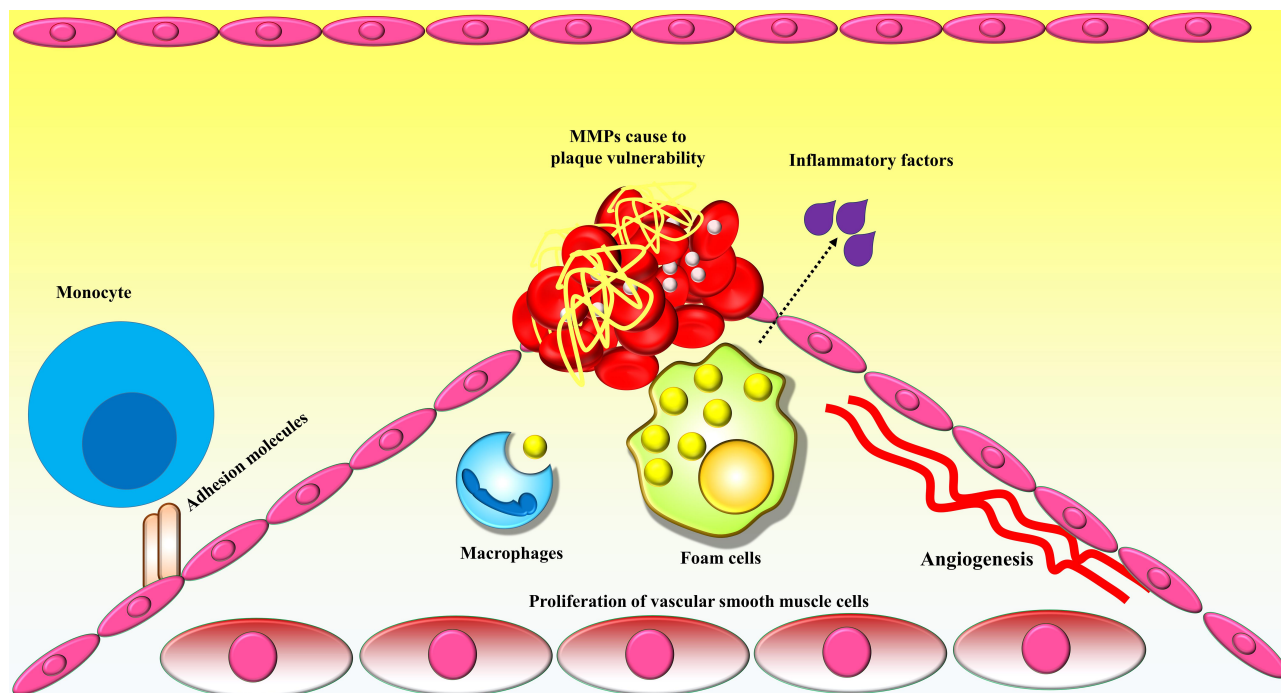


Figure 1 Schematic overview of the process of atherosclerosis. Monocytes infiltrate the inflammation site and differentiate into macrophages, which engulf the ox-LDL, and then develop into foam cells, which in turn increase the size and volume of atherosclerotic plaques. Foam cells, as well as other inflammatory immune cells present in atherosclerotic plaques, release several factors, among which extracellular matrix (ECM)-degrading enzymes such as matrix metalloproteinases (MMPs) contribute to the degradation of the ECM, resulting in loosening of the fibrous cap and plaque instability/rupture. Finally, atherosclerotic plaque rupture culminates in the development of a thrombus.

factors, yet lacking clinical presentations of atherosclerosis, may present with endothelial dysfunction. Such individuals have an abnormal function of vessels in response to endothelial vasodilators.⁹ Therefore, endothelial dysfunction may be considered as a bridge between risk factors for atherosclerotic and atherosclerosis development. Moreover, in patients with risk factors for atherosclerosis and other cardiovascular disorders, endothelial dysfunction may be an independent predictor of future cardiovascular events.¹⁰

