Assessment and pathophysiology of microvascular disease: recent progress and clinical implications

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The development of novel, non-invasive techniques and standardization of protocols to assess microvascular dysfunction have elucidated the key role of microvascular changes in the evolution of cardiovascular (CV) damage, and their capacity to predict an increased risk of adverse events. These technical advances parallel with the development of novel biological assays that enabled the *ex vivo* identification of pathways promoting microvascular dysfunction, providing novel potential treatment targets for preventing cerebral-CV disease. In this article, we provide an update of diagnostic testing strategies to detect and characterize microvascular dysfunction and suggestions on how to standardize and maximize the information obtained from each microvascular assay. We examine emerging data highlighting the significance of microvascular dysfunction in the development CV disease manifestations. Finally, we summarize the pathophysiology of microvascular dysfunction emphasizing the role of oxidative stress and its regulation by epigenetic mechanisms, which might represent potential targets for novel interventions beyond conventional approaches, representing a new frontier in CV disease reduction.
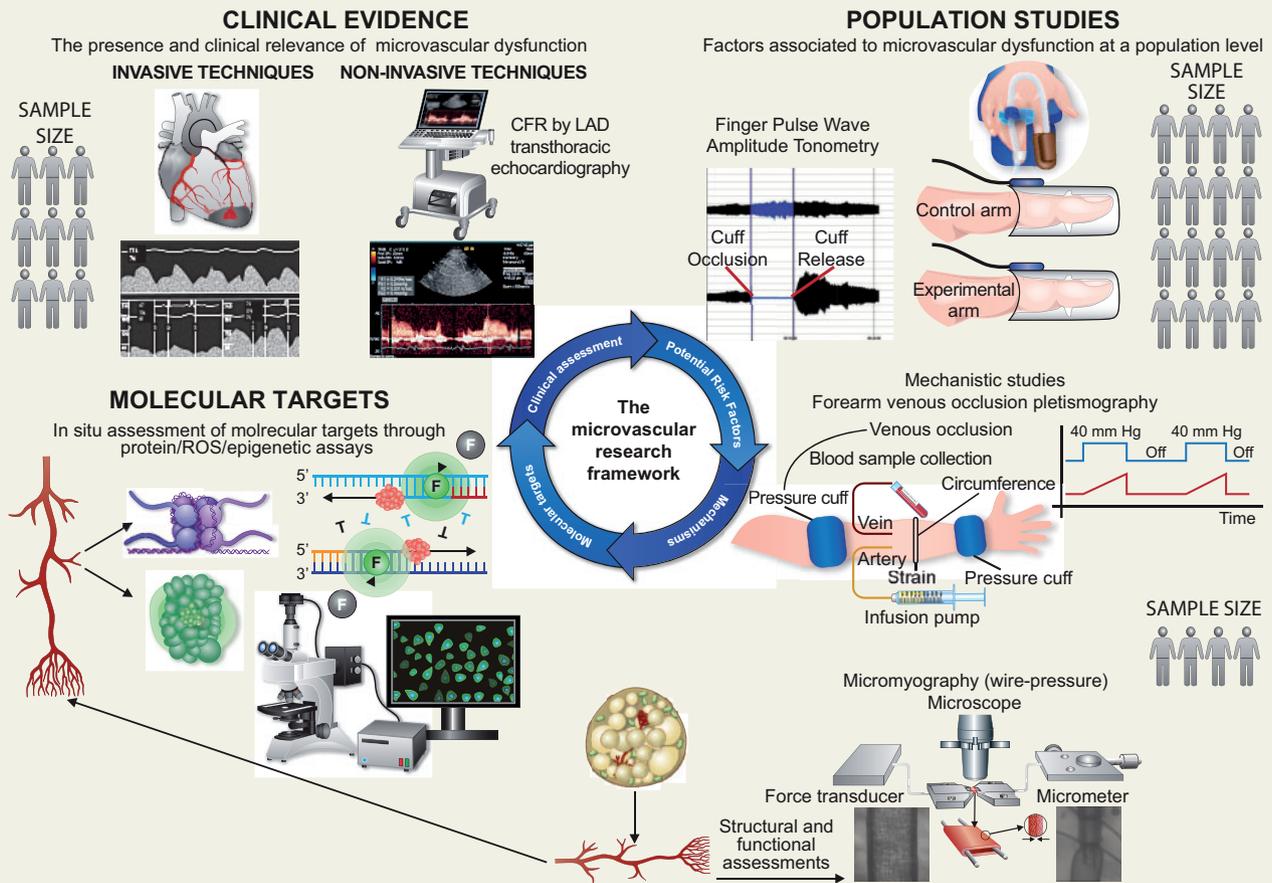
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Graphical Abstract

CLINICAL EVIDENCE
The presence and clinical relevance of microvascular dysfunction
INVASIVE TECHNIQUES   **NON-INVASIVE TECHNIQUES**

SAMPLE SIZE

CFR by LAD transthoracic echocardiography

POPULATION STUDIES
Factors associated to microvascular dysfunction at a population level

SAMPLE SIZE

Finger Pulse Wave Amplitude Tonometry

Cuff Occlusion   Cuff Release

Control arm
Experimental arm

MOLECULAR TARGETS
In situ assessment of molrecular targets through protein/ROS/epigenetic assays

The microvascular research framework

Clinical assessment
Potential Risk Factors
Mechanisms
Molecular targets

Mechanistic studies
Forearm venous occlusion pletismography

Venous occlusion
Blood sample collection
Pressure cuff
Vein
Artery
Infusion pump
Strain
Circumference
Pressure cuff

40 mm Hg   40 mm Hg
Off   Off
Time

SAMPLE SIZE

Micromyography (wire-pressure)
Microscope

Force transducer   Micrometer

Structural and functional assessments

Keywords   Microvascular disease • Oxidative stress • Epigenetics • Clinical manifestations

Introduction

The microcirculation, constituted by pre-arterioles (lumen diameter 100–500 μm), arterioles (lumen diameter <100 μm), capillaries, and venules, is responsible for most part of the resistance to flow that modulates blood pressure (BP) and tissue perfusion. As such, the microvascular structures have key roles in regulating systemic haemodynamics, tissue oxygenation and nutrition, transport of mediators, exchanges of gases and metabolites to and from tissues.[1] Over the years, microvascular dysfunction has been recognized as an early marker of cardiovascular (CV) disease and a key feature of several clinical manifestations (see the paragraph 'Historical Perspectives' in the Supplementary material online, *Addendum*).

