Endothelial Function in Cardiovascular Precision Medicine: A Consensus Paper of the European Society of Cardiology Working Groups on Atherosclerosis and Vascular Biology, Aorta and Peripheral Vascular Diseases, Coronary Pathophysiology and Microcirculation, and Thrombosis

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ABSTRACT

Endothelial cells (EC) are sentinels of cardiovascular health. Their function is reduced by the presence of cardiovascular risk factors, and is regained once pathological stimuli are removed. In this European Society for Cardiology Position Paper we describe endothelial dysfunction as a spectrum of phenotypic states and advocate further studies to determine the role of EC subtypes in cardiovascular disease. We conclude that there is no single ideal method for measurement of endothelial function. Techniques to measure coronary epicardial and microvascular function are well established but they are invasive, time-consuming and expensive. Flow-mediated dilatation (FMD) of the brachial arteries provides a non-invasive alternative but is technically challenging and requires extensive training and standardization. We therefore propose that a consensus methodology for FMD is universally adopted to minimize technical variation between studies, and that reference FMD values are established for different populations of healthy individuals and patient groups. Newer techniques to measure endothelial function that are relatively easy to perform, such as finger plethysmography and the retinal flicker test, have the potential for increased clinical use provided a consensus is achieved on the measurement protocol used. We recommend further clinical studies to establish reference values for these techniques and to assess their ability to improve cardiovascular risk stratification. We advocate future studies to determine whether integration of endothelial function measurements with patient-specific epigenetic data and other biomarkers can enhance the stratification of patients for differential diagnosis, disease progression and responses to therapy.

WRITING PROCESS

This Position Paper was written by the following ESC Working Groups: Atherosclerosis and Vascular Biology, Aorta and Peripheral Vascular Diseases, Coronary Pathophysiology and Microcirculation, and Thrombosis. This topic was discussed at an ESC-sponsored session entitled “Endothelial Cell Dysfucntion” held at the European Vascular Biology Organisation meeting in 2016 (Maastricht, the Netherlands) which was Chaired by Prof. Paul Evans (Sheffield, UK) and Prof. Marie-Luce Bochaton-Piallat (Geneva, Switzerland). This session included talks from Prof. Arno Schmidt-Trucksäss (Basel, Switzerland), Dr Rosa Suades Soler (Karolinska Institutet, Sweden), Prof. Danijela Trifunovic (Belgrade, Serbia), Prof. Michael Shechter (Tel Aviv, Israel), Dr Elena Osto (Zürich, Switzerland), Prof. Yvonne Alexander (Manchester, United Kingdom). It was followed by a writing meeting attended by the Chairs, speakers and Prof Dirk Duncker (Rotterdam, the Netherlands). Other worldwide experts in the field of endothelial function testing were subsequently recruited to the writing process.

1. DEFINING ENDOTHELIAL FUNCTION AND DYSFUNCTION.

1.1 What is endothelial dysfunction?

The vascular endothelium acts a semipermeable barrier to regulate an exchange of fluids, nutrients, and metabolites, and is critical to haemostasis and vascular health. In healthy arteries, endothelial cells (ECs) exist in a quiescent state that is maintained by laminar blood [1, 2], and by circulating cytoprotective factors such as high density lipoprotein (HDL)[3]. However, several stimuli including chronic disease states[4], metabolic conditions (e.g. type 2 diabetes mellitus (T2DM), obesity, dyslipidemia), smoking[5] and disturbed blood flow[6-8] interrupt the quiescent phenotype and drive EC dysfunction[9, 10] In 1998, Hunt and Jurd defined dysfunctional ECs by 5 key characteristic mechanisms: 1) loss of vascular integrity, 2) increased expression of adhesion molecules, 3) pro-thrombotic phenotype, 4) production of cytokines and 5) up-regulation of human leukocyte antigen molecules[10]. It is now known that EC dysfunction is not a single pathological state but instead represents a spectrum of phenotypes associated with pathophysiologically heterogenous alterations in vascular tone, permeability, inflammation and de-differentiation, leading to the loss of homeostatic functions of endothelium (Figure 1). Indeed, recent single cell RNA sequencing studies have revealed multiple distinct EC subtypes for instance with aneurysms and atherosclerosis, thus emphasising the heterogeneity of ECs in diseased tissues[11-14] Aside from tissue-resident endothelium, EC dysfunction also involves changes in circulating endothelial colony forming cells (ECFCs) and endothelial-derived microvesicles (EMVs) that have major roles in cardiovascular health and disease.