Numerous studies have testified that a prolonged inflammation related to innate and adaptive immune abnormality is involved in the development of atherosclerosis; therefore, atherosclerosis is currently regarded as an autoinflammatory disorder.¹¹ Patients with autoimmune disorders have been shown to have an increased rate of subclinical atherosclerosis, suggesting a relationship between atherosclerosis and autoimmunity and a shared mechanism in the pathogenesis of these pathological conditions.^{12,13} During the autoimmune response events in atherosclerosis, several atherosclerosis-related elements, such as ox-LDL, may act as an autoantigen source to trigger inflammatory innate and adaptive cells, such as monocytes, macrophages, T and B cells, that finally promote atherosclerotic plaque formation, plaque expansion, and instability/rupture of the plaques.^{14,15}

Research has shown that ox-LDL plays an important role in atherosclerosis development during autoimmune disorders and should be regarded as a critical triggering factor of chronic inflammation.¹⁶ Interaction of ox-LDL with the vessel wall leads to activation of the endothelial cells and overexpression of several factors, including adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), as well as the release of chemokines and cytokines, resulting in the infiltration of immune cells to the site of vessel involvement.¹⁷ Monocytes recruited to the site of endothelial injury develop into macrophages that internalize the ox-LDL, leading to the formation of foam cells that are crucially involved in the release of several inflammatory factors.¹⁸ Studies have revealed that ox-LDL interacts with C-reactive protein (CRP) to develop the ox-LDL/CRP formations, which stimulate autoimmune reactions, vascular inflammation, and atherosclerosis development.¹⁹

Several inflammatory biomarkers have been developed to assess the endothelial dysfunction during atherosclerosis development and various medications are utilized to

control the inflammatory responses in atherosclerotic individuals,^{20,21} which will be discussed in this article.

Innate and Adaptive Immunity in Atherosclerosis

Innate Immunity in Atherosclerosis

In the early phases of the inflammatory response during atherosclerosis, studies have indicated the involvement of critical cells playing a role in the cellular arm of innate immunity, namely, monocytes/macrophages. Studies on human arterial tissues and animal models of atherosclerosis have demonstrated that the infiltration of monocytes into the intima is the initial event during atherogenesis. The infiltration of monocytes to the region involved in atherosclerosis requires the adhesion of these cells to the activated endothelial cells through adhesion molecules, named selectins and integrins. The inflammatory cytokines and chemokines modulate the infiltration of monocytes into the region involved in atherosclerosis. After migration, monocytes differentiate into macrophages, proliferate, and release a wide range of mediators. These classical events have already been reviewed in detail.^{22–24} Herein, we assess the hypothesis regarding the involvement of innate immunity in the process of atherogenesis.

Research has disclosed new aspects of monocyte infiltration to the intima during atherosclerosis. Surveys on the kinetics of monocyte infiltration to the involved sites in animal models of atherosclerotic indicate that monocytes migrate to the intima layer both in the early phases of lesion development and over the course of an established atherosclerotic lesion.²⁵ This highlights that monocyte trafficking could be targeted for therapeutic purposes in atherosclerosis.

The heterogeneity in the monocyte population has been focused on as a critical issue in determining the severity and outcome of an atherosclerotic lesion.²⁶ Observations from human samples and mouse models propose the double-morphism of monocyte phenotypes according to the disease phase.^{27–29} Experiments in a mouse model indicated that higher lipid levels stimulate the development of a highly pro-inflammatory phenotype of monocytes. In humans, such pro-inflammatory monocytes express high levels of P-selectin glycoprotein ligand (PSGL) on the surface.³⁰ PSGL-expressing pro-inflammatory monocytes tend to migrate to the intima layer in atherosclerotic lesions to produce higher amounts of inflammatory mediators (particularly MMPs),

promoting the inflammatory innate immune response in the intima.