This has led to the development of novel methods and refinement of old techniques for the assessment of microcirculation. Reference values to identify microvascular dysfunction are now being generated and have the potential to promote harmonization in the interpretation of the results between different laboratories worldwide. In parallel, advances have been made in the understanding of the biological processes regulating microvascular function, including cross-talk between inflammation, oxidative stress, and cellular ageing pathways.

In this article, we discuss how coronary and peripheral microcirculation can be investigated, the strengths and limitations of each method, and the reference values that could be used for a correct interpretation of the results. We also provide recent advances on the molecular mechanisms underpinning microvascular dysfunction, with particular emphasis on the role of epigenetic alterations regulating inflammation, oxidative stress, and cellular ageing pathways. We conclude that a more integrated approach to microvascular research should be adopted in the future, whereby central and peripheral assessment methods should be more frequently combined given their capacity to provide important complementary information. Such an integrated approach has the potential to overcome current

Table I Comparative summary of the strength, weakness, reproducibility and operator-dependency of each method used to assess coronary microvascular function in clinical practice

	Availability	Repeatability	Costs	Risks	Reproducibility and operator dependance	CMV dilatation	CMV constriction
Invasive methods[a]	+/-	-	--	+/--	+	+++	+++
TTDE	+++	+++	+++	+++	+/-	++	+/-
MCE	+/-	+/-	+	+	+/-	++	+/-
PET	-	-	-	+/-	++	+++	+/-
CMR	+	+/-	-	+	++	+++	+/-

(-) = poor for the item; (+) = sufficient for the item; (++) = good for the item; (+++) = very good for the item; (+/-) = between sufficient/poor.
CMR, cardiac magnetic resonance; CMV, coronary microvascular; IIM, invasive intracoronary methods; MCE, myocardial contrast echocardiography; PET, positron emission tomography; TTDE, transthoracic Doppler echocardiography.
[a]Include intracoronary Doppler recording of coronary blood flow velocity and thermodilution method (see text for discussion).

limitations in the development of microvascular active drugs (Take home figure).

Methods for assessing coronary microvascular function

Coronary microcirculation can be assessed using invasive and non-invasive techniques. Although each method has its advantages and limitations (*Table 1*), it is now clear that patients may present different types of coronary microvascular dysfunction (CMD), highlighting the necessity of a full assessment of coronary microvascular function.

Invasive assessment of coronary microvascular function

Nearly half of patients undergoing coronary angiography for appropriate indications have normal coronary arteries (NCAs) or non-significant coronary artery disease, as defined by the absence of any stenosis ≥50% of lumen diameter and/or an abnormal fractional flow reserve (FFR <0.80). Most of these patients has microvascular angina (MVA), i.e. angina caused by abnormalities of coronary microcirculation.[2–5]

