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Review

BIOMARKERS OF CORONARY MICROVASCULAR DYSFUNCTION IN PATIENTS WITH MICROVASCULAR ANGINA: A NARRATIVE REVIEW

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ABSTRACT

The current gold standard for diagnosis of coronary microvascular dysfunction (CMD) in the absence of myocardial diseases, whose clinical manifestation is microvascular angina (MVA), is reactivity testing using adenosine or acetylcholine during coronary angiography. This invasive test can be difficult to perform, expensive and harmful. The identification of easily obtainable blood biomarkers which reflect the pathophysiology of CMD, characterized by high reliability, precision, accuracy and accessibility may reduce risks and costs related to invasive procedures and even facilitate the screening and diagnosis of CMD. In this review we summarized the results of several studies that have investigated the possible relationships between blood biomarkers involved with CMD and MVA. More specifically, we have divided the analyzed biomarkers into 3 different groups, according to the main mechanisms underlying CMD: biomarkers of "endothelial dysfunction", "vascular inflammation" and "oxidative stress". Finally, in the last section of the review, we consider mixed mechanisms and biomarkers which are not included in the 3 major categories mentioned above, but could be involved in the pathogenesis of CMD.

Keywords: coronary microvascular dysfunction; microvascular angina; endothelial dysfunction; inflammation; oxidative stress, biomarkers.

INTRODUCTION

Microvascular angina (MVA), firstly described by Kemp in 1973 with the term "Cardiac Syndrome X", is characterized by: a) symptoms of myocardial ischemia: effort and/or rest angina or angina equivalents (i.e. shortness of breath); b) absence of significant flow-limiting obstructive coronary artery disease (CAD) (<50% diameter reduction) on invasive coronary angiography and/or computed tomography angiography; c) evidence of ischemia assessed with non-invasive methods; d) evidence of impaired coronary microvascular function.[1,2] Risk factors most frequently associated with this disorder are aging, hypertension, diabetes, metabolic syndrome, obesity, inflammation and female sex.[3]

It has been proposed that the onset of angina in patients with non-obstructive CAD might be caused by non-ischemic mechanisms, such as neurogenic and behavioral disorders, which cause an increased adrenergic activity and/or abnormal function of sympathetic and nociceptive nerve fibers of the heart, leading to an enhanced pain perception to harmless local stimuli.[4,5] It is also possible that estrogen deficiency in women with MVA contributes to altered chest pain perception.[6] However, the main mechanism which leads to myocardial ischemia in patients with MVA is coronary microvascular dysfunction (CMD), defined as alterations involving the structure or vasoreactivity of the coronary microcirculation.[7]

The microcirculation consists of very small vessels that cannot be visualized by coronary angiography, such as extramyocardial pre-arterioles (300-150 μm), arterioles (150-10 μm), capillaries (8-4 μm) and venules (10-100 μm).[8] These small vessels account for 70% of coronary resistance and are therefore major determinants of coronary blood flow regulation.[8] Structural alterations of the coronary microcirculation may include: vascular wall remodeling, microvascular atherosclerosis and capillary rarefaction.[9] Functional alterations may include a reduction of the vasodilator response and an increased vasoconstrictor activity, i.e. microvascular spasm, mainly due to endothelial and smooth muscle cells dysfunction.[10]

CMD is linked not only to traditional cardiovascular risk factors such as hypertension, smoking, hyperlipidemia and diabetes, but also to several atherogenic factors such as inflammation, oxidative stress and thrombosis.[11-14] Assessment of coronary microvascular function can be carried out using invasive and non-invasive methods, in particular it can be documented by the evidence of one of the following: (a) in the absence of critical stenosis of epicardial vessels, a *coronary flow reserve (CFR) ≤2.0*, defined as the ratio between coronary blood flow (CBF) at peak drug administration (usually adenosine) and at baseline[2]. The evaluation of CFR helps to investigate the intrinsic dilator capacity of the coronary microcirculation; (b) *coronary microvascular spasm*, defined as the onset of ischemic symptoms and ECG ischemic changes, without epicardial spasm, during acetylcholine testing; (c) *abnormal coronary microvascular resistance indices* (IMR >25, a more specific measurement of microvascular function); (d) *coronary slow flow phenomenon*, defined as TIMI (Thrombolysis In Myocardial Infarction) frame count >25, a marker of coronary vascular resistance that reflects the severity of CMD.

We aim to provide a narrative review of the literature in the last 20 years. We used PubMed to find studies that have investigated the possible diagnostic and prognostic potential of several biomarkers involved in different pathways related to CMD and MVA. These markers may allow to better understand the pathophysiological mechanisms that underlie these conditions. The keywords we used to search on PubMed were: endothelial dysfunction, coronary microvascular disfunction, oxidative stress, microvascular angina and biomarkers.

From a pathophysiological point of view, CMD and MVA are intriguing conditions because they are caused by several closely related factors, which form the basis of the structural and functional alterations of the microvascular system. The main mechanisms and related biomarkers are analyzed individually below.