Studies have also suggested the potential involvement of mast cells in the inflammatory process of atherosclerosis. Although mast cells are found less often in atherosclerotic lesions, they may play critical roles in the pathogenesis of atherosclerosis.^{30–32} In atherosclerotic lesions, these cells have been shown to release several mediators, including heparin, histamine, serine proteinases, and leukotrienes, which suit the conditions for inflammation.^{33,34} Although these data have been illustrated in preliminary experiments, further research on human samples is still required. A number of drugs has already been devised to alter the function of mast cells, and hence could be further considered in the treatment of atherosclerosis.³⁵

The involvement of lipoproteins in the modulation of innate immune players has been established. Altered lipoproteins, such as ox-LDL, are able to bind to the scavenger receptors expressed on the macrophages and transduce pro-inflammatory signals into these cells.^{36,37} Some studies have focused on phospholipase A₂ (PLA₂), which is able to generate pro-inflammatory stimulators from the oxidized lipoproteins.^{38,39} PLA₂ is reported to be an inflammatory biomarker of cardiovascular diseases and to be involved in the pathogenesis of atherosclerosis.⁴⁰ In addition, apolipoprotein C₃ has been indicated to be able to ligate to the Toll-like receptors (TLRs), particularly TLR-2, on the immune cells, promoting the inflammatory signals in these cells.^{41,42}

A relationship between inflammation and thrombosis has been suggested.^{43,44} Thrombosis is proposed to be a mechanism of the innate immune system in defending against pathogenic microbes.⁴⁵ For instance, the cyclooxygenase pathway generates prostaglandins that are involved in the modulation of thrombosis and inflammation.⁴⁶ Hence, inhibitors of the cyclooxygenase pathway may increase the risk of thrombosis.^{47,48} Thrombin is a critical protein mediator that modulates coagulation and enables the production of inflammatory mediators from vascular endothelial cells and vascular smooth muscle cells (VSMCs).^{49,50}

Activated platelets produce and release inflammatory mediators and can upregulate the surface expression of inflammatory molecules, such as the ligand of CD40 (also known as CD154).^{51–53} Myeloid-related protein (MRP)-8/14, which is an inflammatory mediator, can also be generated and released by platelets.^{54,55} This molecule

has been indicated to be a biomarker for the prognosis of adverse events of the cardiovascular system in survivors of acute coronary syndrome.⁵⁶ Investigations have demonstrated that MRP-8/14 can ligate with TLR4, resulting in the activation of the innate immune system.⁵⁷ MRP-8/14 is also involved in the apoptosis of endothelial cells, an event that has been associated with thrombosis in the atherosclerotic plaque.⁵⁸ Taken together, the current knowledge implies a relationship between thrombosis and inflammation, proposing the involvement of these pathways in the development of atherosclerosis.

Adaptive Immunity in Atherosclerosis Cellular Immunity

A large amount of data has stressed the critical modulatory function of adaptive immunity in atherosclerosis.⁵⁹ Here, we discuss the recent findings on the implications of the adaptive immune system during atherosclerosis. A previous review has extensively described this pathway.⁶⁰

T cells are activated after recognizing antigens expressed on the dendritic cells (DCs).⁶¹ DCs are frequently found in atherosclerotic plaques as well as in the nearby draining lymph nodes, and play a role in the activation of T cells through presenting antigens and costimulatory molecules to these cells.⁶² The probable antigens that can trigger T-cell activation in the lymph nodes in the vicinity of the atherosclerotic plaques are microbial components, heat-shock proteins, and a number of plasma lipoproteins.^{63–65} T cells recognizing these antigens proliferate and cause the amplification of the immune response.⁶⁶ Then, interactions of the T cells with the same antigens culminate in the generation of the mediators and the development of inflammation. Cytotoxic T cells may also kill the cells expressing the antigens.⁶⁷

After activation, CD4⁺ T cells may develop a distinct subclass of the effector helper T (Th) cells. The Th1 responses are generally involved in the development of the inflammatory pathways through the production of cytokines, particularly interferon (IFN)- γ . Investigations indicate that both the Th1 and the Th17 response aggravate atherosclerosis.^{24,68} Th2 cells may develop a reaction that modulates the inflammation; nonetheless, research on the function of Th2 in atherosclerosis is conflicting.^{69–73} Some studies propose that Th2 responses may stimulate the development of aortic aneurysm formation.^{74–76} On the other hand, regulatory T (Treg) cells are involved in the suppression of the immune responses through different

mechanisms.^{12,77,78} Studies found that genetic manipulations of Treg cells (to interrupt their function) resulted in exacerbated atherogenesis in animal models, as presented by an increment in the size of lesions with upmodulated inflammation and the development of thrombosis.^{12,79} As a result, the responses by Treg and Th2 cells in relation to the inflammatory responses by Th1 and Th17 cells may contribute toward the improvement of atherosclerotic lesions.⁸⁰