Coronary microvascular function in the catheterization laboratory can be assessed by intracoronary vascular reactivity tests, that can be performed with high reproducibility and safety.[6,7] The evaluation usually begins with the assessment of coronary flow reserve (CFR), the maximal dilator response to an arteriolar vasodilator, usually adenosine. Furthermore, the response to acetylcholine (Ach) is also assessed. In normal vessels Ach at lower doses causes vasodilatation through release of nitric oxide (NO) by the endothelium; at higher doses, however, it also exerts direct constrictor effects on small coronary arteries via muscarinic receptors on vascular smooth muscle cells (VSMCs), which might occur at lower doses in dysfunctional vessels.[8,9] Accordingly, an abnormal coronary microvascular response to Ach may result from endothelial dysfunction, an increased reactivity of VSMCs, or both.
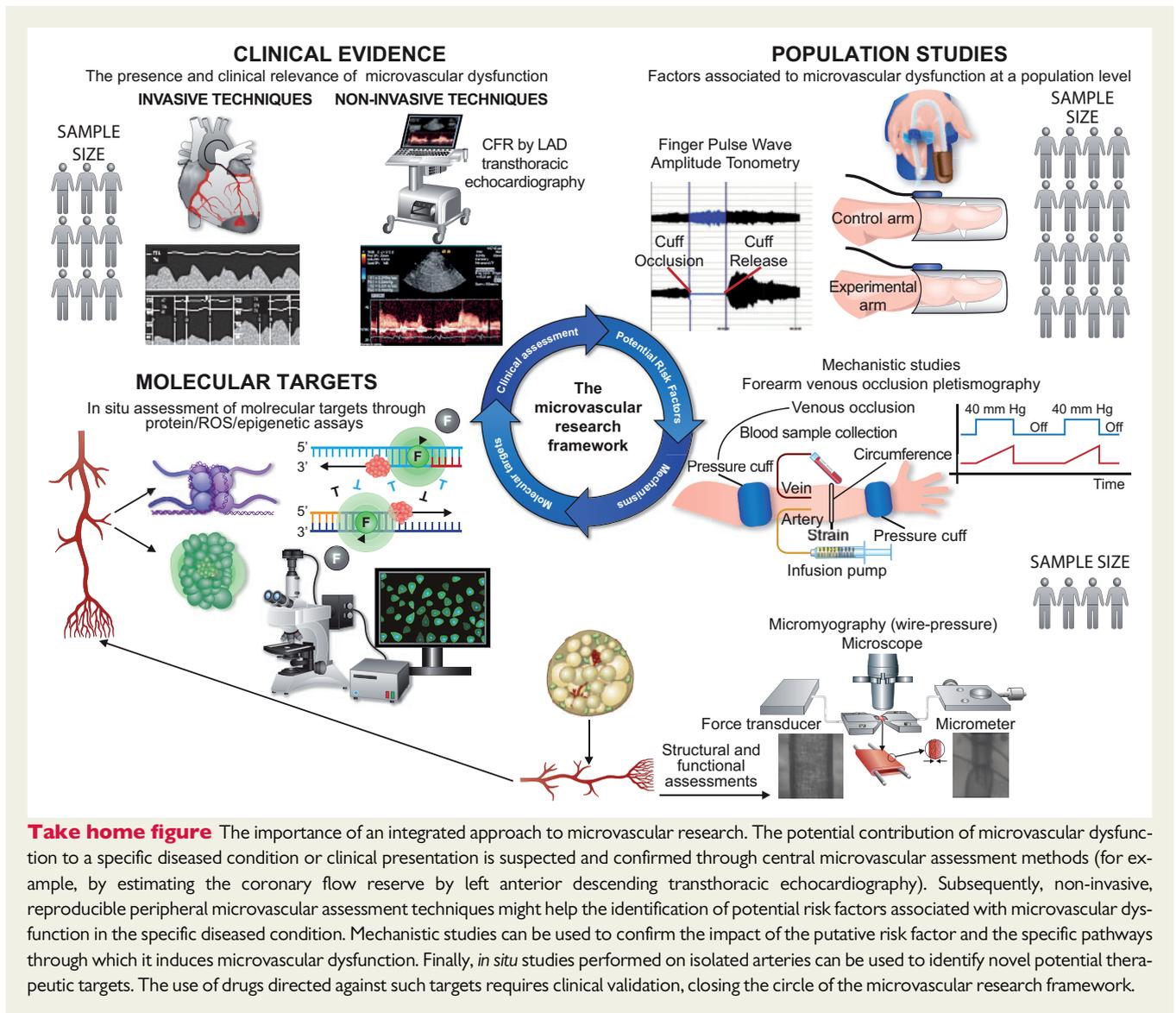
Coronary flow reserve
CFR in the cath lab can be assessed by measuring coronary blood flow (CBF) velocity by an intracoronary Doppler flow wire placed in the studied coronary artery [usually the left anterior descending (LAD) artery, Supplementary material online, *Figure S1*] or using a temperature sensor-tipped guidewire (CFR$_{thermo}$) (see the Supplementary material online, *Addendum*). Both CFR$_{Doppl}$ and CFR$_{thermo}$ have been shown to predict clinical events[10,11] and, therefore, in the absence of large comparative studies, both methods can be used to measure CFR. In a recent small study, CFR$_{Doppl}$ showed a better correlation, compared to CFR$_{thermo}$, with CFR assessed by positron emission tomography (PET), in particular in arteries with no-flow-limiting stenosis. Poor quality traces that did not allow a reliable assessment of CFR were, however, more frequent with the Doppler than the thermodilution method.[12] An advantage of the thermodilution method is that it allows a simultaneous assessment of FFR to exclude or confirm the presence of haemodynamically significant epicardial stenosis.

Adenosine-induced CFR is considered normal when >2.5. Although some differences in cut-offs may be found among individual studies, there is now large consensus that an adenosine CFR <2.0 should be considered as definitely demonstrative of an impaired dilatation of coronary microcirculation.[13] Values between 2.0 and 2.5 are of borderline clinical significance.

Index of microcirculatory resistance
Coronary microcirculatory function can also be tested by obtaining the index of microcirculatory resistance (IMR) with the pressure-thermodilution guidewire catheter (see above and the Supplementary material online, *Addendum*). IMR is a measure of coronary microvascular resistance during hyperaemia (usually obtained with adenosine). Assuming a zero pressure in the coronary vein/right atrium, IMR is calculated with the formula: IMR = P_d × Tmn$_{Hyp}$, where P_d is the mean coronary pressure in the distal part of the epicardial artery and Tmn$_{Hyp}$ the Tmn during hyperaemia.

IMR has been shown to be independent of epicardial vascular function and reproducible. In STEMI patients, IMR provides important prognostic information regarding the post-MI recovery of ventricular

Take home figure The importance of an integrated approach to microvascular research. The potential contribution of microvascular dysfunction to a specific diseased condition or clinical presentation is suspected and confirmed through central microvascular assessment methods (for example, by estimating the coronary flow reserve by left anterior descending transthoracic echocardiography). Subsequently, non-invasive, reproducible peripheral microvascular assessment techniques might help the identification of potential risk factors associated with microvascular dysfunction in the specific diseased condition. Mechanistic studies can be used to confirm the impact of the putative risk factor and the specific pathways through which it induces microvascular dysfunction. Finally, *in situ* studies performed on isolated arteries can be used to identify novel potential therapeutic targets. The use of drugs directed against such targets requires clinical validation, closing the circle of the microvascular research framework.

function.[14,15] Despite reasonable prediction of events and outcomes following PCI,[16,17] there are no guideline-based recommendations for its regular utilization in the catheterization laboratory at present.

Acetylcholine testing

To assess the microvascular response to Ach, this endothelial agonist is infused at increasing doses while CBF velocity is measured by Doppler guidewire. The effect is expressed as the ratio of average peak velocity (APV) of CBF during Ach administration over basal APV. A concomitant assessment of the epicardial response to Ach is usually done, to exclude the occurrence of epicardial spasm. Further details on Ach test can be found in the Supplementary material online, *Addendum*.