[A] BIOMARKERS OF ENDOTHELIAL DYSFUNCTION

The vascular endothelium regulates blood vessel function, contributing to the maintenance of their homeostasis.[15] Many factors can directly interact with the endothelium, such as vascular inflammation, activated platelets, coagulation factors and circulating cells, and in some cases, they can impair its function.[16] Endothelial dysfunction is not only considered as the initial mechanism that occurs in the cascade of events that will lead to atherosclerosis and the development of CAD, but it also plays a pathogenic role in MVA.[17,18] Several studies have investigated on the role of different biomarkers related to endothelial dysfunction in patients with MVA. We consider the main ones below.

➢ ENDOTHELINS

Endothelial dysfunction is a key mediator in the pathogenesis of CMD. This has been shown in experimental studies, where the attenuation of endothelium-dependent vasodilation results from reduced nitric oxide bioavailability and increased vasoconstrictor responses to endothelin-1 (ET-1), prostaglandin H2 and thromboxane A2.[19]

The endothelins (ETs) are a family of endothelial peptides with a strong vasoconstrictor and neuromodulator effect, released as a consequence of endothelial stimulation and activation by several stimuli, which sometimes can be harmful to the endothelium. Indeed, increased plasma ETs levels are found in acute coronary syndrome, atherosclerosis, hypertension and renal failure.[20-23] ETs exert their vasoconstrictor effect mainly on the microcirculation and are also able to stimulate nociceptors, thus these peptides may play a role in the pathophysiology of MVA[24]. Indeed, experimental studies have shown that structural inward remodelling of

arterioles can be produced at low intraluminal pressure in vitro and is enhanced by ET-1 and prevented by the calcium-antagonist amlodipine.[13,19]

Kaski et al. found that in 40 patients with MVA who underwent treadmill exercise testing and SPECT (single photon emission computed tomography) coronary perfusion assessment, plasma ET-1 levels were significantly higher than those in 20 healthy controls[25]. Moreover, in patients with "high" ET-1 concentrations (n=23), levels were positively correlated with an earlier onset of chest pain during treadmill stress testing. In another study plasma ET levels were assessed in 54 patients with MVA[26].

MVA patients were subdivided into 4 subgroups according to the presence of a left bundle branch block (group A), history of previous myocardial infarction (group B), positive ECG response to exercise stress testing (group C) and negative ECG response to exercise stress testing (group D) compared with a group of healthy controls (E). Only subgroups A, B and C had significantly higher ET levels than the group of healthy controls; in particular the highest levels were found in subgroups A and B. Cox et al. enrolled 19 patients with chest pain and normal coronary arteriograms who underwent radioimmunoassay measurement of arterial and coronary sinus ET levels and invasive assessment of coronary sinus blood flow during rapid atrial pacing[27]. They found that the percentage fall in coronary vascular resistance (d.CVR%) was higher in female patients than in males; in particular, women with high arterial ET levels had lower levels of coronary vascular resistance (%d.CVR). Further investigations would be helpful to understand whether treatment with endothelin antagonists could improve endothelial function in these patients.

PLATELETS

Platelets play a role in the pathogenesis of microvascular dysfunction by forming distal microemboli and by adhering to the endothelium or to attached leucocytes, contributing to the

release of vasoconstrictor or toxic molecules and turning platelets into boosters of the inflammatory process.[16,28] Oxidative stress reduces NO levels and increases ET-1 expressions in the endothelial cells, enhancing vasoconstriction, proliferation of fibroblasts and vascular smooth muscle cells, triggering inflammatory pathways and platelets adhesion.[18,29] Indeed, Oxidative stress, endothelial cell activation, and the recruitment of rolling leucocytes, contribute to the interaction with intact inflamed microvessels without the need of exposed extracellular matrix material.[28] Molecules mediating this interaction are P-selectin and P-selectin glycoprotein ligand-1 or glycoprotein-Ib and von-Willebrand factor.[29] Proteins from preformed granules, synthetized bioactive molecules (ROS, thromboxane) such as chemokines, TNF superfamily factors and coagulation factors or shed them from the membrane (e.g. CD40L), are released from platelets adhered to the endothelial cell lining.[28] In this way, platelets provide leucocytes/monocytes recruitment and activation promoting inflammation.

ENDOCAN

Endocan is a soluble dermatan sulfate proteoglycan produced by the endothelium as a result of its activation and dysfunction. This molecule seems to participate in the regulation of cell adhesion process, inflammation, tumor progression, angiogenic switch in stem cells and endothelial-mesenchymal transition processes.[30-32] Endocan may be a predictor of cardiovascular disease events[33] and its plasma levels are associated with the presence and severity of CAD in hypertensives.[34-35] Çimen et al. measured plasma endocan levels in 40 MVA patients, 120 obstructive CAD patients and 53 healthy controls[36]. They found that CAD and MVA patients had similar endocan levels that were higher than those of controls. Furthermore, plasma endocan levels were independent predictors of the presence and severity of CAD in both hypertensive and normotensive patients. Efe et al. also found higher plasma endocan levels in 50 patients with MVA compared to 28 healthy controls[37]. A diagnosis of

MVA was based on: typical exercise-induced angina, positive exercise stress electrocardiography (ECG; >0.1 mV ST-segment depression at 80 ms after the J point in 2 or more contiguous leads), or myocardial perfusion scintigraphy (MPS) and angiographically normal coronary arteries.