T cells expressing the surface marker of CD8 are involved in the recognition of antigens expressed by the major histocompatibility complex (MHC) class I. CD8⁺ T cells kill the target cells by triggering apoptosis in the target cells.⁸¹ Several chemokines released in the atherosclerotic lesion milieu are involved in the recruitment of the CD8⁺ T cells, which may then stimulate apoptosis in other immune cells and VSMCs, resulting in the development of lesions and related adverse complications.^{82,83} One of the functions of the natural killer (NK) T (NKT) cells is to recognize lipid antigens presented by CD1 molecules on several cells. The activated NKT cells generate diverse inflammatory mediators that augment the development of atherosclerosis.^{84,85}

Humoral Immunity

Several lines of evidence have indicated that the humoral arm of the adaptive immune system, mediated by the antibodies produced by B cells, plays a critical role in the process of atherogenesis.^{86,87} Research has suggested the protective role of B cells in the development of atherosclerosis. It was shown that depletion of the B-cell colonies using splenectomy in animal models led to the exacerbation of atherosclerosis.⁸⁸ In animal models with hypercholesterolemia, a humoral immune response develops against ox-LDL, resulting in decreased establishment of inflammation by atherosclerotic macrophages.^{89,90} In addition, immunization of animals with ox-LDL, resulting in antibody production against these molecules, has been associated with the exacerbation of atherosclerosis. In a mouse model, the antibodies produced against ox-LDL also targeted a bacterial antigen on the pneumococcus.^{91,92} This observation casts doubt on the notion that the host immune responses against infectious antigens may overlap with inflammatory pathways during atherogenesis. The involvement of humoral immunity in targeting ox-LDL and probable protection against atherosclerosis may open up new horizons in devising therapeutic tools to prevent or mitigate the inflammatory state in atherosclerotic patients.

Inflammatory Biomarkers in Atherosclerosis

Upon identification of the inflammatory pathways involved in the etiology and pathogenesis of atherosclerosis, it makes sense to translate the biology of these events into clinical applications. The identification of bona fide inflammatory implications has led to novel potential therapeutic strategies being designed in the context of atherosclerosis.^{93,94} Several currently available medications used to suppress inflammation, such as glucocorticoids,⁹⁵ non-steroidal anti-inflammatory drugs (NSAIDs),⁹⁶ anti-inflammatory cytokine therapy,⁹⁷ and monoclonal antibodies,^{98,99} may be linked to the development of adverse complications that hinder their use as long-term treatments for the improvement of atherosclerosis. Several promising anti-inflammatory compounds developed for atherosclerosis therapy have not yet been approved for clinical application.¹⁰⁰ However, the application of inflammatory biomarkers to estimate the disease risk and monitor the efficacy of therapeutics, and in personalized therapy, has indicated their significant potential for clinical utilization.¹⁰¹

Inflammatory Biomarkers in Prediction of Atherosclerosis Risk

An association between diverse inflammatory biomarkers and estimation of the risk of cardiovascular events has been reported. The clinical applicability of a biomarker for the prediction of a disease risk relies on several factors, including reproducibility, practicability, ease of implementation, and cost-effectiveness.¹⁰² Of the numerous inflammatory biomarkers suggested for the diagnosis of atherosclerosis, most attention has been paid to lipoprotein-associated phospholipase A₂ (Lp-PLA₂),¹⁰³ pentraxin-3,^{104,105} myeloperoxidase,^{106,107} cytokines such as interleukin (IL)-6,¹⁰⁸ CRP or highly sensitive CRP (hs-CRP),¹⁰⁹ and proteases such as MMP-9.^{110,111}