Result interpretation and cut-offs of acetylcholine-induced changes in coronary blood flow

The coronary microvascular response to Ach is considered normal when CBF increases by >50% compared to baseline,[2,4,9,18,19] in

absence of significant epicardial constriction. Thus, an increase lower than 50% indicates impaired coronary microvascular dilatation, but some patients show CBF reduction, which suggests an abnormal coronary microvascular constrictive response. Coronary microvascular spasm (CMS) can also be diagnosed when angina and ischaemic electrocardiogram (ECG) changes occur during Ach test in the absence of significant epicardial spasm.[13,20]

Non-invasive assessment of coronary microcirculation

In angina patients in whom flow-limiting coronary stenoses have been excluded by angiography and by FFR, coronary microvascular dilator function can be assessed non-invasively by various methods able to provide estimates of coronary/myocardial blood flow at rest and during maximal hyperaemia. In all cases, adenosine (140 µg/kg/min intravenously) or dipyridamole (0.56–0.84 mg intravenously) CFR is usually measured and criteria for normal/abnormal values are the same of those indicated for the invasive assessment, while an

Table 2 Strengths and weakness of the most commonly used central and peripheral microvascular assessment methods

	Strengths	Weakness	Reproducibility	Inter-observer variability	Recommended	Not recommended
Micromyography	• Direct and reproducible definition of microvascular structure (more precise with wire micromyography) and function (better evaluation with pressure micromyography) • The media-to-lumen ratio has prognostic significance • Definition of the patterns of microvascular remodelling (eutrophic/hypertrophic) • Opportunity for *in situ* assessment of gene/protein expression and epigenetic assays	• Micro-invasive as it requires bioptic species (limited availability) • Adequate training needed to ensure acquisition of reliable results and minimize the risk mechanical damage of the vessel	The intra-assay coefficient of variation of the media cross-sectional area calculation is 10.4% (6 vessels, 10 measurements in each vessel in a single session)[27]	The interassay coefficient of variation is 11.2% (6 vessels, 10 measurements in each vessel performed in 2 sessions by 2 different observers)[27]	Studies requiring: • Accurate assessment of microvascular structure together with the endothelial function, definition of the prevalent remodelling pattern • *In situ* evaluation of genetic/epigenetic/protein information • Acquisition of prognostic information	• Lack of biopsy samples or lab technicians expert in handling biopsy samples/small arteries • Studies requiring repeated microvascular assessments • Studies assessing the dynamic interaction between blood flow and vascular wall responses
Forearm venous plethysmography	• Easy access • Relatively cheap • Enables non-invasive acquisition of structural information (i.e. forearm minimum vascular resistance); • *In vivo* assessment of endothelial-dependent and in-dependent pathways accounting for microvascular dysfunction • Enables combined assessment of different aspects of endothelial physiology	• Adequate training needed to ensure standardization • The assessment of the vascular response to endothelial stimuli requires invasive protocols • Time-consuming (long time necessary for each acquisition)	Within-subjects coefficient of variation of baseline forearm blood flow ratio is around 20%.[28,29] Between-day reproducibility of hyperaemic vascular resistance is around 10%.[30]	Not available	Studies requiring: • Assessment of the dynamic interaction between blood flow and vascular wall responses under different stimuli • Non-invasive and (although indirect) evaluation of the microvascular structure • information on pathways involved in endothelial dysfunction *in vivo* • *in vivo* assessment of the potential impact of specific	Studies requiring: • Accurate assessment of microvascular structure or remodelling patterns • *In situ* evaluations of genetic/epigenetic/protein information related to microvascular dysfunction • Acquisition of prognostic information • Multiple microvascular assessments

Continued

Table 2 Continued

	Strengths	Weakness	Reproducibility	Inter-observer variability	Recommended	Not recommended
	in vivo (e.g. vascular tone, coagulation system)				drug/molecules on different aspects of the endothelial physiology	
Finger plethysmography	• The reactive hyperaemia index has prognostic significance • Relatively operator-independent • Availability of standardized protocols for signal acquisition • Requires minimal training • Non-invasive	• Microvascular structure not evaluable • The information acquired on microvascular reactivity does not reflect only NO availability • Impossible to assess potential pathways accounting for microvascular dysfunction • The finger probes are expensive • Prone to artefacts due to movements during acquisition	The interclass correlation for repeated assessments is reported between 0.61[31] and 0.74.[32]	Not available	Studies requiring: • Repeated measures of endothelial function • Limited influence of the operator on the acquired results • The acquisition of prognostic information • The inclusion of vulnerable populations (e.g. children)	Studies requiring: • Information on pathways involved in endothelial dysfunction • Microvascular remodelling information • Assessment of subjects with tremor or unable to maintain a stable position during acquisitions • Assessment of the integrated vascular wall-blood responses to different compounds

NO, nitric oxide.

increased reactivity to constrictor stimuli, as indicated by a critical reduction of CBF in response to their administration, cannot adequately be assessed by non-invasive methods due to the impossibility to exclude with certainty an involvement of epicardial vessels in the constrictor response.

Transthoracic Doppler echocardiography of the LAD coronary artery is a largely available and potentially widely applicable method to assess CFR,[21] but its use can be limited by technical issues and operator variability.[22] Myocardial contrast echocardiography, on the other hand, is a promising more objective ultrasound method to assess CFR, but it has been used in only few studies until now and requires further validation.[23,24]

PET and cardiac magnetic resonance (CMR) are the most reliable methods for non-invasive assessment of coronary microvascular dilation as they allow valuable measurements of global and regional myocardial blood flow. Both methods have been validated in animal models by microspheres blood flow studies, providing data for physiological classification and diagnosis of perfusion abnormalities.[25] Compared to PET, CMR shows a higher spatial resolution, lack of ionizing radiations and guarantees an assessment of cardiac structure and function (*Table 1*). However, the limited availability, high costs and long execution times of these methods, make them poorly suitable for serial assessment of CMV function (*Table 1*). Further limitations include radiation exposure for PET and, for CMR, contraindication to gadolinium use in patients with glomerular filtration rate <30 mL/min, claustrophobia, arrhythmias and implanted devices.[26]

Methods of assessing peripheral microvascular function/structure

The most validated and largely used techniques to measure microvascular function and/or structure in the peripheral microcirculation include forearm plethysmography, micromyography, finger plethysmography, and laser-Doppler flowmetry (LDF). Practical recommendations for the selection of the most appropriate peripheral vascular technique based on their strengths and limitations, as well as the research question to be addressed, are provided in *Table 2* and in the Supplementary material online, *Addendum*.