In particular, in ROC (receiver operating characteristic) curve analysis plasma endocan levels >2072 ng/l had 72% sensitivity and 54% specificity to predict a diagnosis of MVA.

Endocan may be considered as a surrogate indicator of early endothelial dysfunction, therefore it might be a hypothetical therapeutic target for the primary prevention of cardiovascular diseases. Endocan might be a marker of microvascular disease and is an indicator of pathophysiology of MVA.

- However further studies are needed to confirm that endocan could be a common predictor of the endothelium-dependent inflammatory processes.

- **ANGIOGENIC FACTORS**

Angiogenic factors, such as vascular endothelial growth factor (VEGF), tyrosine kinase-2 receptor (Tie-2) and its ligands, angiopoietin-1 (Angp-1) and angiopoietin-2 (Angp-2), are important proteins which regulate the processes of vascular development and angiogenesis.[38] In patients with cardiovascular diseases, such as congestive heart failure and acute coronary syndrome, higher levels of Angp-1, Angp-2 and Tie-2 have been reported.[39,40] Moreover, angiogenic factors have been investigated for their possible role in affecting endothelial function and integrity, leading to CMD and MVA. Asl et al.[41] measured angiogenic factors levels in 30 MVA patients and 20 healthy controls; they found that the patients had significantly higher levels of Angp-2 and Tie-2 than controls. Afterwards, patients with MVA

underwent 4 weeks of metoprolol therapy, and at the end of this period no significant changes were found in the levels of angiogenic factors compared with baseline values.

Changes in plasma levels of angiogenic factors in patients with MVA require further investigations, in order to better understand their role in the pathogenesis of MVA and possibly implement targeted treatment.

[B] BIOMARKERS OF VASCULAR INFLAMMATION

Systemic inflammation has a pathogenic role in endothelial dysfunction and atherogenesis.[42,43] Several inflammatory markers have been investigated for their possible pivotal role leading to CMD.

➢ C-REACTIVE PROTEIN (CRP)

CRP, a sensitive nonspecific marker of systemic inflammation, is a cardiovascular risk predictor associated with endothelial dysfunction and atherogenesis.[42-45] Thus, its possible role in MVA has been investigated.

Arroyo-Espliguero et al. studied for the first time the association between chronic inflammation and increased arterial stiffness in 30 patients with MVA compared with 30 healthy controls, by evaluating high sensitivity CRP (hsCRP) levels and ultrasound parameters of arterial elasticity[46]. Cosin-Sales et al. measured plasma hsCRP levels in 137 patients with typical chest pain and angiographically normal coronary arteries who completed a standardized angina and cardiovascular risk factor questionnaire, and underwent treadmill exercise stress testing and 24-h continuous ambulatory ECG monitoring (Holter)[47]. They found that hsCRP levels were higher in patients with prolonged (>20 min) and frequent (>5/week) angina episodes, with a positive exercise stress testing and with ST-segment

changes during 24-h Holter monitoring. In addition, they also noticed a direct correlation between hsCRP levels and the severity of ischemic responses on stress testing and with the number of ST-segment changes during 24-h Holter monitoring. Recio-Mayoral et al.[48] measured plasma hsCRP levels in 21 patients with MVA without traditional cardiovascular risk factors and 21 healthy volunteers who underwent PET (positron emission tomography) assessment of myocardial blood flow and CFR (coronary flow reserve). Patients were subdivided into 2 groups according to hsCRP levels (≤3 or >3 mg/l). MVA patients had lower CFR values compared with controls; moreover, patients with hsCRP >3 mg/l had a greater impairment of CFR and more frequent ischemic ECG changes during adenosine infusion. As a result, it was reported the correlation between inflammation, CMD and the occurrence of ischemic episodes, even in the absence of conventional cardiovascular risk factors.

➢ CELL ADHESION MOLECULES

Cell adhesion molecules, such as ICAM-1 and VCAM-1, are trans-membrane proteins that allow blood circulating cells to adhere to other cells or to the extracellular matrix. VCAM-1 (vascular cell adhesion molecule-1) and ICAM-1 (intercellular cell adhesion molecule 1) are expressed by the endothelium as it becomes inflamed and activated by cytokine stimuli, and promote the adhesion of circulating leukocytes on endothelial cells, their migration and atherosclerosis.[49] Tousoulis et al. measured circulating VCAM-1 and ICAM-1 levels in 36 patients with CAD, 21 patients with MVA and 11 healthy volunteers[50]. CAD and MVA patients showed higher levels of these molecules compared with controls, although only the differences in ICAM-1 levels were significant ($p<0.05$). Moreover, in patients with MVA there was no significant evidence of correlation between circulating cell adhesion molecules levels and the magnitude of ST-segment depression. Shim et al. found that in 135 patients with MVA, among several inflammatory biomarkers of early coronary atherosclerosis

