REVIEW

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Novel biomarkers of coronary microvascular disease

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Coronary microvascular disease in the absence of myocardial diseases has traditionally been diagnosed through coronary reactivity testing in the cardiac catheterization laboratory. Compared with invasive procedures, blood-based biomarkers may have reduced cost, less risk of physical harm and greater accessibility, making them ideal for an outpatient management strategy. There are a variety of biomarkers available with potential utility in the management of microvascular disease; however, none have yet been extensively validated or established in this clinical patient population.

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Background

Biomarkers have generated significant interest because they can add independent prognostic value over traditional risk factors. The most useful biomarkers are easily obtainable, demonstrate high accuracy, precision and reliability, and reflect the underlying pathophysiology of the disease process in question. For the purposes of this review, we examine blood-based biomarkers that demonstrate potential in the management of coronary microvascular disease (CMD).

• CMD

Patients with angina but no significant flow-limiting obstructive coronary artery disease (CAD) experience a high rate of myocardial infarction or cardiovascular death [1–4], which is comparable to those with obstructive CAD. Nonobstructive CAD processes that lead to myocardial ischemia, in other words, the imbalance of the myocardial supply and demand relationship, include CMD, endothelial dysfunction and vasospasm. CMD can be categorized into four types [5]: in the absence of myocardial diseases and obstructive CAD; in myocardial diseases without obstructive CAD; in obstructive CAD; and iatrogenic. While there is likely no difference in the development of CMD between those with or without obstructive CAD, for the purposes of this review, we will focus mostly on the first category, CMD in the absence of myocardial diseases and obstructive CAD.

The microcirculation includes extramyocardial prearterioles (100–500 μm), arterioles (<100 μm) and capillaries. These compartments account for 90% of coronary resistance and are therefore the major determinants of coronary blood flow regulation. Structural alterations in the coronary microcirculation may include luminal obstruction from microemboli and external compression from left ventricular hypertrophy. Alterations that lead to coronary microvascular impairment include dysfunction of the vascular smooth muscle cells or endothelium (Figure 1) [6].

KEYWORDS

• biomarkers • coronary flow reserve • coronary microvascular disease

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Figure 1. Schematic representation of the contributions of endothelial and smooth muscle cell dysfunction to coronary microvascular disease. Impaired coronary microvascular function can arise from several mechanisms related to endothelial and/or smooth muscle cell dysfunction. Infections, inflammation or trauma can damage endothelial cells, leading to dysregulation of vasoconstriction and vasodilation, NO imbalance that contributes to reactive oxygen species production and LDL oxidation, leukocyte adhesion and platelet aggregation. Smooth muscle cell dysfunction can manifest as abnormal responses to vasodilation or vasoconstrictor stimuli, or as cell hypertrophy, migration and proliferation. Smooth muscle cell proliferation also results in increased production of endothelin and other proinflammatory molecules. CMD: Coronary microvascular disease.

There are several methods of diagnosing CMD. Coronary reactivity testing, including adenosine-induced hyperemia and acetylcholine-induced endothelial response, is commonly used to assess the functionality of the coronary microvasculature [7]. Primarily through its vasodilator action on vascular smooth muscle cells, adenosine produces endothelium-independent vasodilation of the coronary vessels. The ratio between the coronary blood flow during maximal hyperemia and at baseline is defined as the coronary flow reserve (CFR), which represents the capacity of the coronary circulation to respond to a physiologic increase in oxygen demand with a corresponding increase in blood flow. In healthy adults, an appropriate CFR is over 3.0. Decreased CFR

without any observed flow-limiting stenosis can be attributed to CMD, but may not necessarily be predictive of cardiovascular events. Since CFR is affected by both epicardial and microvascular disease, it is difficult to differentiate between epicardial and microvascular flow limitation if a hemodynamically significant stenotic epicardial artery is present. Newer methods offering more specific measurements of microvascular function include the index of myocardial resistance (IMR) and hyperemic microcirculatory resistance [8,9].