Forearm plethysmography

This technique allows measurements in forearm blood flow (FBF) changes by venous plethysmography before and after the infusion of endothelium-dependent and -independent vasoactive substances into a cannulated brachial artery.[33] The absence of systemic effects of the vasoactive drugs being tested is guaranteed by the lack of FBF changes in the contralateral forearm and the constant rate of arterial inflow.[34,35] Details are reported in the Supplementary material online, *Addendum*.

Venous plethysmography can also provide indirect information on the structure of small resistance arteries by measuring the maximum post-ischaemic flow. This is obtained by occlusion of the brachial artery of the dominant arm, followed by dynamic exercise (see the Supplementary material online, *Addendum* file for further details). Once the arterial occlusion is rapidly removed, the venous occlusion is maintained. The increased volume of the forearm is proportional

to the maximal arterial flow capacity. The minimum vascular resistances are obtained by dividing the mean BP for the maximum arterial flow. This measure correlates well with more direct measurements of resistance vessel structure, such as the media-to-lumen ratio (MLR) obtained with micromyography,[36] and is not influenced by sympathetic tone or administration of vasodilators.[37]

Cut-off of forearm blood flow and minimum forearm vascular resistance

Although some studies have documented a relationship between forearm endothelial function and risk of CV events,[38,39] at present clear cut-off values for abnormal FBF and minimum vascular resistance are not available. Accordingly, the use of this technique remains mainly limited to mechanistic studies.

Micromyography

This technique is performed in two major variants: wire and perfusion-pressure micromyography. Both methods enable the assessment of both functional and structural characteristics of isolated resistance arterioles (100–300-μm lumen diameter), obtained from subcutaneous tissue biopsies from the glutaeal or anterior abdominal region.[40–43] After isolation, vessels are cannulated with stainless steel wires (wire micromyograph) or slipped into two glass microcannulae contained in a pressurized chamber (pressurized myograph). Under the direct visualization of the vessel by a microscope connected to a computer, the endothelium-dependent and -independent relaxations are assessed by measuring the dilatory responses to cumulative doses of endothelial agonists (i.e. Ach) and sodium nitroprusside, respectively.[43,44] Details on the recommended protocols are reported in the Supplementary material online, *Addendum*.

The micromyography is the most widely used approach to evaluate microvascular structural changes. The total wall thickness, as well as the internal diameter of the vessels, can be evaluated by computer-assisted video analysers.[44] These data inform on the severity and the pattern of vascular remodelling induced by CV risk factor exposure. Given its high sensitivity and specificity, MLR is the most commonly used index of resistance artery remodelling, as it is independent from the vessels' dimensions,[45,46] with no difference between wire or pressure micromyography.[45,47,48] Conversely, media thickness and internal diameters are dependent on vessel dimensions;[45] therefore, comparisons between such parameters need caution.

Cut-off of media-to-lumen ratio

Age- and sex-specific percentiles of MLR in a healthy population have been recently provided and should be used to discriminate healthy subjects from those with early or advanced microvascular remodelling (Supplementary material online, *Table S1*).[40] Moreover, a cut-off value of 0.098 has been shown in two studies to distinguish between people with vs. those without CV events.[49,50] In contrast, no studies have documented a prognostic role for endothelial function, as evaluated by micromyography.

Finger plethysmography

This technique provides observer-independent measures of endothelial function using peripheral arterial tonometry (PAT). The

technique captures beat-to-beat recordings of the finger arterial pulse wave amplitude with pneumatic probes.[51] A pressure cuff is placed on one forearm and inflated to a supra-systolic pressure to produce 5 min of ischaemia followed by reactive hyperaemia. The pneumatic probe applied to the fingertip of the same arm records the increase in arterial blood volume, which is reflected by an increase in pulsatile arterial column changes. The derived reactive hyperaemia index (RHI) is considered a marker of endothelial function. Details of the recommended protocol are reported in the Supplementary material online, *Addendum*.

An inverse relation between RHI and multiple CV risk factors has been described,[51–54] and a systematic meta-analysis has documented that the RHI is as effective as flow-mediated dilation in predicting CV events.[55] However, it has been objected that RHI does not reflect only endothelial function, as augmentation of the pulse amplitude after reactive hyperaemia involves changes in flow that are only partly (50%) dependent on NO availability.[56] In addition, a poor correlation has been reported between PAT and flow-mediated dilation in various clinical settings.[57,58]

Cut-off of reactive hyperaemia index

Early studies have associated an RHI of >2.1 with a low risk of CV events,[59,60] while values <1.5 have been associated with an increased risk of CV events,[61] particularly in subjects at moderate-to-high CV risk or with angina symptoms. Other studies have confirmed that the cut-off value of RHI that independently discriminate between subjects at high or low risk for CV events ranges between 1.60 and 1.75.[62,63]

Laser-Doppler flowmetry

LDF provides a non-invasive real-time quantification of relative changes in skin microcirculation. It is based on light-beating spectroscopy and the detection of a Doppler shift of the laser light backscattered from moving red blood cell in cutaneous microcirculation.[64,65] The relative modifications in skin blood flow in response to endothelial stimuli [usually post-occlusive reactive hyperaemia (PORH), local thermal hyperaemia, Ach, or sodium nitroprusside iontophoresis] are considered. LDF shows a good reproducibility (coefficient of variation <10%).[66] Early studies suggested that PORH area is mainly driven by the activation of sensory nerves and myogenic response.[67] In turn, more recent reports have suggested that time to half before and after hyperaemia (TH2) may represent a reliable marker of endothelial function.[68,69]

Local thermal hyperaemia approach leads to a temperature-dependent sustained increase in skin cutaneous flow. The plateau is mostly mediated by NO and endothelium-derived hyperpolarizing factor (EDHF),[70] while the initial rapid phase relies predominantly on local sensory nerves.[71] The contributions of neural reflex, NO, and EDHF vary according to the target temperature and rate of heating. A rapid heating to 39°C seems to cause an almost exclusively NO-dependent hyperaemia.[72]