C-Reactive Protein

According to numerous pieces of evidence, CRP has been highlighted as a critical inflammatory biomarker for clinical use.^{112,113} In healthy subjects without inflammatory disorders or acute infections, investigations have observed stable levels of hs-CRP (compared to cholesterol) for long periods.^{114,115} The beneficial points of CRP as a biomarker relative to other molecules include its remarkable stability, the fact that no invasive approaches are needed for sampling, and its comparably long half-life,

with no diurnal variation. Several large prospective cohort investigations have demonstrated that hs-CRP estimates the incidence of myocardial infarction, cardiovascular-related death, and stroke, even after adjusting for traditional risk factors.¹¹⁶ In subjects with psoriasis, the sub-clinical atherosclerosis risk was reported to be high and hs-CRP was found to be a beneficial biomarker for the prediction of the future risk of cardiovascular complications in these subjects.¹¹⁷ Unbiased computational methods have shown that hs-CRP, accompanied by parental history, augments the predictive ability of the traditional risk factors in detecting cardiovascular events.^{118,119} According to the Reynolds risk scores, hs-CRP plus parental history is able to perfectly recategorize subjects classified as having intermediate risk based on the traditional Framingham criteria.¹²⁰ hs-CRP and fibrinogen, as inflammatory biomarkers, have been implicated in fatty liver. Non-alcoholic fatty liver disease may predict future atherosclerotic cardiovascular disease, and the predictive value increases with the addition of inflammatory biomarker levels.¹²¹ Therefore, hs-CRP has the potential to be a useful auxiliary tool to the Framingham score to predict cases at increased risk of cardiovascular complications.

Highly Sensitive C-Reactive Protein

Research has also evaluated the potential of hs-CRP as a biomarker in evaluating therapeutics in cardiovascular events.¹²² In the clinic, several biomarkers are being followed as an approach to monitor the efficacy of the medications and to determine the optimal dose of drugs in patients with cardiovascular diseases.¹²³ For instance, the levels of LDL are routinely measured after the administration of lipid-lowering drugs. Considering that inflammation is involved in the different phases of atherosclerosis, it seems that hs-CRP could be used as an inflammatory biomarker to monitor the efficacy of treatments in soothing the inflammatory indices and clinical manifestations. In the Pravastatin or Atorvastatin Evaluation and Infection Therapy–Thrombolysis In Myocardial Infarction 22 (PROVE IT-TIMI 22) trial, it was proposed that statins may decrease LDL levels and soothe the inflammation in the context of cardiovascular diseases, as mirrored through the decreased level of hs-CRP in patients.^{124,125} According to this trial, statins were suitable compounds to reduce the hs-CRP levels to below 2 mg/L.¹²⁶ Other studies on the survivors of acute coronary syndromes supported the remarkable effect of statin therapy by lowering the hs-CRP levels.^{127,128} Despite the

endorsement of this evidence on the potential of monitoring hs-CRP to measure the efficacy of statin treatment, further explorations may demonstrate more advantages of this biomarker by following the efficacy of medications in lowering inflammation in patients with cardiovascular disorders.

Neutrophil Gelatinase-Associated Lipocalin

Neutrophil gelatinase-associated lipocalin (NGAL), an acute-phase protein that regulates inflammation,¹²⁹ is increased in cardiovascular disease¹³⁰ and has been investigated in the context of atherosclerosis. It was reported that the NGAL level was higher in patients with high total atherosclerotic plaque volumes compared to those with low plaque volumes. In addition, a high predictive score of NGAL was seen in patients without established cardiovascular disease. Therefore, it seems that NGAL could be an encouraging inflammatory biomarker to predict cases with asymptomatic atherosclerosis.¹³¹

Endothelial Dysfunction Biomarkers

There is a need to develop efficient biomarkers to monitor the change from normal endothelium to endothelial dysfunction. The adhesion molecules expressed on the endothelium, such as ICAM-1 and VCAM-1, have been regarded as well-accepted biomarkers to survey the endothelial dysfunction.¹³² It was also observed that ICAM-1 and VCAM-1 were associated with the development of new post-acute myocardial infarction symptoms in patients with heart failure.¹³³ Several inflammatory mediators, such as tumor necrosis factor (TNF)- α , are able to enhance the surface expression of adhesion molecules on the vessel endothelial cells.¹³⁴ Another study suggested the potential of soluble biomarkers in monitoring endothelial dysfunction, including E selectin, von Willebrand factor, soluble endothelial cell protein C receptor, thrombomodulin, and tissue plasminogen activator.¹³⁵