Endothelial dysfunction of the microcirculation is the most common cause of CMD [10,11]. The endothelial layer of the coronary vasculature performs several essential functions including the synthesis of vasodilators, such as prostacyclin and

nitric oxide (NO), release of those vasodilators in response to stimuli, such as inflammation, stress or exercise, and the regulation of vascular remodeling and hemostasis. In response to stress, the diseased endothelium and small arterioles are unable to properly dilate and can even paradoxically constrict, resulting in hypoperfused myocardium and the clinical manifestations of ischemia and angina [12–14]. The principal cause of endothelial impairment is an imbalance in NO production and consumption, favoring consumption and reduced production. In addition, endothelial dysfunction creates a milieu favorable for platelet aggregation and leukocyte adhesion (**Figure 2**).

The NO imbalance may also be reflected in abnormal microvascular responses arising from smooth muscle cell dysfunction. Vascular smooth muscle cells usually dilate in response to NO and other vasodilator stimuli. Blunted dilatation or vasoconstrictor response can be attributed to either a lack of circulating NO or altered receptor and cell signaling pathways. Smooth muscle proliferation can also contribute to CMD through vascular remodeling and increased secretion of endothelin and cytokines. Biomarkers involved in the inflammatory, oxidative stress and coagulation pathways may therefore be useful to investigate against CMD.

Markers of vascular inflammation

Inflammation and immune dysregulation play a pivotal role in endothelial dysfunction and CAD pathogenesis [16–18]. Consistent with these observations, CMD has been associated with several inflammatory biomarkers.

• CRP

CRP is a nonspecific systemic marker of inflammation that has been well validated in the prediction of cardiovascular risk for women [19–21]. The Reynolds Risk Score, developed from the Women's Health Study that followed 24,558 initially healthy women for a median of 10.2 years, incorporates CRP in addition to traditional risk factors, such as age, smoking status, family history and cholesterol levels to estimate 10-year risk of major adverse cardiac events [20]. As CMD has conventionally been considered a woman's disease, a reasonable inference from these population studies would be that CRP may be a suitable biomarker for CMD. Indeed, compared with normal controls, patients with CMD generally have significantly higher CRP levels, which may even be comparable to those with chronic stable angina [22–26].

However, as CRP reflects the metabolic changes of many pathways involved in endothelial cell function [27,28], its level can fluctuate throughout the day, making it difficult to interpret over long periods of time.

• suPAR

suPAR is a proinflammatory biomarker and chemotactic agent that is released by cleavage of the uPAR, which is expressed on hematopoietic, endothelial and smooth muscle cells [29,30]. In contrast to CRP, suPAR is highly stable and not subject to circadian variation [30]. Although suPAR modestly correlates with CRP, several longitudinal population-based studies have linked plasma suPAR levels with increased risk of cardiovascular disease and mortality independent of traditional risk factors and CRP [31–35]. In a small study of 47 patients, higher plasma suPAR level was associated with lower CFR in patients with nonobstructive CAD [36].

• Heat shock proteins

Heat shock proteins are a family of essential intracellular chaperone proteins that are upregulated and presented onto the cell surface when cells are exposed to stressful conditions. In the atherosclerotic process, the activation of heat shock proteins has been hypothesized to reflect early pathophysiologic mechanisms that link infective-metabolic insults to the consequent inflammatory reaction, resulting in both endothelial and microcirculatory damage [37–39]. Early studies have suggested that elevated levels of heat shock proteins are associated with greater subclinical atherosclerosis [39] and may portend increased risk of cardiovascular events [40,41]; however, there have been no studies specifically using heat shock proteins as a biomarker for CMD.

• Cell adhesion molecules

Cell adhesion molecules are transmembrane proteins that help cells bind to other cells or to the extracellular matrix. Studies have reported mixed results regarding the diagnostic and prognostic value of ICAM-1 and VCAM-1. One study of 68 patients reported that, compared with healthy control cohorts, patients with microvascular angina have both elevated ICAM-1 and VCAM-1 [42]. Another reported that only circulating levels of ICAM-1 were elevated in CMD patients, but that there were no significant differences in VCAM-1 levels [26]. Another suggested that there is no difference in the levels of adhesion