Iontophoresis can be used to test the influence on the LDF signal of vasoactive compounds. Ach and sodium nitroprusside might be used to generate endothelium-dependent and -independent vasodilatation, respectively. Some authors, however, showed that a large part of the Ach-related increase in blood flow is mediated by prostanoids with only a modest attenuation by NO synthase inhibition.[73] Others, instead, confirmed that Ach-mediated vasodilation is highly NO dependent, showing also an important interactions of NO with prostaglandins.[74] Of note, methacholine seems to induce a more specific NO-dependent vasodilation.[75]

Cut-off of laser-Doppler flowmetry

The LDF does not provide measures of absolute perfusion values. Measurements are expressed as arbitrary perfusion unit (PU) or in millivolts (1 PU = 10 mV). To account for BP variations, most authors present results as cutaneous vascular conductance, dividing blood flow by mean BP values (mV/mm Hg).[76] The non-specific responses to iontophoresis and different methods used to acquire and elaborate the results make difficult the identification of normal values for LDF; furthermore, it remains unclear whether LDF measurements have any additional value in predicting CV events.

Molecular mechanisms of microvascular disease: oxidative stress and inflammation drive microvascular ageing

Reactive oxygen species (ROS) and the inflammatory response can be considered final common pathways leading to microvascular dysfunction consequent to CV risk factor exposure. Increasing evidence supports a tight and highly dynamic regulation of inflammatory and redox-signalling pathways induced by inflammatory, metabolic, and haemodynamic changes at the level of the microcirculation. Many of these pathways are also involved in the control of cellular ageing and apoptosis and, in animal experiments, their modulation can induce substantial changes in the lifespan, ultimately supporting a relevant contribution of an inflammageing process to the evolution of microvascular dysfunction.

The mitochondrial adaptor p66^{Shc} has emerged as a pivotal modulator of mitochondrial ROS production, inflammation, and vascular aging.[77] Several CV risk factors have been shown to induce p66^{Shc} up-regulation, leading to premature ROS accumulation, senescence, and endothelial dysfunction in mice and humans[77] (*Figure 1*). Genetic deletion of p66^{Shc} prevents age-dependent endothelial dysfunction and extends lifespan by 30% in mice.[78–80] Activation of Arginase II (Arg II), the enzyme that metabolizes the eNOS substrate L-arginine to urea and L-ornithine, also represents an important link between CV risk factors and microvascular aging. Arg II activity is increased in patients with type 2 diabetes (T2DM) and hypertension, where accounts for reduced L-arginine availability, decreased NO production, and endothelial dysfunction.[81] It also triggers vascular senescence in VSMCs and endothelial cells.[82] Arginase inhibition restores endothelial-dependent vasorelaxation in small arteries of obese patients, although this effect is attenuated in ageing due to a high pro-oxidant environment sustained by hyperactivation of the NADPH oxidase (Nox).[41,83]

The family of Nox enzymes is another major source of microvascular ROS. Clinical conditions associated to the perturbation of microvascular haemodynamic and metabolic homeostasis are characterized by increased vascular reactivity and overexpression of
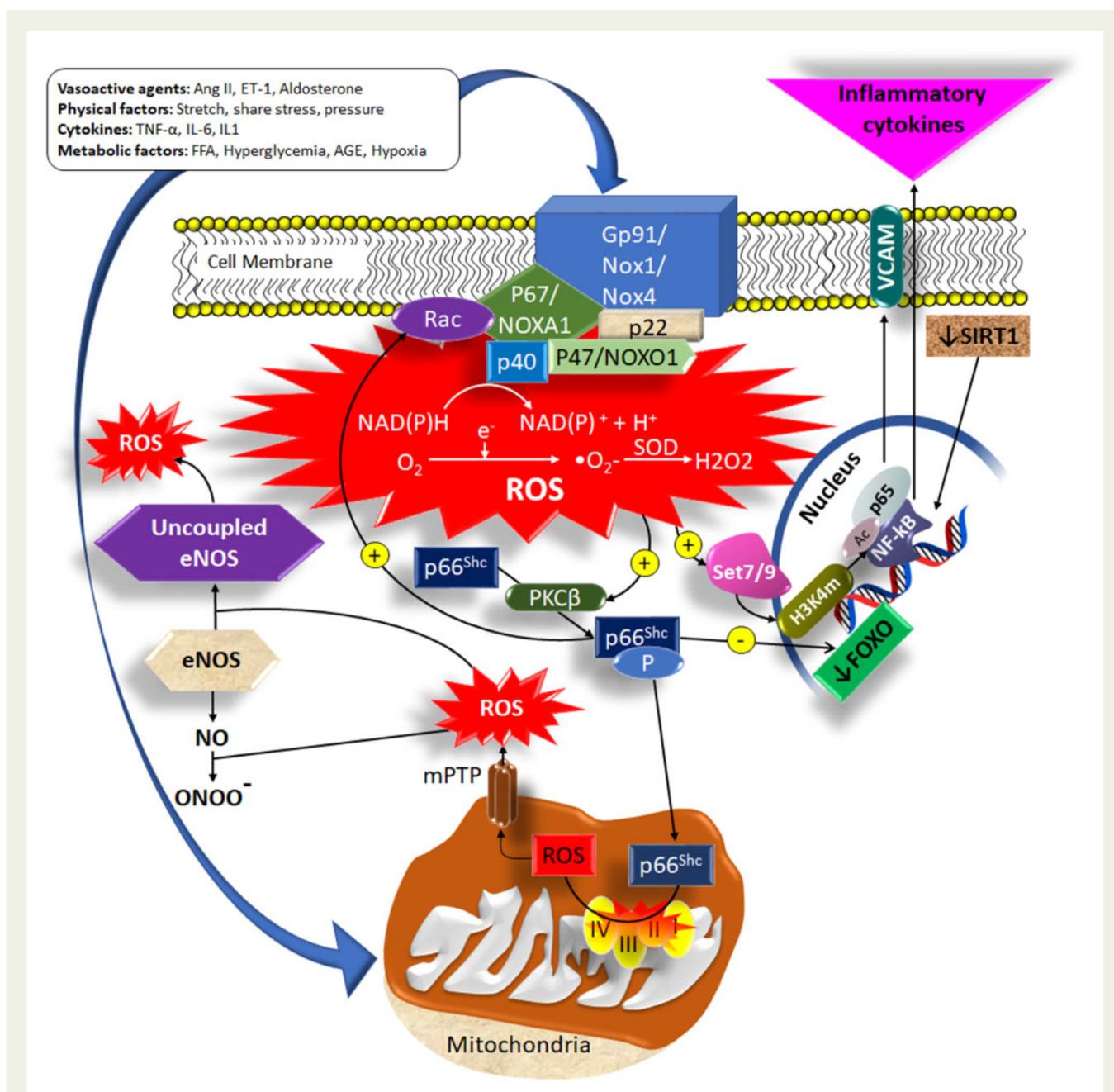
Figure 1 Major pathways leading to ROS-mediated microvascular dysfunction. Nox isoforms and mitochondria represent major systems accounting for the dynamic regulation of ROS production within the microvascular wall. The activation of Nox leads to ROS production and triggers p66^{Shc} phosphorylation and translocation within the mitochondria, where it further promotes ROS generation by altering the function of the mitochondria respiratory chain and opening the mitochondria permeability transition pore. In turn, p66^{Shc} activation stimulates the activity of Nox, thus generating a vicious cycle that amplifies ROS production. The increased concentration of intracellular ROS promotes the transformation of NO in peroxynitrite radicals and uncouples the eNOS, switching its activity from a NO to a ROS producing enzyme. Epigenetic modifications commonly detected in ageing, such as a reduced SIRT1 expression, sustain a further increase in intracellular oxidative stress, reducing the expression of antioxidant enzymes (secondary to FOXO inhibition) and promoting pro-inflammatory cytokine production through activation of the NF-kB and expression of adhesion molecules. NO, nitric oxide; NoX, NADPH oxidase; ROS, reactive oxygen species.