1.2 Vascular tone, nitric oxide and superoxide anion.

In physiological conditions, maintenance of appropriate endothelial function provides vasorelaxant and protective properties through release of vasoactive substances such as nitric oxide, prostacyclin (PGI_2), and/or endothelium-derived hyperpolarizing factor[4,9,10]. For their discovery of nitric oxide (NO) as a signalling molecule in the cardiovascular system, Ferid Murad, Robert Furchgott and Louis Ignarro earned the Nobel Prize for medicine in 1998. NO, a gaseous mediator produced by ECs, is generated from the nitrogen atom of L-arginine and O_2[15], and catalysed by endothelial nitric oxide synthase (eNOS)[16]. While primarily defined through its regulation of vasorelaxation and vascular tone, NO exerts several atheroprotective effects including protection against oxidative stress, platelet activation and aggregation, inflammation and smooth muscle cell (SMC) proliferation[17]. However, the bioavailability of NO is reduced by numerous cardiovascular risk factors[18]. eNOS dimer is the main contributor to homeostatic NO in healthy cells. In pathology, when important co-factors such as tetrahydrobiopterin (BH4) are depleted, eNOS becomes uncoupled and it's monomers contribute to reactive oxygen species (ROS) production[19] which is a driver of EC dysfunction. Reduced bioavailability of NO can also be due to oxidative inactivation. Indeed, loss of

BH4 is often linked to vascular oxidative stress, characteristic for all major clinical risk factors for atherosclerosis, including diabetes, hypertension, hypercholesterolemia and smoking[20]. In human vasculature, endothelial and smooth muscle NADPH oxidases contribute to superoxide anion production[21-23]. Rapid scavenging of NO by superoxide with generation of strongly prooxidant peroxynitrite ($ONOO^-$) remains the principal mechanism of endothelial dysfunction in wide range of clinical conditions. $ONOO^-$ is in turn able to oxidize BH4 to BH2 contributing to eNOS uncoupling[24]. In some cases, eNOS substrate L-arginine or co-factor NADPH bioavailability may be limited, or eNOS expression inhibited by epigenetic modifications, including miRNA. The relative importance of these distinct mechanisms of loss of NO bioavailability may differ in individual patients leading to the need for precision medicine approaches. These could be achieved by biomarker screening such as for example plasma BH4, miRNA, L-Arginine allowing for targeted approach to pathology underlying the dysfunction. Lack of bioavailability of other mediators such as prostacyclin can provide an important factor in the pathogenesis of vascular disease. For example, cardiovascular effects of Cox-2 inhibitors have been linked to loss of vascular PGI_2 production which promoted development of endothelial dysfunction[25].

1.3 Vascular permeability and inflammation.

It is well recognised that pro-atherogenic stimuli and cardiovascular risk factors, including diabetes, obesity and smoking cause functional and structural changes in the permeability properties of the endothelium. These alterations are characterised by a rise in the movement of plasma across the vessel wall and into the surrounding tissues, comprehensively reviewed elsewhere[26]. Numerous studies show that endothelial permeability, inflammation and atherosclerosis are inextricably linked. Interleukin (IL)-1β is a cytokine that is activated by the NLRP3 inflammasome, a master regulator involved in innate immunity. Thus, as a result of NLRP3 inflammasome/IL1β activation by cholesterol crystals, lipids and triglyceride-rich lipoproteins, pathogen-associated molecular patterns and disturbed blood flow[27-34], ECs will be activated and express adhesion molecules (e.g. vascular adhesion molecule-1, intercellular cell adhesion molecule-1, E-selectin) to drive vascular inflammation and atherosclerosis initiation and progression. Mendelian Randomization (MR) studies have now shown strong links between specific inflammatory proteins and lipid metabolism in patients with an inflammatory status, providing the impetus to develop novel systemic and vascular immunomodulatory approaches to address the public health challenge of cardiovascular disease (CVD). Thus, genetic screens linking metabolic and plasma proteomic profiles with causal effects, are becoming an attractive approach in the cardiovascular precision medicine arena[35, 36] and may provide both novel targets and/or an improved prognostic tool for stroke, ischemic heart disease and T2DM[37].