measured, such as soluble vascular cell adhesion molecule (sVCAM-1), interleukin 6 (IL-6), monocyte chemoattractant protein-1 (MCP-1) and lipoprotein-phospholipateA2 (Lp-PLA2), only plasma sVCAM-1 levels influenced the CFR, as demonstrated by the evidence of an inverse correlation between these 2 parameters[51]. In particular, sVCAM-1 levels were significantly higher in patients with CFR <2.0, leading to the idea that because sVCAM-1 is expressed in subclinical atherosclerosis and endothelial activation, patients with MVA might also manifest higher levels of this biomarker. Liang et al. compared serum levels of hsCRP, ICAM-1, VCAM-1, MCP-1 and soluble CD40 ligand (sCD40L) in 92 MVA patients and 145 CAD patients without known diabetes, finding that the CAD group had higher levels of VCAM-1 and ICAM-1 compared with the MVA group. Moreover, they found that VCAM-1 levels might be a predictor of the differential diagnosis of CAD *vs* MVA[52].

➢ INFLAMMATORY CYTOKINES

Cytokines are small proteins produced by a variety of cells (i.e. mononuclear cells, activated T-cells, platelets, endothelial cells and smooth muscle cells) in response to several stimuli; they can act as signals that allow the communication between the immune cells and between these cells and different organs and tissues.[53] Cytokines can be involved in several inflammatory pathways and can also have pro-atherogenic effects, affecting endothelial function and increasing leukocyte recruitment.[53] Therefore, they may also be involved in the pathogenesis of CMD and MVA.

MCP-1 was one of the first inflammatory cytokines evaluated in the possible association with CMD, as it stimulates monocytes to infiltrate the arterial wall in early atherogenesis.[54] Deo et al. were the first to evaluate the correlation between high serum MCP-1 levels with subclinical atherosclerosis and traditional cardiovascular risk factors[55]. On et al. assessed the total serum antioxidant status, plasma CRP levels and inflammatory cytokines (P-selectin,

MCP-1, IL-6, IL-10) in 36 patients with MVA, 36 patients with CAD and 24 controls who had experienced atypical chest discomfort with normal coronary angiograms and without the evidence of ischemic episodes in non-invasive tests[56]. Patients with MVA had higher CRP levels compared with the other groups, whereas controls had higher total serum antioxidant status levels but lower serum MCP-1 concentration. Blood levels of the other cytokines were similar in the entire population of the study. Li et al. measured plasma CRP and IL-6 levels and circulating leukocytes in 36 female patients with MVA and 30 female healthy controls[57]. Patients with MVA had significantly higher peripheral circulating leukocytes and monocytes counts and 2.2-fold higher CRP and IL-6 levels compared with controls. Moreover, CRP was independently associated with MVA. Other cytokines studied were suppression of tumorigenicity 2 (sST2) and sCD40L, which are involved in inflammation and fibrosis. Aslan et al. evaluated serum sST2 and sCD40L levels in a population of 91 patients with MVA and 61 controls with story of chest pain but with normal coronary angiograms and absence of ischemic evidence on non-invasive tests[58]. Serum sST2 and sCD40L levels resulted significantly higher in patients with MVA compared with controls; there was also a significant positive correlation between sST2 levels with low density lipoprotein-cholesterol (LDL-C) and sCD40L. Moreover, sCD40L levels were also positively correlated with hsCRP levels. Finally, only sCD40L and hsCRP levels were found to be independently associated with MVA. In order to better understand the internal relationships between different biomarkers involved in common pathophysiological pathways that possibly can lead to CMD, Schroder et al. measured a panel of 92 biomarkers related to cardiovascular or other diseases in 174 women with angina-like symptoms and no significant obstructive CAD, who underwent transthoracic doppler echocardiography to measure their coronary flow velocity reserve (CFVR). Only 28% of patients had a CFVR <2.0, which was indicative of CMD[59]. On a regression analysis, 18 biomarkers were significantly related to CFVR; by using the

statistical technique PCA (principal component analysis), which allowed a simplification of the large number of variables analyzed in order to explore possible common interrelations within the group of biomarkers significantly associated with CFVR, it was found that 8 biomarkers such as chemokine ligand 16 (CCL16), C-X-C motif chemokine ligand16 (CXCL16), growth differentiation factor-15 (GDF15), peptidoglycan recognition proteins (PGLYRP1), suppression of tumorigenicity-2 (ST2), tumor necrosis factor receptor-1 (TNFR1), soluble urokinase-type plasminogen activator receptor (suPAR) and TNF receptor Superfamily member 10c (TNFRSF10C) were interrelated and most of them were involved in TNF-α and IL-6 pro-inflammatory pathways.

- **MONOCYTE TO HIGH DENSITY LIPOPROTEIN-CHOLESTEROL (HDL-C) RATIO (MHR)**

The MHR is considered a predictor of major cardiovascular events.[60,61] Monocytes represent the majority of the inflammatory cells infiltrating the arterial wall in early atherogenesis and are involved in the release of pro-inflammatory and pro-oxidative cytokines at sites of inflammation; thus, they play an important role in chronic inflammation and cardiovascular diseases. HDL-C has anti-inflammatory and antioxidant effects, preventing the migration of macrophages, removing cholesterol molecules from these cells and inhibiting monocyte production and mobilization. Dogan et al. found that the MHR can be used in clinical practice as an easy detectable marker of inflammation in the diagnosis of MVA patients. Indeed, comparing the MHR in 105 patients with MVA and 125 healthy controls, the patients had significantly higher values of this ratio than controls, due to an increased monocyte count and decreased HDL-C levels[62].