different Nox isoforms, which has been implicated in the development of an early microvascular ageing phenotype.[41,84] Furthermore, inflammatory cytokines [i.e. tumour necrosis factor alpha (TNF-α) and interleukin-6] are often elevated in patients with cardiometabolic disturbances and associate with the augmented activity of Nox and

vascular aging.[85] Sirtuins (a family of nicotinamide adenine dinucleotide-dependent proteins) represent another example of the connection between oxidative stress and ageing pathways at the level of the microcirculation. Age-related loss of SIRT1 leads to functional deficits, diminishes stress response, reduces capacity for migration/

proliferation, and increases senescence by modulating several downstream effectors including LKB1, AMPK, and FOXO.[86] Moreover, microRNA-217 suppresses SIRT1-dependent eNOS functionality and triggers endothelial senescence.[87]

The increased ROS generation at the level of the microvascular wall is also a potent activator of the inflammatory response. ROS activate mitogen-activated protein kinases (ERK1/2, p38MAPK, and JNK), tyrosine kinases, and tyrosine phosphatases, which control vascular cell proliferation, migration, hypertrophy, and inflammation.[88–90] Important downstream redox targets are transcription factors (NF-kB, AP-1 and HIF-1) and pro-inflammatory genes, critically involved in chemokine and cytokine production, recruitment, and activation of inflammatory and immune cells, which promote microvascular inflammation and fibrosis in response to CV risk factors.[89] Indeed, human studies showed an inverse relationship between peripheral microvascular structural changes and circulating Treg lymphocytes.[91] In addition, subpopulations of circulating T lymphocytes are associated with microvascular oxidative stress in peripheral human arteries.[91]

Epigenetic regulation of microvascular function

Epigenetic changes or mutations are emerging as key mechanisms underpinning the dynamic ability of the microcirculation to modulate inflammation, oxidative stress, and ageing signalling in response to changes in the local metabolic and haemodynamic environment.[92] Some of most intensively studied epigenetic changes involved in oxidative stress-mediated microvascular dysfunction relate to the p66^{Shc} gene. Epigenetic remodelling of p66^{Shc} promoter by DNA methylation and histone modifications is involved in obesity and diabetes-induced oxidative stress, endothelial dysfunction, and vascular hyperglycaemic memory, the phenomenon responsible for persistent oxidative stress and vascular dysfunction despite the achievement of an optimal glycaemic control.[93–95] Reduced DNA methylation and increased methylation of histone at lysine 4 (H3K4) were found on the promoter of TNFα, a pro-inflammatory cytokine involved in obesity and hypertension-related microvascular dysfunction.[96,97] Similarly, activation of the methyltransferase SUV39H1 in VSMCs from diabetic mice was found to regulate the pro-inflammatory transcription factor NF-kB p65.[98] Furthermore, mono-methylation of histone 3 at lysine 4 (H3K4me1) by the methyltransferase SETD7 fosters endothelial inflammation, senescence, and vascular dysfunction via up-regulation of NF-kB p65 and p53.[99] Besides the modifications in chromatin, several miRNAs (miR-92, miR-126, miR-195, miR 26a, and miR-155) are implicated in microvascular dysfunction by targeting relevant pathways involved in endothelial cell and VSMC damage (i.e. eNOS, RhoA, Smad1, Sirt1, Bcl-2).[100] The importance of these epigenetic discoveries is supported by the recent development of epigenetic pharmacological intervention. The histone deacetylase inhibitor Vorinostat has shown to prevent NF-kB activation, eNOS uncoupling, and vascular oxidative stress in diabetic mice.[101] Similarly, histone deacetylase blockade by Trichostatin A and sodium butyrate prevented TNF-α transcription while promoting angiogenic response.[102] Apabetalone—an epigenetic regulator targeting bromodomain and extra-terminal proteins—modulates vascular inflammation and reduces CV events.[103] Also, miRs targeting, by mimics or antagomiRs, is a promising epigenetic therapy currently tested in clinical trials.[104,105]