1.4 Phenotypic plasticity.

The full details of EC plasticity are outside the scope of this ESC Position Paper but have been extensively reviewed elsewhere[38-40]. It is now established that endothelial-to-mesenchymal transition (EndMT), a process characterised by loss of EC markers, gain of mesenchymal markers, activation and delamination, is of particular relevance in atherosclerosis[40]. Endothelial-lineage tracing studies from the Simons laboratory revealed that ECs activated in response to TGFβ signaling undergo EndMT leading to migration, dedifferentiation and contribution to plaque formation and progression[41]. Kovacic and his team used endothelial-specific lineage-tracking, to show that EndMT-derived fibroblast-like cells are present in atherosclerotic lesions, and coexpress endothelial and fibroblast/mesenchymal proteins, a recognised hallmark of EndMT[42]. Further studies have revealed that EndMT is driven by disturbed flow, oxidative stress and hypoxia [42-44]; all of which trigger progression of atherosclerosis. However, the contribution of EndMT-derived cells to plaque development is an area of ongoing investigation.

1.5 Organ-specific endothelial cell specialization.

ECs are specialized in different vascular beds, such as the unique vasculature of the kidneys or the blood-brain barrier. Specific subtypes of endothelial cells have also been found in adipose tissue, gut and other tissues, and multiple distinct EC subtypes have been revealed by single cell sequencing in aorta[13] and atherosclerotic plaques[11]. Unravelling the as yet unknown molecular pathways that specify and sustain each organ functional and structural diversity will set the stage for deciphering the pathogenesis of several disorders, allowing future attempts at reversal of endothelial dysfunction and improved patient outcome. At the same time, while molecular mechanisms appear to differ between different vascular beds, clinically measured vascular dysfunction can correlate between different arterial beds[45] as well as between arterial and venous endothelium[46] suggesting that endothelial dysfunction can be systemic.

1.6 ECFCs and EMVs.

ECFCs comprise a heterogenous population of cells that have distinct roles in angiogenesis and vascular repair. A number of reports suggest their numbers increase with disease activity in patients with vasculitis and other vascular disorders and that progenitor repair cells become exhausted in disease (Figure 2)[47, 48]. ECFCs can be cultured from patients and analysed for pathophysiological properties and epigenetic markers and this approach has the potential to inform precision cardiovascular medicine. EMVs are extracellular vesicles of 0.2-5μm diameter that are produced by ECs in response to a variety of stimuli[49]. They can exert paracrine and autocrine actions on vascular cells with the potential to modulate key intracellular signalling pathways, promoting disease

progression via transfer of a range of bioactive molecules (growth factors, proteases and microRNAs) to adjacent cells (Figure 2).

1.7 Summary.

Although a conventional definition of endothelial dysfunction has focused on NO dysregulation and an altered redox status[50], EC dysfunction involves a range of plastic phenotypic states with inflammation and enhanced permeability. Indeed, it is clear from recent studies that ECs assume multiple diverse phenotypes associated with various disease states including hypertension, atherosclerosis and the development of heart failure (Figure 1). This is exemplified by fate mapping and single cell RNAseq studies that have revealed multiple EC phenotypes associated with health and disease[11-14] and recent insights into the function of ECFCs and EMVs in CVD (Figure 2). Together, these findings have led to growing interest in assessing endothelial function by a range of traditional and novel methods, discussed below, to inform about individual patient risk, to guide best therapy, clinical management and ultimately to establish whether it is feasible to target endothelial dysfunction and attenuate CVD progression[51, 52]. This could enhance the field in applying novel endothelial function tests and endothelial damage biomarkers to innovate a more personalised approach to cardiovascular medicine.