- **PENTRAXIN-3 (PTX-3)**

PTX-3 is a marker of systemic inflammation structurally and functionally similar to CRP, produced by a variety of inflammatory cells.[63] It is a marker of vascular inflammation associated with increased cardiovascular risk.[64] Büyükkaya et al. measured serum PTX-3 and hsCRP levels in 41 MVA patients, 41 stable CAD patients and 40 controls with angina symptoms without evidence of myocardial ischemia[65]. MVA and CAD patients had higher PTX-3 and hsCRP levels than controls; however, no differences were observed between MVA and CAD groups. Serum PTX-3 and hsCRP levels were positively correlated.

SOLUBLE UROKINASE-TYPE PLASMINOGEN ACTIVATOR RECEPTOR (suPAR)

suPAR is a biomarker with pro-inflammatory and chemotactic effects which promote immune activation and fibrinolysis inhibition. This molecule is released by cleavage of uPAR, which is expressed on the membrane of hematopoietic, endothelial and smooth muscle cells.[66] Unlike CRP, suPAR is a very stable biomarker which is not subject to circadian variations.[66] Plasma suPAR levels have been shown to predict cardiovascular outcomes independently of CRP or traditional risk factors. Indeed, elevated systemic plasma suPAR levels have been associated with increased risk of major adverse cardiovascular outcomes in advanced CAD.[67,68]

Mekonnen et al. measured plasma suPAR levels in 66 patients with an abnormal non-invasive stress test, stable angina or stabilized acute coronary syndrome, but without angiographic evidence of CAD[69]. They demonstrated for the first time that plasma suPAR levels were associated with CMD: indeed, suPAR correlated negatively with CFR and, after adjustment for cardiovascular risk factors and hsCRP levels, it remained an independent predictor of CFR impairment. Corban et al. hypothesized that local production of suPAR and plasminogen activator inhibitor-1 (PAI-1) is associated with coronary endothelial

dysfunction; thus, they measured left main and coronary sinus suPAR and PAI-1 levels, as well as cross-coronary suPAR and PAI-1 production rates, in 79 patients with angina who had angiographically normal coronary arteries or mild epicardial CAD (<40% stenosis)[70]. All patients underwent acetylcholine coronary vasomotor testing to assess the presence of epicardial endothelial dysfunction (EED, defined as a decrease in coronary artery diameter >20% after acetylcholine), and microvascular endothelial dysfunction (MED, defined as a change <50% in coronary blood flow after acetylcholine infusion). Patients with MED had significantly higher local median coronary suPAR production compared with all other patients. Conversely, patients with EED had significantly higher median local coronary PAI-1 production compared with those with normal epicardial endothelial function. This is the first study to link local coronary suPAR production to endothelial dysfunction at the microvascular level.

- **HEAT SHOCK PROTEINS (Hsp)**

Hsp are a family of intracellular chaperone proteins with cytoprotective functions essential for cell survival in both physiological and stress conditions. The increased production of these proteins following stressful events, can lead to the activation of the immune system with the onset of an inflammatory reaction.[71] Hsp60 and Hsp72 have been linked to the atherosclerotic process and to ischemic myocardial damage.[72,73] Hsp60, produced by endothelial cells and myocytes in response to biochemical and/or infective insults, seems to participate in inflammatory processes leading to early atherogenesis.[71] Hsp72 is involved in the ‘‘hibernation” of myocytes, a myocardial adaptive process to chronic ischemia, with the role of myocardial cells protection.[72]

Even if there are no studies that can specifically validate the role of Hsp as possible markers of CMD, Giannessi et al. found a relationship between Hsp levels with the extent of

myocardial and coronary microvascular damage[73]. They enrolled 60 patients with idiopathic left ventricular dysfunction and angiographically normal coronary arteries (subdivided in 2 groups according to the severity of impairment of left ventricular ejection fraction (LVEF) and 22 healthy controls. There was a positive correlation between both the grade of left ventricle (LV) systolic dysfunction and the severity of CMD, assessed by rest/stress PET myocardial blood flow study**,** with plasma levels of Hsp60, Hsp72, anti-Hsp60 auto-antibody, CRP and IL-6.