Clinical implications of microvascular dysfunction

Coronary microcirculation

Many studies have shown that CMD can be responsible for both chronic and acute myocardial ischaemic syndromes.[106,107] The microvascular origin of ischaemic symptoms should be suspected in patients with the evidence of myocardial ischaemia at non-invasive investigation who show no obstructive stenosis (usually ≥50%) and no other specific causes of ischaemia (e.g. epicardial spasm, intracoronary thrombus, coronary dissection) at coronary angiography, but it is definitely demonstrated by confirming the presence of CMD.[13]

MVA [and asymptomatic myocardial ischaemia with non-obstructed coronary arteries (INOCAs)] may occur in the context of various heart diseases,[108] but it also represents the main or specific cardiac issue for many patients (primary MVA). Stable MVA, mainly related to efforts, is the most typical clinical presentation.[106] Both an impairment of coronary microvascular dilatation and an increased coronary microvascular constriction have been demonstrated and may variably contribute to the symptoms in these patients.[109–112]

The initial treatment of stable MVA is based on traditional anti-ischaemic drugs (beta-blockers, calcium antagonists, nitrates). These, however, fail to obtain a satisfactory control of angina symptoms in a sizeable proportion of patients.[113,114] Thus, several additional drugs (e.g. ranolazine, xanthines, ivabradine, trimetazidine) and non-pharmacological therapies (e.g. spinal cord stimulation, enhanced external counterpulsation) have been proposed and may help achieve significant angina relief.[115,116] Importantly, the recent CorMicA trial has shown that, in patients with angina but non-obstructive CAD, pharmacological therapy guided by the results of invasive provocative testing, aimed to identify the pathophysiological mechanism(s) responsible for the symptoms, may result in better symptom control, as compared with empiric choices.[117]

Prognosis of stable MVA is debated. Studies including patients with NCAs have consistently shown good clinical outcome.[113,114,118,119] Some reports, however, have described increased rates of clinical events in angina patients with non-obstructive coronary stenoses[120,121] or a relation between CMD and events.[9,122,123] The interpretation of these results is complicated by the recruitment of heterogeneous populations, in whom the role of CMD as an independent causal factor was not assessed appropriately. Furthermore, the relation of CMD with outcome was not consistent among studies.[9,122–125] Thus, whether CMD adds prognostic information in patients with non-significant stenosis requires further assessment.[126]

Most recently, CMS has been suggested to be a cause of the clinical picture of acute coronary syndrome (ACS) with non-obstructed coronary arteries at angiography, who constitute 10–15% of those admitted with a diagnosis of ACS and include patients with myocardial infarction with non-obstructed coronary arteries (MINOCAs).[13,106,127] The mechanisms of these ACSs, however, are heterogeneous, and the prevalence of CMS-related ACSs remains to be established.[128] In a study, 16.3% of 80 MINOCA patients[129] had

evidence of CMS on Ach testing, which was associated with a worse clinical outcome. However, most patients with events also had epicardial spasm and, therefore, the exact prognostic role of CMS in this context also remains to be better defined.

No established treatment for either acute INOCA or MINOCA is available at present, due to the different pathophysiological mechanisms responsible for the syndrome and the lack of clinical trials. Some benefits on CV events have been reported in a retrospective study by statins, beta-blockers, and inhibitors of the renin–angiotensin system,[130] but calcium antagonists are expected to be the main treatment for recurrent angina episodes related to CMS.[131]

Finally, recent data suggest that CMD may be involved in the mechanisms responsible for heart failure with preserved left ventricular ejection fraction,[132] although prospective studies relating CMD to the development of this clinical condition are still lacking.

Peripheral microcirculation

Some studies showed that peripheral microvascular endothelial dysfunction might be a powerful predictor of CV events in both subjects at high or low CV risk.[38,39] In particular, the RHI and the hyperaemic velocity (considered an indirect marker of microvascular function) confirmed that the severity of endothelial dysfunction at the forearm microcirculation can predict the risk of future CV events.[61,133,134]

Microvascular structural changes also provide reliable prognostic information. Structural alterations in the subcutaneous vascular district are, indeed, a good reflection of those detectable in target organs, including heart[135] and brain.[136] This might lead to impaired organ flow reserve and, as such, could account for the faster progression of organ damage and the increased CV risk of diabetic and hypertensive patients.[137,138] An increased MLR is also a powerful and independent predictor of adverse CV outcome.[49,50,139]

There is evidence suggesting that interventions on advanced microvascular disease burden are less effective than early treatments.[140] Indeed, individuals with high MLR remain at increased CV risk despite normalization of BP values.[141] Instead, early CV interventions have significant impact on peripheral artery remodelling,[142–144] potentially leading to improved tissue perfusion and regression of organ damage.[142] Also, more aggressive interventions are needed to reduce the burden of microvascular remodelling in patients with advanced disease.[145]

Conclusions

The increasing use of invasive and non-invasive coronary and peripheral microvascular assessment methods has unravelled the role of microvascular dysfunction in several CV disease manifestations. In parallel, a better understanding of the biological mechanisms involved in microvascular dysfunction has shown that regulation of the microvascular phenotype is highly dynamic and tightly regulated by local levels of inflammation and oxidative stress. Epigenetic modifications are emerging as key mechanisms of post-natal microvascular adaptation to different environmental factors and have highlighted the substantial overlap between inflammatory, oxidative stress, metabolic and cellular ageing pathways in the regulation of the microcirculation phenotype. Despite these advancements, no microvascular active drugs are available at present. This is related to several factors,

including the difficulties in creating large datasets of microvascular data acquired using standardized protocols and the limited integration of the data obtained from central and peripheral microvascular methods. The refinement of microvascular assessment techniques and standardization of their protocols, combined with the availability of specific prognostic cut-off values, provide now a unique opportunity to move microvascular research from the bench to a clinical setting, potentially allowing the earlier identification of patients at high CV risk. Furthermore, the adoption of a more integrated approach to microvascular research in the future, whereby central and peripheral microvascular assessments are performed as part of the same study, has the potential to accelerate the identification of novel and disease-specific therapeutic targets and the development of drugs that could be used to prevent the evolution and clinical consequences of microvascular disease.

Supplementary material

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