Studies have demonstrated that vascular homeostasis is manipulated by a balance between injury and repair of the endothelium.^{136–139} Reports show that the level of endothelial injury-related factors, such as endothelial microparticles (EMPs), is increased in the serum of individuals at risk for cardiovascular diseases, while the level of factors associated with endothelial repair, such as endothelial progenitor cells (EPCs), is reduced.^{137–139} As a consequence, enhanced EMP and decreased EPC may

have potential in estimating endothelial dysfunction and the increased risk of future cardiovascular events.^{140,141}

Despite progress in developing biomarkers of endothelial dysfunction, there are several caveats and points limiting their use in the clinic. Application of each biomarker individually may confer little efficacy; hence, a combination panel of biomarkers may offer better outcomes in distinguishing disease types, stratifying cardiovascular risk factors, revealing disease origin, and devising potential new targets for disease treatment.

Prospective and Future Directions Biomarkers for Anti-Inflammatory Therapy in Atherosclerosis

Many investigations have endorsed the theory that statin therapy decreases the level of inflammation in patients with cardiovascular complications. Advances in determining the etiology and pathogenesis of the underlying inflammatory mechanisms in atherosclerosis have revealed potential new tools to modulate inflammation in the context of atherosclerosis. To date, no large clinical trial has demonstrated that anti-inflammatory therapy could improve the cardiovascular complications, regardless of unaltered lipid profile. Even though some systemic anti-inflammatory treatments, such as NSAIDs or corticosteroids, do not seem to be hopeful compounds for the treatment of atherosclerosis, other treatments in this category appear to be promising.

Several clinical trials are under consideration to probe the potential of inhibiting PLA₂ as an anti-inflammatory treatment, even though the initial data were discouraging.¹⁴² On the other hand, several strategies to interrupt the trafficking of inflammatory cells to the atherosclerotic milieu and to inhibit the production of or neutralize the released inflammatory cytokines have gained remarkable attention for the treatment of atherosclerotic patients.^{143,144} Lipoprotein peptides have also been used as therapeutic vaccination in clinical trials.¹⁴⁵ Nonetheless, many clinical considerations need to be addressed before these anti-inflammatory modalities can be introduced as inflammatory biomarkers in the practical setting.

The conventional imaging of cardiovascular complications concentrates on anatomical observations. Many functional aspects of the cardiovascular system can be investigated using magnetic resonance and nuclear imaging methods. That notwithstanding, determining the molecular players of inflammation that are involved in the process of atherogenesis

has led to remarkable interest in their use as strategies for imaging. For instance, integrins, adhesion molecules (such as ICAM and VCAM), phagocytosis and glucose uptake (the function of inflammatory macrophages) evaluated using ¹⁸F-fluorodeoxyglucose, microvessels recognized via integrin-directed compounds, accumulation of modified LDL in atherosclerotic lesions, and ECM proteinases involved in plaque vulnerability have gained significant attention.^{146,147} Several investigations have indicated the applicability of these targeted imaging modalities and some of them are close to being applied in real clinical practice.^{148,149}

Reactive Oxygen Species Detection as a Biomarker in Atherosclerosis

Reactive oxygen species (ROS) are unstable and highly reactive by-products of oxygen that are generally produced as metabolites of endogenous processes involving molecular oxygen.¹⁵⁰ Studies have indicated the involvement of ROS produced by vessel wall cells in the inception and development of cardiovascular events. Among the pathophysiological complications resulting from high levels of ROS that have been implicated in the initiation and perpetuation of atherosclerosis are upregulation of adhesion molecules, apoptosis of vessel endothelial cells, activation of MMPs, and lipid oxidation.^{151–154} All of these events may interconnect with the elevated inflammatory state and exacerbate atherosclerosis. Therefore, a sophisticated system for the detection of ROS in the vessels seems to be critical in determining cardiovascular complications in atherosclerotic subjects.