Figure 2. Electron micrographs of endothelial cells in the presence and absence of oxidative stress. (A) The normal (control) capillary wall has a uniform thickness of endothelial cells (modified with permission from [15]). (B) In ischemic conditions, capillaries increase the endothelial cell layer to lumen ratio. (C) Capillaries reperfused in the absence of antioxidants are exposed to significant amounts of reactive oxygen species, resulting in swollen endothelium and floating membrane blebs in the lumen. (D) A capillary reperfused in the presence of the antioxidant α -tocopherol, which has mitigated the damage observed in panel C. (E) A capillary reperfused in the presence of the antioxidant ascorbate, which has also mitigated the damage observed in panel C. (F) A capillary reperfused in the presence of both α -tocopherol and ascorbate, demonstrating reduced endothelial swelling and large bleb-free lumen that is similar to the control capillary.

molecules in patients with CMD when compared to healthy controls [43].

• Cytokines

A broad category, cytokines are cell signaling small proteins that modulate between

humoral and cellular immune responses and include chemokines, interferons, interleukins and TNF. Compared with healthy controls, CMD patients may have elevated levels of MCP [22,44]; endothelin-1 [45–50] or IL-6 [26]. Larger cohort studies are warranted to validate

the use of these cytokines for diagnosis or prognostication.

Markers of oxidative stress

Oxidative stress plays a key role in the impairment of endothelium-dependent vasodilation, the pathophysiology of CMD and the pathogenesis of atherosclerosis. It occurs on the cellular level when redox signaling and control are disrupted, resulting in pro-oxidant forces overwhelming antioxidant defense mechanisms [9]. Decreased production and increased consumption of NO, the major endogenous vasodilator, lead to endothelial dysfunction and anginal symptoms, even in patients without findings of atherosclerosis on angiography.

In the endothelium, eNOS catalyzes the synthesis of NO from L-arginine and BH₄ [51–53]. This enzyme plays a crucial role in maintaining normal endothelial function by modulating vasodilatory tone, regulating local cell growth and protecting the vessel from injury [54]. An increase in reactive oxygen species (ROS) leads to disturbances of this NO production pathway by depleting cofactors for eNOS. As a result, eNOS uncouples and synthesizes superoxide and hydrogen peroxide in preference to NO (Figure 3). Therefore, uncoupling not only leads to decreased bioavailable NO, but also further contributes to the oxidative stress and worsens endothelial dysfunction [52,55–57]. Various blood-based biomarkers associated with the disturbance in the balance of NO production have been identified and may be considered for markers of CMD (Figure 3).

• Red blood cell morphology

In the microvasculature, blood flow depends on red blood cell (RBC) deformability, which has been related to NO release. RBC morphology can be measured in terms of amount of erythrocytes, mean RBC size and/or RBC distribution width (RDW). A couple of small studies have suggested that CMD patients have higher RDW values compared with healthy control cohorts [58,59]

• Amino thiols

Glutathione maintains thiol groups of enzymes and other biomolecules in their reduced states and prevents peroxidation of membrane lipids [15]. Glutathione is also thought to transport NO from larger epicardial coronary vessels to the distal smaller vessels and microcirculation [60]. A positive correlation between glutathione levels and CFR has been observed [9], suggesting that

higher glutathione levels are indicative of healthier microvasculature and that lower glutathione levels reflect higher amounts of oxidative stress and are associated with CMD [9,58].

• L-arginine

Decreased amounts of L-arginine, the substrate for NO, would be suspected in those with endothelial dysfunction and in theory may be used as a biomarker for endothelial dysfunction. However, conflicting evidence exists to suggest that plasma levels of L-arginine correlate with the severity of CMD. While one study found a difference in L-arginine levels between CMD patients with and without anginal symptoms [61], others found similar levels of L-arginine between the control group and CMD patients [62,63].

• Asymmetric dimethylarginine

Asymmetric dimethylarginine (ADMA) is a naturally occurring metabolic byproduct of L-arginine (Figure 4) [12] that can be elevated in patients with hypertension, hyperlipidemia, atherosclerosis and renal failure [64–67]. The vascular effects of ADMA operate through multiple mechanisms, including competitive binding to eNOS to result in diminished NO production and bioavailability, promotion of atherogenesis [64], ACE upregulation, enhanced superoxide production [68] and increased expression of LOX-1, the receptor for oxidized LDL in endothelial cells [67]. Plasma ADMA levels have been found to correlate with endothelial dysfunction [65], CMD [63] and subclinical atherosclerosis [61]. Numerous studies have shown increased ADMA levels and decreased L-arginine to ADMA ratios in patients with CMD [61–63]. Studies have been conflicted, however, about whether ADMA levels predict risk of future cardiovascular events, and so its prognostic value may depend on the target patient cohort [65].