[C] BIOMARKERS OF OXIDATIVE STRESS

Increased oxidative stress occurs in case of an imbalance between anti-oxidant defenses and excessive pro-oxidant reactive molecules, and it plays an important role in inflammation, endothelial dysfunction and atherogenesis.[74-76] Oxidative stress seems to be to be involved in the pathophysiology of MVA, by leading to an impaired endothelium-dependent vasodilatation of the coronary microvasculature and to the consequent decrease of CFR.[77,78]

- **SUPEROXIDE FREE RADICALS and MONONUCLEAR CELL ACTIVITY**

Free radicals are highly reactive molecules that result from the increased oxidative stress and can impair cellular structure and function, causing cell death. The evaluation of the amount of superoxide free radicals in whole blood, as well as the measurement of superoxide production by mononuclear cells during basal activity and after induced stimulation, are valid methods to assess intravascular oxidative stress in patients with atherosclerosis or subclinical atherogenesis.[79,80] In order to understand the possible role of mononuclear cell activity, endothelial inflammation and oxidative stress in MVA, Lin et al. evaluated inflammatory biomarkers, plasma total nitrite/nitrate level, superoxide free radicals in whole blood and

mononuclear cell activity in 32 patients with MVA, 34 with CAD and 17 healthy volunteers[81]. MVA and CAD patients had different degrees of oxidative stress. In particular, only patients with MVA had significantly increased levels of superoxide free radicals in whole blood; conversely, only patients with CAD had an increased production of free radicals after the induced activation of monocytes. These results suggested that in MVA there is no significant activation of circulating mononuclear cells, although there is evidence of an increased oxidative stress.

- **NITRIC OXIDE (NO) SYNTHETIC PATHWAY, L-ARGININE and ASYMMETRIC DIMETHILARGININE (ADMA)**

NO is a gaseous molecule with vasoactive action and vascular protective effects, which are mediated by the promotion of vasodilation, inhibition of platelets aggregation, adhesion of circulating leukocytes to the endothelium and proliferation of smooth muscle cells. NO is produced by the action of the enzyme NO synthase (NOS), which converts L-arginine to NO and L-citrulline.[82,83] ADMA is an endogenous methylarginine analogue of L-arginine which acts as a NOS competitive inhibitor, leading to a decrease in NO production and bioavailability. Other endogenous methylarginines, i.e. symmetric dimethylarginine (SDMA) and monomethylarginine (MMA), inhibit the NO pathway.[84] L-arginine facilitates endothelium-dependent vasodilation, with protective effects on the endothelium; therefore, a reduced availability of L-arginine could be related to endothelial dysfunction. Conversely, ADMA, which can be elevated in patients with hyperlipidemia, atherosclerosis and hypertension, prevents NO-mediated vasodilatation with a role in endothelial dysfunction.[85,86] NO in the vasculature is mainly produced by endothelial cells, though circulating cells are able to produce it as well. Indeed, red blood cells (RBCs) can produce NO, playing an important role in the regulation of systemic blood pressure.[87] Moreover, RBCs also express the enzyme arginase I, which regulates the function of NOS expressed in erythrocytes by

competing with NOS for its common substrate arginine to form L-ornithine.[88] Oxidative stress can reduce NO bioavailability by interfering with NO metabolism, thus leading to impaired endothelium dependent vasodilation and an increase in cardiovascular risk.[89] Consequently, these mechanisms can cause the onset of angina symptoms even in patients with normal coronary angiograms. Okyay et al. evaluated the correlations between plasma L-arginine and ADMA levels and their ratio with epicardial coronary blood flow, assessed by TIMI frame count method (TFC), and the grade of myocardial perfusion, valued by myocardial blush grade method (MBG)[90]. They enrolled 32 patients with MVA and 17 controls who complained of chest discomfort but with normal coronary angiograms and negative exercise stress testing. Both groups had similar L-arginine levels; however, MVA patients showed higher ADMA levels and lower L-arginine/ADMA ratio than controls. Moreover, ADMA levels were negatively correlated with MBG, whereas there was no evidence of correlations between TFC and other variables. Sen et al. studied possible correlations between plasma nitrate/nitrite (NOx), L-arginine and ADMA levels with common carotid intima-media thickness (cIMT)[91]. The study population included 30 patients with MVA and 30 controls with angina pain and normal coronary angiograms and no evidence of ischemic episodes in non-invasive tests. MVA patients had a higher cIMT, number of carotid atherosclerotic plaques and ADMA levels, but lower plasma NOx and L-arginine level, compared with controls. cIMT was positively correlated with ADMA levels, but negatively with plasma NOx and L-arginine levels. In order to assess the degree of oxidative stress and the impairment of NO pathway of patients with MVA, Porro et al. measured the principal metabolites involved in Arg/NO metabolic pathway (in both plasma and lysed RBCs), oxidative stress and RBC-NOS and arginase expression, in a population of 25 patients with MVA, 22 patients with CAD and 20 healthy controls[92]. Oxidative stress was evaluated by the ratio between oxidized and reduced forms of glutathione. It was reported

that MVA and CAD patients had similar inhibitor levels of the NO pathway (i.e. ADMA), in both plasma and RBCs, which were higher ($p<0.05$ and $p<0.001$ respectively) than those of controls. Conversely, controls had higher NOS expression in RBCs compared with patients. Finally, CAD patients had higher oxidative stress status and arginase I levels compared with other groups, even if oxidative stress of MVA patients was higher than in controls.

Further studies with larger cohorts are needed to better understand the clinical relevance of ADMA and L-arginine levels as prognostic factors that correlate with the severity of CMD.