Several approaches have been developed to assay mitochondrial superoxide (using the MitoSOX method) and superoxide (using dihydroethidium [DHE]) in mammalian cells and mouse aorta. Measurement of ROS can be accomplished using fluorescence dye-based techniques¹⁵⁵ or flow cytometry.¹⁵⁶ In the DHE-based detection of ROS, DHE permeates the cell membrane, leading to its oxidization via endogenous O₂^{•-}. When DHE is oxidized, two red-fluorescent compounds are generated: one is ethidium, which is produced by a non-specific redox reaction, and the other is 2-hydroxyethidium (a specific adduct of cellular O₂^{•-}). As a limiting point, these products have similar fluorescent spectra which overlap, making it difficult to apply fluorescence-based microscopic assays or confocal microscopy to detect them. As a result, a modified high-performance liquid chromatography (HPLC) method has been developed to accurately measure both ethidium and 2-hydroxyethidium. HPLC also has a limitation in its inability to discriminate the origin of the cell type that produced ROS in

Table 1 Inflammation-Related Biomarkers for Prediction of Cardiovascular Diseases

| Biomarker | Category | Involvement/Function | Implications | References |
|--|---|---|--|------------|
| Lipoprotein-associated phospholipase A ₂ (Lp-PLA ₂) | Innate/ adaptive immunity | Chemotactic activity, promotion of generation of inflammatory mediators | Lp-PLA ₂ is associated with coronary artery calcification, endothelial dysfunction, and carotid intima-media thickness | [103] |
| Myeloperoxidase (MPO) | Innate/ adaptive immunity | Contributes to inflammation | pro-MPO is involved in oxidative modifications of proteins and contributes to innate and adaptive inflammatory responses in cardiovascular disease | [107] |
| Interleukin-6 (IL-6) | Innate/ adaptive immunity | Involvement in various aspects of inflammation | In intermediate-risk patients, a serum IL-6 level >1 pg/mL is predictive of significant coronary artery disease | [108] |
| C-reactive protein (CRP)/highly sensitive CRP (hs-CRP) | Humoral innate immunity | Acute-phase protein that is produced during inflammatory conditions | Independent predictor of future cardiovascular events | [116] |
| Matrix metalloproteinase (MMP)-9 | Innate/ adaptive immunity | MMPs degrade collagen (vascular remodeling and endothelial dysfunction) and cause accumulation of inflammatory cells in the vessel wall | Strong predictor of events during heart failure | [110] |
| Neutrophil gelatinase-associated lipocalin (NGAL) | Innate/ adaptive immunity | Acute-phase protein that regulates inflammation | NGAL level is higher in patients with high total atherosclerotic plaque volumes. NGAL confers a high predictive score for future cardiovascular events in individuals without established cardiovascular disease | [130,174] |
| Intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion protein-1 (VCAM-1) | Innate/ adaptive immunity and endothelial dysfunction | Upregulation of ICAM-1 and VCAM-1 on endothelial cells contributes to infiltration of inflammatory cells to vessel wall | Increased serum levels of ICAM-1 and VCAM-1 in patients with heart failure. ICAM-1 and VCAM-1 are associated with the development of new post-acute myocardial infarction symptoms in heart failure subjects | [133] |
| Endothelial microparticle (EMPs)/endothelial progenitor cells (EPCs) | Endothelial dysfunction | EMPs are secreted during endothelial injury | Levels of endothelial injury-related factor EMPs are increased in the serum of individuals at risk for cardiovascular diseases, while the quantity of endothelial repair-associating factor EPCs is reduced | [137–139] |
| Reactive oxygen species (ROS) | Endothelial dysfunction | High levels of ROS promote endothelial dysfunction | High levels of ROS lead to upregulation of adhesion molecules, apoptosis of vessel endothelial cells, activation of MMPs, lipid oxidation, and endothelial dysfunction | [151–154] |
| Chromosome 9p21 (Chr9p21) genotype | Genetics | Expression of transcripts adjacent to the Chr9p21 locus of coronary artery disease is affected by the genotype at this locus and associated with atherosclerosis risk | Antisense non-coding RNA in the INK4 locus (ANRIL, which is adjacent to the Chr9p21 locus) is associated with atherosclerosis and Chr9p21 genotype | [168–170] |

(Continued)

Table 1 (Continued).

| Biomarker | Category | Involvement/Function | Implications | References |
|--|----------|---|---|------------|
| <i>Phosphodiesterase 4D</i> (PDE4D) gene rs966221 polymorphism | Genetics | In vascular smooth muscle and endothelial cells, PDE4D participates in intracellular signaling pathways that modulate the concentration of cyclic adenosine monophosphate (cAMP), a secondary messenger critical to vascular function | rs966221 polymorphism may be a valuable biomarker for predicting the risk of recurrence in ischemic stroke patients with large artery atherosclerosis | [172] |

response to the factors triggering atherosclerosis.¹⁵⁷ However, fluorescent microscopy is typically used in the DHE system to detect the concentration of ROS in the vessels of atherosclerotic patients.¹⁵⁸