• LDL

Historically, LDL cholesterol levels have been the prototypical biomarker for CAD [27], and elevated LDL cholesterol levels have direct correlation with coronary microvascular function, quantified by IMR, regardless of the presence of coronary atherosclerosis [70]. Oxygen-free radicals react with methylene carbons on polyunsaturated fatty acids in the vessel wall and form oxidized LDL [27], which serves as a stimulus for chronic inflammation and atherosclerotic plaque development. After oxidized LDL is formed in the artery walls, the expression of adhesion molecules is upregulated

Figure 3. Oxidant stress-induced endothelial dysfunction in coronary artery disease. eNOS converts L-arginine to NO in the endothelial cell. The presence of systemic risk factors, such as hypercholesterolemia, hypertension or diabetes mellitus can increase reactive oxygen species production leading to oxidation of BH4 to BH2, eNOS uncoupling and further production of superoxide. Inflammatory factors, such as IL-1 β and VCAM-1, also affect eNOS expression and NO production.

Modified with permission from [52].

and chemokines that recruit circulating leukocytes are secreted. Monocytes and macrophages then infiltrate the plaque, accelerating atherosclerosis [67]. Oxidized LDL's unique role of linking lipoprotein disorders and inflammation that drives atherosclerosis is well established, and it may also be considered as a potential biomarker for CMD.

Infiltration of oxidized LDL into the endothelium upregulates the gene expression and post-transcriptional activity of major oxidative enzymes, such as NADPH oxidase and xanthine oxidase, that are located on the endothelial cell membranes [71], which leads to even greater production of ROS. Oxidized LDL has also been found to increase the release of Von Willebrand factor and to be directly cytotoxic to endothelial

cells [65]. The endothelial cell membrane integrity becomes impaired with self-propagating reactions of lipid peroxides that result in increased membrane permeability and irreversible cell damage [15]. Unfortunately, oxidized LDL levels are difficult to assess *in vivo* and are minimally present in plasma [65], thereby limiting its utility as a biomarker.

• ROS & isoprostanes

ROS, such as superoxide and lipid radicals produced from the oxidation of LDL, have been found to be elevated in hypercholesterolemia, diabetes, hypertension and cigarette smoking [55–57], conditions that predispose towards CMD development. Excessive endogenous ROS reacts with

NO to form peroxynitrite. As this reaction is three times faster than catabolism of superoxide by SOD, NO is rapidly and preferentially converted to peroxynitrite, thereby decreasing the availability of biologically active NO [52,72].

Free-radical catalyzed peroxidation of arachidonic acid can produce isoprostanes, which are stable unique end products of lipoprotein metabolism that circulate in plasma and are excreted in urine. Isoprostanes can be measured with high sensitivity and specificity, so they are frequently used to measure oxidative stress *in vivo*; however, they are not specific to lipoprotein oxidation [27]. Despite promising preclinical data, no information is currently available with respect to their use in CMD diagnosis or management.

Markers of the coagulation cascade

Coagulation cascade factors are active during acute coronary syndromes; however, there is less information about their use in patients with stable ischemic heart disease.

• Fibrin & fibrinogen degradation products

Fibrin and fibrinogen degradation products (FDP) are byproducts of thrombin breakdown and include the well-known d-dimer and other soluble fibrin fragments and monomers. Higher serum FDP levels have been associated with increased incidence of CAD and adverse outcomes [40,73]. The only available data regarding the relationship between FDP and CMD is from a study of 75 patients who underwent comprehensive intravascular imaging and physiology investigation. While this study observed a relationship between higher levels of FDP and imaging features of vulnerable plaque, it failed to show a significant relation between FDP and CFR [74].