2. MEASURING ENDOTHELIAL FUNCTION

The ideal method to assess endothelial function should be non-invasive, easy to use, prospectively validated in different cohorts and ethnic groups, with an incremental value over standard, clinically established risk markers, cost-effective, measured according to methodological consensus and providing reference values as a basis for treatment[53, 54]. Both invasive and non-invasive methods to assess vascular endothelial function have their advantages and disadvantages (Table 1). The basic principle of these methods, however, is similar. Healthy arteries dilate in response to reactive hyperaemia via increased shear stress (flow-mediated vasodilatation) or in response to endothelium-dependent vasodilators such as acetylcholine (Ach), bradykinin or serotonin, via release endothelium-derived vasoactive substances e.g. NO[55]. In disease states, this process of endothelial-dependent dilatation may be reduced or absent. It should be noted that vascular responses are not only determined by local function at the point of measurement, but also by the structure and physiology of resistance arteries and microvasculature. Furthermore, vascular dysfunction can also be endothelium-independent function via alterations in vascular structure and SMC function rather than changes in EC. Responses to exogenous NO donors (e.g. glycerol-trinitrate) or vasodilators acting directly on vascular smooth muscle (e.g. adenosine) can be compared to differentiate endothelium-dependent from endothelium-independent responses. This section focuses on methods which are already established and which have the perspective to be

implemented in clinical practice. Research methods such as infusion of NO synthase inhibitors such as L-NMMA are out of the scope of this paper and will not be discussed in detail. It should be noted that our assessment of the various methods for quantifying endothelial function should not be interpreted as a recommendation of any particular product or technology manufacturer.

2.1 Coronary circulation.

2.1.1 Coronary epicardial function

Coronary endothelial function is assessed by performing measurements in both epicardial as well as resistance vessels. Although these methods are invasive, they have the advantage of measuring EC function directly in a clinically-important vascular bed. Vasomotor responses of epicardial coronary arteries are measured using quantitative coronary angiography or intravascular ultrasound (IVUS) to quantify changes in vessel diameters in response to endothelium-dependent pharmacological interventions. Vessels and segments with an intact endothelium vasodilate in response to Ach and other endothelial-stimulating substances, whereas vessels with dysfunctional endothelium will exhibit reduced vasodilatation or vasoconstriction due to a direct activation of muscarinic receptors on vascular SMCs[56, 57]. The observation that endothelial-dependent flow-mediated dilatation of coronary epicardial vessels is impaired in atherosclerosis[58, 59] inspired studies of responses to flow in the peripheral vasculature later as a potential surrogate indicator of CAD (see below). Finally, it should be mentioned that this technique should be used with caution, as serious (although rare) side effects may occur that carry risks for the patient, such as severe coronary vasoconstriction or the induction of arrhythmias.

2.1.2 Coronary microvascular function

Changes in coronary blood flow (CBF) have been suggested as a surrogate parameter for microvascular function[60]. Coronary flow reserve (CFR) is defined as the ratio of maximal CBF during maximal coronary hyperemia in response to a stimulus (such as adenosine infusion, pacing or exercise), divided by the resting CBF. This maximal blood flow response (CFR) is both endothelium and non-endothelium dependent and a CFR below 2.0 is considered abnormal[61]. There are no invasive methods for measuring CBF directly in clinical practice. Instead, wire-based Doppler flow velocity or thermodilution techniques are used as surrogates but these are technically challenging and can lack reproducibility. Thus, there is a need for an accurate method for measurement of volumetric CBF. To determine endothelium-dependent microvascular function, the percentage increase in CBF in response to endothelial-dependent vasodilation is analysed. Non-invasive functional tests have also been developed to assess the coronary microvasculature, which include positron emission tomography[62], myocardial perfusion imaging[63], blood oxygen level-dependent (BOLD) MRI[64] and echocardiography[63].

2.2 Peripheral techniques to assess endothelial function

The abovementioned invasive techniques may be suitable for patients requiring coronary angiography for other clinical indications. However, invasive functional coronary angiograms may not be indicated or feasible for assessment of vascular function in the asymptomatic patient. Because of this, non- or less invasive surrogate techniques have been developed to quantify macrovascular as well as microvascular endothelial function[65-67]. While these techniques can be used to assess the general function of the vasculature, they do not provide information on local vascular dysregulation e.g. dysfunction at branch and bends exposed to disturbed shear stress[68-70].