In the context of atherosclerosis, endothelial dysfunction (and the resulting inflammation) may underlie the high production of ROS, predominantly by mitochondria from various cells.¹⁵⁹ The red fluorescence in the MitoSOX approach is a modified analog of DHE that is generated via the addition of a triphenylphosphonium (TPP) group. In this technique, the mitochondrial $O_2^{\bullet-}$ is a specific target of TPP. In analogy with the DHE method, oxidization of MitoSOX leads to the constitution of two fluorescent compounds, namely mito-ethidium and 2-hydroxy-mito-ethidium. In living cells, Mitotracker green and MitoSOX red are used for two-color staining to detect mitochondrial $O_2^{\bullet-}$ by means of confocal fluorescent microscopy.¹⁶⁰ Nonetheless, owing to limitations of the MitoSOX approach, a combined approach of MitoSOX/HPLC is frequently utilized to measure mitochondrial ROS.^{161,162}

Genetic Biomarkers of Inflammation in Atherosclerosis

Genetic association studies have also been promising in the prediction of inflammation in the context of atherosclerosis.^{163,164} Advances in genetics have facilitated the possibility of devising genetic biomarkers for estimating the risk of atherosclerosis. Evaluation of diverse biomarkers, referring to the already recognized familial history of cardiovascular disorders in parents along with hs-CRP, improved the potential of the traditional risk factors in the prediction of the cardiovascular risk.^{118,119} As a consequence, genetic factors have a potential application in predicting the cardiovascular

risk, which may not be reflected by the traditional risk factors.

The first line of investigations studied genetic variations such as single-nucleotide polymorphisms (SNPs) in the allele, genotype, haplotype, as well as inheritance models,¹⁶⁵ which were accomplished through both small and genome-wide studies.¹⁶⁶ *CRP* gene rs1205 SNP was detected to be associated with low-grade chronic inflammation.¹⁶⁷ In multiple studies, a genetic locus on chromosome 9 was associated with susceptibility to cardiovascular disease,^{168–170} suggesting the potential for variations in defining novel biomarkers for cardiovascular diseases. More importantly, genome-wide association studies (GWASs) have identified the potential genetic loci in atherosclerosis with higher validity.¹⁷¹ A GWAS in the Han Chinese population suggested that *Phosphodiesterase 4D* (*PDE4D*) gene rs966221 polymorphism may be a valuable biomarker for predicting recurrent risks in ischemic stroke patients with large artery atherosclerosis.¹⁷² On the other hand, an individual SNP evaluation indicated the involvement of polymorphism in the receptor activator of NF- κ B (RANK) as a predictor of blood pressure and, hence, cardiovascular risk.¹⁷³ Identification of the functional implications of the genomic variations implicated in inflammation may disclose the corresponding biological pathways revealed by GWAS. This may increase the potential of genetic predisposing factors in identifying the inflammatory biomarkers in the context of atherosclerosis (Table 1).

Conclusions

Over the past few years, our understanding of the implications of the innate and adaptive immune inflammatory responses in the etiology and pathogenesis of atherosclerosis has improved. We are currently on the brink of translating our knowledge on inflammation from the bench to bedside. Among the

inflammatory molecules involved in the different steps of atherogenesis, hs-CRP has shown promising potential to be used as a biomarker not only in the prediction of future atherosclerosis development, but also in the recurrence of cardiovascular disorders and in the estimation of medication efficacy. The urge to translate the laboratory findings into clinical application has challenged laboratory research to revolutionize the insights to understand different aspects of the interactions between inflammation and atherosclerosis. The necessity for a fast-track utilization of the laboratory progress in clinical cardiovascular medicine is inspiring the design of sophisticated strategies for the diagnosis, prognosis, management, and monitoring of patients who are at probable risk of atherosclerosis in the years to come.

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Disclosure

The authors report no conflicts of interest in this work.

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