• PAI-1

Produced by the vascular endothelium, PAI-1 is a fast-acting inhibitor of plasminogen activation and plays an important role in regulation of fibrinolysis process. In a small study of 30 patients with untreated essential hypertension and ten controls,

Figure 4. Arginine/NO cycle. Methylation of L-arginine occurs through N-methyltransferases, which utilize S-adenosyl methionine as a methyl group donor. After proteolysis of proteins containing methylated L-arginine, ADMA is present in the cytoplasm and acts as a competitive inhibitor of NO synthase, thereby increasing the risk of endothelial dysfunction and atherosclerosis development. ADMA is eliminated from the body via urinary excretion and can be metabolized by the enzyme DDAH to citrulline.

ADMA: Asymmetric dimethyl arginine.

Modified with permission from [69].

PAI-1 activity was inversely correlated with PET-derived CFR, such that elevated PAI-1 activity was an independent determinant of CMD [75].

Other markers

• Natriuretic peptides

Two familiar natriuretic peptides are ANP, secreted in response to the stretching of the atrial wall, and BNP, secreted in response to the stretching of the ventricular wall.

The vasoactive effects of ANP infusion appear to be dose dependent. In humans consuming a high-salt diet, low-dose ANP infusion resulted in vasoconstriction of skin microvasculature, a functional decrease in conjunctival capillary density, and increased renal vascular resistance [76]. The authors argued that ANP probably had a direct effect on the microvasculature because the observed microvascular changes did not correlate with other confounding factors such as blood pressure, heart rate or the activity of the renin–angiotensin–aldosterone system. Infusion of higher ANP doses appears to lead to vasodilation of the skin with concomitant decrease in blood pressure and increase in heart rate.

Although it has been well established that elevated levels BNP have prognostic value in patients with systolic heart failure, there are limited studies associating BNP levels with CMD. Higher resting NT-proBNP were observed in patients with CMD complicated by left bundle branch block and left ventricular diastolic dysfunction in comparison to normal subjects; however NT-proBNP levels were more comparable to controls in CMD patients without left bundle branch block [46]. A small but comprehensive imaging study of ten controls and 18 patients with symptomatic hypertrophic cardiomyopathy and normal coronary arteries used PET-derived hyperemic myocardial blood flow as a marker of CMD. In this study, while hyperemic myocardial blood flow was blunted in patients with hypertrophic cardiomyopathy compared with the controls, NT-proBNP was inversely and independently correlated with hyperemic myocardial blood flow [77]. Another measurement of CMD, hyperemic myocardial perfusion reserve, was assessed by MRI in 184 asymptomatic adults without overt CAD from the multi-ethnic study of atherosclerosis. This study also showed a correlation between higher levels of NT-proBNP and reduced myocardial perfusion reserve [78].

• Cardiac enzymes

A highly sensitive marker and the most common one for myocardial necrosis, troponin has been studied in patients with iatrogenic CMD (category 4) after percutaneous coronary intervention (PCI). In patients with stable angina, post-PCI IMR values were significantly higher (therefore indicating more microvascular dysfunction) in patients with abnormal troponin I elevation than in those without abnormal troponin I elevation [79]. Another study of 55 patients undergoing PCI also demonstrated a positive correlation between post-PCI CFR and cardiac enzymes [80]. The relationship between higher post-PCI IMR values and higher levels of peak CK and CK-MB was similarly observed in a smaller study of 24 patients [81]. Finally, a study of 29 patients after primary PCI for STEMI reproduced this correlation between peak CK and post-PCI IMR but not between peak CK and CFR [82].

Although cardiac enzymes have been well validated as a biomarker for acute myocardial injury, there is less information about its role in the management of stable CMD. In a small study, 58 patients with heart failure were evaluated for CMD (CFR <2.0) and the authors observed that troponin levels were higher in patients with CMD than those without [83]. Another study of 19 stable patients who underwent troponin measurements and coronary physiological interrogation in the same visit reported poor correlation between troponin values and CFR, and only modest correlation between troponin and IMR [8]. Finally, a larger study followed 761 patients with suspected but not overt CAD for a median of 2.8 years and reported that patients with at least one positive troponin value had lower CFR compared with those patients with negative troponin, and those with both lower CFR and positive troponin were at higher risk of major adverse cardiovascular events [84].