2.2.1 Plethysmography of the Forearm Circulation

This technique measures changes in forearm blood flow by plethysmography in both arms in response to vasoactive substances which are introduced via a cannulated brachial artery[71, 72]. An advantage is that responses to vasoactive hormones or drugs can be quantified therefore providing information on both endothelial-dependent and endothelial-independent vasodilation. The infused hormones and drugs have negligible systemic effects, and therefore the contralateral limb can be used as an internal control. A disadvantage is that this technique is considered semi-invasive nature due to the reliance on arterial cannulation.

2.2.2 Flow-mediated vasodilation of brachial artery

Due to its non-invasive approach, flow-mediated vasodilatation (FMD) of the brachial artery has become the most widely used technique to evaluate endothelial function. The technique quantifies the ability of larger conduit arteries to dilate in response to reactive hyperaemia after a brief (5 min) suprasystolic occlusion of the brachial artery using a blood pressure cuff. The resultant reactive hyperemia causes an increase in endothelial shear stress in upstream artery, which in turn stimulates release of NO. Celermajer, Deanfield and colleagues were the first to evaluate this response *in vivo* by measuring changes in the diameter of the brachial artery by ultrasound[73], later shown to be NO-dependent[74-76], although other vasodilator pathways may be involved[77]. Of note, FMD-assessment of peripheral endothelial function has been shown to correlate with coronary artery endothelial function[65, 67]. Although FMD may appear to be a simple technique, it is challenging and necessitates extensive training of operators and standardization[78-81]. This is outlined in dedicated guidelines[78, 81-83] which highlight the critical importance of image acquisition and site selection, study preparation, cuff occlusion time, sphygmomanometer probe position, and application of edge-detection software. These guidelines are of critical importance, as they emphasize the need to standardize protocols and technology to improve reproducibility and data interpretation of FMD[84]. The semi-automatic measurement of brachial FMD with self-adjusting

ultrasound probes and automatic edge detection of the arterial wall will likely facilitate the usage in clinical practice and has already established reference values for a Japanese population[85]. While FMD assesses the function of conduit arteries, the stimulus for FMD is an important parameter of peripheral microvascular function because reactive hyperaemia is dependent on maximal forearm resistance[86, 87]. Indeed, shear stress and velocity changes induced by hyperaemia have shown stronger correlations with cardiovascular risk factors than FMD[88] and these parameters also predict cardiovascular outcomes[89, 90]. Moreover, baseline brachial artery diameter measurements per se have been shown to correlate with clinical outcomes [91, 92]. This finding reveals a significant limitation of the in vivo assessment of endothelium-dependent vasodilation. In contrast to the ex vivo situation, the baseline arterial tone cannot be standardised. Therefore, the amount of additional dilatation depends on the initial diameter of the vessel and could paradoxically show poor FMD in a situation of initial vasodilation due to a well-functioning endothelium (e.g. in pregnancy or in hyperthermia). These influencing factors strongly warrant a strict standardization of the measurement environment (e.g. room temperature, resting phase) and the consideration of clinical conditions that may influence baseline diameter and vasodilation[81].