➢ AMINOTHIOLS

Aminothiols are biomolecules with a sulfhydryl group which have important antioxidant effects.[93] Among them, glutathione is located intracellularly, whereas cysteine is located extracellularly and can be present in an oxidized disulfide form, cystine, which is a surrogate marker for oxidative stress. Low glutathione levels or high cystine levels are associated with increased oxidative stress. Changes in plasma aminothiols levels are related to diabetes, impaired endothelial function and atherosclerosis.[93] Moreover, a recent study found that disulfide/thiol homeostasis can be used as a novel parameter that reflects oxidative stress.[94] Dhawan et al. investigated on the possible role of plasma aminothiols levels in CMD and in abnormal coronary arteries[95]. In particular, they evaluated the association between CMD, epicardial plaque burden, plasma aminothiols and inflammation in 47 patients undergoing cardiac catheterization. Lower plasma glutathione levels correlated with a greater plaque burden and plaque necrotic core content, as well as impaired microvascular function, expressed by lower CFVR and higher hyperemic microvascular resistance (HMR). Cystine was not related to any parameters, whereas higher cystine/glutathione ratio correlated to a lower CFVR and greater plaque necrotic core content.

➢ LDL-C

LDL-C levels are important risk factors for the development of CAD. In particular, oxidized LDL, which are formed in the artery walls due to the oxidative effect of ROS (reactive oxygen species), stimulate chronic inflammation and epicardial coronary atherosclerosis.[96] Moreover, LDL-C levels inversely correlate with CFR in hypercholesterolemic subjects, therefore these particles might play an important role in the impairment of coronary microcirculation[97]. Mangiacapra et al. enrolled 95 patients with stable CAD who underwent coronary angiography and intracoronary functional evaluation[98]. They found a significant positive correlation between total cholesterol and LDL-C with the index of microvascular resistance (IMR); in particular, LDL-was an independent predictor of IMR.

- **PARAOXONASE-1 (PON-1)**

PON-1 is an enzyme associated with HDL-C which has 3 functions: PON, arylesterase and dyazoxonase.[99] It seems to have anti-inflammatory and anti-oxidative effects, by catabolizing highly reactive oxidized lipids in lipoproteins and cells. Hence, a decrease in PON-1 activity is associated with an increase in oxidative stress and atherosclerosis.[100] In line with these data, Gur et al. enrolled 41 patients with MVA, 33 patients with atypical chest discomfort (but with normal coronary angiograms and without evidence of ischemic episodes in non-invasive tests) and 20 healthy controls[101]. They found that serum PON-1 activity and total antioxidant status were significantly lower in the MVA group than in other groups; conversely, MVA patients had significantly higher lipid hydroperoxide levels. Moreover, serum arylesterase activity of PON-1 was negatively correlated to the grade of ST-segment depression in MVA group. Further studies are needed to better evaluate the possible diagnostic and prognostic importance of this enzyme in patients with CMD and MVA.

- **LYMPHOCYTE DNA DAMAGE**

Lymphocyte DNA damage, which is mainly caused by oxidative stress and inflammation, is associated with atherosclerosis and increased levels of this marker were found in patients with CAD.[102,103] Gur et al. investigated, for the first time, the possible association between lymphocyte DNA damage and MVA in 23 patients with MVA, 21 patients with atypical chest discomfort and 20 healthy controls[104]. MVA patients showed higher lymphocyte DNA damage levels and lower total antioxidant status (TAS) levels, compared with other groups. Particularly, in patients with MVA, DNA damage levels had an independent correlation with TAS and hsCRP.

[D] MIXED MECHANISMS AND BIOMARKERS

Several biomarkers, which are not included in the 3 major areas described above, could be involved and considered in mixed pathogenic mechanisms underlying MVA and CMD. Among these, we mention:

a) Plasminogen activator inhibitor type-1 (PAI-1): increased levels have been associated with endothelial dysfunction and inflammation in patients with MVA.[105]

b) Adiponectin and lipoprotein(a): are associated with endothelial dysfunction, pro-thrombotic effects and premature atherosclerosis.[106,107]

c) Galectin-3: a member of the lectin family, has a possible role in the pathogenesis of microvascular dysfunction in term of endothelial dysfunction, pro-inflammatory and pro-fibrotic effects.[108]

d) Endothelial progenitor cells (EPCs): EPC numbers are inversely correlated with risk factors for atherosclerosis, and reduced levels of EPCs are associated with endothelial dysfunction.[109]

e) Hyperinsulinism and hyperglycemia: are the main protagonists of MVA and correlate extensively with endothelial damage, neurohormonal dysfunction, and inflammation as well as oxidative and wall stress.[110-112]

f) Neuropeptide Y (NPY): a peptide endogenous to human coronary arteries, seems to cause constrictor effects on the coronary microcirculation. NPY can induce transient myocardial ischemia and can play a role in the genesis of angina in patients with MVA.[113-117] Table 1 summarizes the main mechanisms, related biomarkers and reference analyzed above.