• Endothelial microparticles & progenitor cells

Microparticles are vesicles (100 nm–1 µm in diameter) shed from plasma membranes following cell activation, oxidative stress or apoptosis [65]. Microparticles originating from endothelial cells have been hypothesized to decrease endothelial function by impairing endothelium-dependent dilation and the endothelial NO pathway. Circulating endothelium derived microparticles increase in several cardiovascular and atherothrombotic diseases and correspond with the

severity of CAD in patients presenting with acute coronary syndromes [65].

Endothelial progenitor cells (EPC) play an important role in endothelial cell regeneration and can be measured from peripheral blood. Current studies that have attempted to correlate the amount of circulating endothelial progenitor cells (EPC)s and endothelial function have reported mixed results [65]. However, early results from a substudy of the REPAIR-AMI trial suggested that EPCs may improve microvascular function of the infarct-related arteries after bone marrow stem cell transfusion [85]. While microparticles and progenitor cells have understandably generated substantial excitement, their quantification

and accuracy remain at the nascent stages and will need to significantly improve before they can be considered as biomarkers.

Conclusion

Coronary microvascular abnormalities contribute to angina and worsen ischemia. Accurate diagnosis and prognostication of CMD remain crucial to the management of this clinical population; however, there remains significant uncertainty and challenges to the evaluation of CMD. Although contemporary techniques for CMD assessment include non-invasive imaging and invasive physiologic measurements, these methods are currently impractical for routine surveillance. Blood-based

EXECUTIVE SUMMARY

Background

- Coronary microvascular disease (CMD) is an important contributor to angina and myocardial ischemia in patients without flow-limiting epicardial coronary atherosclerotic lesions.
- Coronary reactivity testing includes adenosine-induced hyperemia and acetylcholine-induced endothelial response and are commonly used to assess the functionality of the coronary microvasculature. These procedures can be expensive and time consuming.
- Biomarkers can add independent prognostic value over traditional risk factors and generally augment outpatient clinical management. The most useful biomarkers are easily obtainable, accurate and reliable, and reflect the underlying pathophysiology of the disease process in question.

Markers of vascular inflammation

- Several inflammatory biomarkers have been associated with CMD, including CRP, suPAR, cell adhesion molecules and cytokines.
- CRP and suPAR are among the most established biomarkers for CMD.

Markers of oxidative stress

- The imbalance in nitric oxide production and consumption results in impairment of endothelium-dependent vasodilation.
- Although there is promising preclinical data on various biomolecules, such as aminothiols, asymmetric dimethylarginine, oxidized LDL cholesterol, superoxide and isoprostanes, there are limited clinical data available supporting their use in the management of CMD. Glutathione and asymmetric dimethylarginine are the most promising oxidative stress markers to demonstrate clinical utility in the CMD patient population.

Markers of the coagulation cascade

- Coagulation cascade factors are active during acute coronary syndromes; however, there is less information about their value in patients with stable CMD.

Other markers

- Natriuretic peptides are well established in heart failure management. There have been small studies demonstrating the correlation between brain natriuretic peptide levels and myocardial blood flow in patients with CMD and other heart disease; further studies in patients with only CMD will help strengthen this association.

Conclusion & future perspective

- Several inflammatory and oxidative stress pathways have been implicated in the pathophysiology of CMD. Biomolecules from these pathways have potential utility in diagnosing and managing those with microvascular disease; however, none have yet been extensively validated or established in this clinical patient population.

biomarkers offer several advantages, including less risk of physical harm compared with invasive procedures, potentially reduced cost, and greater availability and accessibility [27]; however, as this review demonstrates, no biomarker has been extensively validated in this clinical patient population. There are a variety of available biomarkers with plausible utility in the management of CMD and their theoretical connections will likely become more established with our improving understanding of the multiple pathophysiological mechanisms responsible for the development of CMD.

Future perspective

In 5 or 10 years' time, we will have gained a deeper understanding of the pathophysiology of CMD, which will translate into the development of better diagnostic tools and therapies for patients with CMD. The use of blood-based biomarkers specific for CMD will serve this clinical population

well in differentiating between patients with epicardial CAD, CMD and without any coronary flow abnormalities, thereby directing appropriate therapy for each cohort.

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