2.2.3 Finger plethysmography

Endothelial function measurement using peripheral arterial tonometry (PAT) was first used by Bonetti et al to identify patients with early coronary atherosclerosis. A device has been developed to quantify pulsatile arterial volume changes by finger plethysmography[93, 94]. Plethysmographic recordings of the finger arterial pulse wave amplitude are captured with pneumatic probes[94]. In this technique, increased arterial blood volume in the fingertip leads to increased pulsatile arterial column changes, thereby increasing the measured signal. Similar to FMD, a pressure cuff is placed on the arm and used to induce reactive hyperaemia in one arm. It is notable that measurements in the contralateral arm serve as an internal control that can be used to correct for any changes in vascular tone that may occur during the test. An index between the two arms is therefore calculated as a marker for endothelial function. It should be noted however that pulse amplitude augmentation in response to reactive hyperaemia is a complex response because it integrates changes due to altered flow in addition to vessel dilatation and it is only partially NO-dependent[95]. Further studies demonstrated that impaired digital EC function correlates with coronary microvascular function in p atients with early atherosclerosis[66] and that this parameter predicts cardiovascular events[96]. In two large cross-sectional studies (more than 1900 patients in the Framingham cohort[97, 98] and more than 5000 individuals in the Gutenberg Heart Study[99]) vascular dysfunction measured by digital plethysmography was associated with multiple cardiovascular risk factors but had little or no correlation with FMD, suggesting that these measurements provide information on different aspects of vascular biology. A disadvantage of the proprietary device is the high cost per measurement

system, the lack of reusability and the limited parameters offered for further analysis. Moreover, we recognise that technologies such as finger plethysmography that rely on a small number of device manufacturers may more vulnerable to commercial factors.

2.2.4 Retinal endothelial function

For the assessment of the retinal endothelial function, several types of provocation are possible including flicker light[100]. The vessel's reactions are at least partially dependent on NO release and partially attributed to neurovascular coupling[100, 101]. Solid data for patient groups are still lacking, partly due to variation in the flicker response between individuals due to variation in the baseline diameter of retinal vessels[102, 103]. Moreover, a consensus on the protocol used in order to achieve a better comparability of study results, is still lacking[104]. These concerns should be addressed, as should the study of larger and more representative groups of individuals and patient cohorts before recommendations for wider use in clinical practice and prevention can be made. Nevertheless, flicker-induced dilatation of retinal vessels has been shown to depend on age and gender in individuals free of major risk factor burden and prevalent disease[105]. It is impaired in patients with obesity[104, 106] renal disease[107] and diabetes compared to age-matched healthy controls[108, 109]. In hypertension, flicker-induced dilation is also reduced[110, 111] and it is associated with an increase of inflammatory biomarkers[110]. Since many of the studies of retinal flicker rely on a single commercial device, it should be noted that retinal flicker measurement may be considered less resilient from a commercial perspective than those associated with multiple industry products.

2.3 Summary. There is not an ideal method for empirical measurement of endothelial function. Techniques to measure coronary epicardial and microvascular function are well established but they are invasive, time-consuming and expensive. Several techniques are available for measurement of reactive hyperaemia in peripheral arteries, which provide a less-invasive assessment of endothelial function. FMD of the brachial arteries is the most commonly used, but it is technically demanding and requires a high degree of training and experience to ensure accurate measurements, but semi-automatic, easier to use tools are approaching. Techniques, such as finger plethysmography, are easier to use; however, the utility of newer methods is restricted because of a lack of methodological consensus, lack of reference values in healthy individuals and limited validation in large clinical trials.

3 ENDOTHELIAL DYSFUNCTION AND ARTERIAL DISEASE

3.1 Arterial hypertension.

Hypertensive patients have impaired endothelial-dependent vasodilatation both in coronary arteries[112] and in the forearm[113] (Supplementary Table 1), and data from the Framingham offspring

cohort suggest that the degree of endothelial dysfunction is positively associated with the severity of hypertension[114]. However, in a cohort of 3500 ethnically diverse persons from the Multi-ethnic Study of Atherosclerosis (MESA), until now the largest clinical study in the field, impaired FMD was not a significant independent predictor of hypertension development, after adjustment for co-variables[115]. A possible explanation for these seemingly disparate observations is that the interaction between EC function and hypertension may vary between populations. This underscores the importance of developing reference FMD values for different populations. It is also plausible that stratification of patients (e.g. using omics/epigenetics data or via analysis of ECFCs or EMVs) may identify sub-groups where FMD values are more accurately coupled to disease risk[116] (see Section 4).