CONCLUSION

CMD is the main mechanism which underlies microvascular angina. Invasive methods, such as intracoronary Doppler wires or the intracoronary thermodilution-derived method, are considered the gold standard to measure the response to vasoactive stimuli of the coronary microcirculation; however, they can be difficult to perform, expensive, risky for the patient and can also be associated with the prolongation of diagnostic angiography.[118] In the absence of obstructive CAD, coronary microvascular dilator function can be investigated by several non-invasive methods that allow measurement of the coronary blood flow, but some of them, i.e. cardiac PET and cardiac RMN, cannot be used routinely in clinical practice due to their high cost and limited availability. Another non-invasive method is transthoracic Doppler echocardiography which is relatively inexpensive and more available, but it is operator dependent and sometimes it is not possible to obtain good echocardiographic windows. Compared with these methods, the introduction in clinical practice of blood biomarkers detection in patients with suspected CMD and MVA can facilitate the diagnosis of these pathological conditions, offering several advantages, such as reduction of costs, less risk of physical harm, a greater availability/accessibility, and prognostic value.

As reported in this review, there are a variety of biomarkers involved in possible pathophysiological mechanisms responsible for the development of CMD and MVA; however, none of them has yet been validated for routine surveillance in this patient population and their management. In the near future, it is probable that the difficulties that at present prevent the introduction of blood biomarkers in the clinical routine will be overcome, following more evidence. Therefore, the use of biomarkers specific for CMD will be helpful in the differentiation between patients with epicardial CAD, CMD and without any coronary flow abnormalities, as well as in the implementation of a specific target-therapy.

Author contribution

All authors contributed to: (1) substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, and, (3) final approval of the version to be published.

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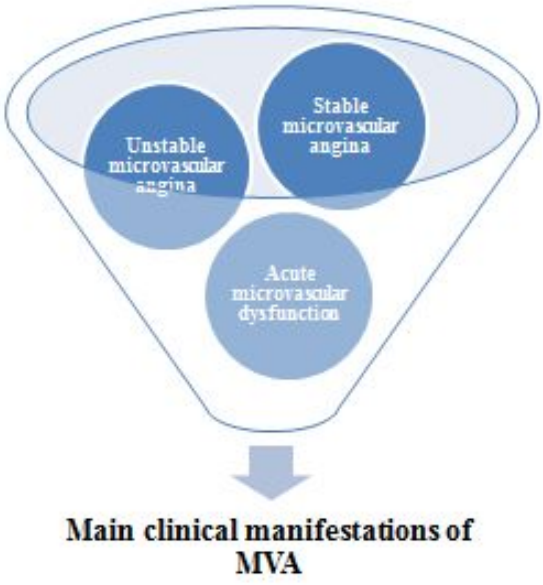

Fig. 1: Main clinical manifestations of microvascular angina (MVA)

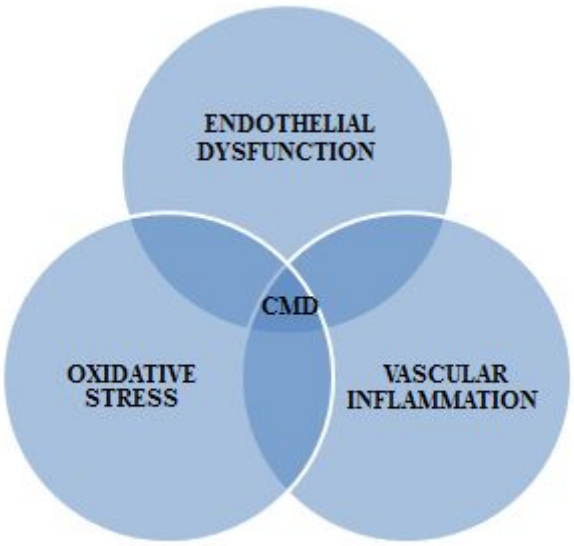

Fig. 2: Close correlation of main microvascular dysfunction (CMD) mechanisms.

TABLE 1. Main pathophysiological mechanisms, related biomarkers and references of CMD and MVA		
Pathophysiological mechanisms	***Biomarkers***	***References***
ENDOTHELIAL DYSFUNCTION	• Endothelins	[8, 12-19, 96-101]
	• Endocan	[20-27]
	• Angiogenetic factors	[7, 28-31]
VASCULAR INFLAMMATION	• C-Reactive Protein	[7-8, 32-38]
	• Cell adhesion molecules	[7-9, 39-42]
	• Inflammatory cytokines	[7-9, 43-47, 96-101]
	• Monocyte to HDL-C ratio	[48-50]
	• Pentraxin-3	[51-53]
	• SuPAR	[54-58]
OXIDATIVE STRESS	• Superoxide free radicals	[59-63]
	• Mononuclear cells activity	[59-66]
	• Nitric oxide synthetic pathway,	[7-9, 67-75]
	• L-arginine	[67-75]
	• Asymmetric dimethilarginine	[67-75, 96-97]
	• Aminothiols	[76-78]
	• LDL- cholesterol	[79-82, 96-97]
	• Lymphocyte DNA damage	[83-84]
MIXED MECHANISMS	• Plasminogen activator inhibitor type-1	[86]
	• Adiponectin	[87-88, 93-94]
	• Lipoprotein(a)	[89]
	• Galectin-3	[89]
	• Hyperinsulinism and hyperglycemia	[91-95]
	• Neuropeptide Y	[96-97]

Abbreviations: microvascular dysfunction (CMD); microvascular angina (MVA);

Tab.1. Main pathophysiological mechanisms, related biomarkers and references of CMD and MVA.