3.2 Diabetes.

Diabetes is associated with a two- to four-fold increased risk of CVD, mainly attributable to hyperglycaemia, dyslipidaemia and oxidative stress[117]. Endothelium-dependent vasodilation in peripheral[118] and coronary[119] arteries of patients with T2DM is blunted (Supplementary Table 2), principally due to loss or reduction of NO[120]. The relationship between insulin resistance and endothelial dysfunction is complex and endothelial dysfunction probably precedes the onset of T2DM. Indeed, polymorphisms of eNOS are multivariable predictors of incidence of T2DM[121]. Several mechanisms of endothelial dysfunction are proposed in the setting of DM including: increased oxidative stress[122], uncoupling of eNOS[123], pro-inflammatory activation of EC[124], mitochondrial dysfunction[125], impaired endothelial repair potential[126], and increased permeability[127, 128]. Although the role of endothelial dysfunction in pathogenesis of micro- and macrovascular complications is well documented, endothelium-dependent peripheral vascular tests do not appear to improve risk stratification in patients with T2DM [129, 130]. However, given the range of endothelial mediators and their multiple mechanisms of action which contribute to endothelial abnormalities, FMD may not represent the most appropriate measure of the early signs of endothelial metabolic disturbances.

3.3 Coronary artery disease (CAD).

Multiple studies have addressed the hypothesis that endothelial dysfunction may improve risk stratification above well-established risk scores/factors for CAD (Supplementary Table 3), thereby offering the possibility of early and personalised therapy. Consistent with this concept, peripheral macrovascular endothelial dysfunction, estimated by FMD[91, 131, 132] or finger plethysmography[133] was demonstrated to independently predict major adverse cardiac events (MACE) in several populations at risk for CAD. Moreover, a recent systematic review and meta-analysis including 35 FMD studies and 6 PAT studies found that these tests provided a similar prognostic value in predicting

cardiovascular events[134]. In contrast, three large prevention trials failed to confirm the predictive value of FMD (macrovascular endothelial dysfunction), but instead found that markers of *microvascular* endothelial dysfunction (hyperaemic velocity in FATE[89] and invasive forearm technique with Ach in PIVUS[135] were associated with increased MACE risk and improved risk discrimination substantially, independent of established risk scores. The reason why FMD had prognostic value in some, but not in all populations is uncertain, but could be related to differences in the age and physical activity of the populations that were studied[89, 131, 132, 136]. It is also plausible that FMD may predict cardiovascular risk in a proportion of patients but not in others. It follows that integrating FMD measurements with patient-specific genetic and epigenetic characteristics may provide a personalised approach for predicting cardiovascular risk.

3.3.1 Ischemia and no obstructive coronary artery disease.

A substantial proportion of patients, especially women with anginal symptoms and myocardial ischemia, have an absence of flow-limiting obstruction in the epicardial arteries at coronary angiography[137, 138]. This syndrome has been increasingly recognised and recently termed as Ischemia and No Obstructive Coronary Artery disease (INOCA) or Angina and No Obstructive Coronary Artery (ANOCA) disease. The pathophysiology of INOCA includes dysfunctionality in coronary macrovascular (i.e. epicardial coronary arteries) and/or microvascular compartment (i.e. small intramural pre-arteriolar coronary arteries). Bairey Merz *et al* have identified a series of investigations to define INOCA including measurement of endothelial dysfunction[139]. A panel of invasive measurements includes coronary vasomotor testing with intracoronary adenosine (to measure CFR, that estimates endothelium-independent microvascular function), Ach (to measure endothelium-dependent coronary vasoreactivity) and nitroglycerin (to measure endothelium-independent macrovascular function; Supplementary Table 4). Coronary endothelial dysfunction is defined as microvascular, if the change in coronary blood flow in response to Ach is <50%, and macrovascular in case of Ach-induced epicardial vasoconstriction[140]. The results of such testing should help to guide therapy in individual patients e.g. to determine whether microvascular endothelial dysfunction is involved.

3.3.2 Chronic coronary syndromes and progression to plaque instability.

Coronary macro- and microvascular endothelial dysfunction can predict acute vascular events independently of conventional CAD risk factors and angiographically-proven coronary atherosclerosis. For example, although patients with high-risk coronary anatomy (left main stenosis and three vessels with CAD) were excluded[141], the detection of microvascular endothelial dysfunction was associated with a 2.4-fold increase in event rates, while the detection of epicardial endothelial dysfunction was associated with a 1.4-fold elevation of event rates (independently from

other risk factors and presence of CAD). These associations point to the importance of coronary endothelial dysfunction for the transition from a stable to unstable form of atherosclerotic disease. Furthermore, peripheral endothelial dysfunction (brachial plethysmography) distinguished subjects at a higher risk for cardiac and total vascular events in populations with documented CAD, highlighting the importance of systemic endothelial changes in plaque progression[142]. Atherosclerosis is a focal disease[143] and it is therefore noteworthy that coronary segments with a higher degree of endothelial dysfunction are associated with more vulnerable plaque containing a necrotic core (evaluated by IVUS[70]) suggesting that localised EC dysfunction may predict focal progression into culprit lesions and acute coronary syndromes.

3.3.3 Acute coronary syndromes (STEMI, NSTEMI, MINOCA).

The pathophysiological mechanism underlying type 2 myocardial infarction (MI) is an acute mismatch between oxygen supply and demand, leading to acute ischaemic myocardial injury[144]. Mechanisms include coronary artery spasm and/or coronary microvascular dysfunction[144]. During the course of atherosclerosis, local inflammation and oxidative stress affect endothelial function and promote plaque vulnerability, with consequent platelet adhesion, vasospasm, stasis and coronary thrombosis, leading to acute coronary syndrome[145]. Importantly, endothelial dysfunction is present not only at the site of the culprit lesion, but also in distant, non-culprit coronary arteries, even with normal angiographic appearance[146]. Aggravation also occurs in peripheral endothelial dysfunction after acute coronary syndrome and its normalization predicts a lower risk of future events[147]. Relatively few studies have correlated endothelial dysfunction with MI with Non-Obstructive Coronary Arteries (MINOCA) which arises, due to either atherosclerotic plaque disruption and coronary thrombosis (i.e. type 1 MI), or coronary vasospasm (i.e. type 2 MI), along with other possible causes. In the Stockholm Myocardial Infarction with Normal Coronaries (SMINC) study, peripheral microvascular endothelial function was normal in MINOCA patients compared to controls[148]. Overall, although microvascular dysfunction is presumed to be a causal component in ACS, both in type 1 and even more frequently in type 2 MI, there are uncertainties whether it is a contributor or a biomarker of disease risk.

3.4 Summary. There is a wealth of evidence that endothelial dysfunction is a key player in the initiation of atherosclerosis and plaque progression. Endothelial dysfunction in coronary macrovascular or microvascular compartments may predict and/or drive disease progression into culprit lesions, acute coronary syndromes and INOCA. Consistent with this, endothelial dysfunction has been demonstrated in asymptomatic individuals with risk factors for atherosclerosis (i.e. before clinical manifestation of the diseases) and, large clinical trials demonstrated that microvascular endothelial dysfunction can independently predict MACE in populations at risk for CAD. However,

there are several examples in the literature where the correlation between EC dysfunction and disease risk varies considerably between studies. This can be partly attributed to technical considerations, thereby underlying the importance of methodological consistency, but may also be related to biological factors that vary between individuals and/or between populations therefore requiring a precision medicine approach.

4 ENDOTHELIAL FUNCTION IN PRECISION MEDICINE

4.1 Can endothelial function measurements be used to stratify patients for therapy?

Results from most clinical trials have documented that despite the successful control of cardiovascular risk factors achieved with cardiovascular drugs, the impact on cardiovascular morbidity and mortality reduction is limited (around 20-45%). Consequently, tools that might enable identification of those patients who develop future events, despite optimal treatment are urgently needed. Several findings support the possibility that endothelial function could be used to identify patients that remain at high cardiovascular risk. For example, among 251 Japanese men with newly diagnosed stable CAD and concurrent impaired brachial artery FMD, those whose endothelial function did not improve after six months of optimised pharmacological treatment showed a significant higher event rate of cardiovascular events (26%) in the 31 months follow-up compared to those with improved endothelial function (10%)[149]. On the other hand, EC function can also identify patients with favourable responses to lifestyle changes, such as increased exercise, or pharmacological interventions. For example, moderate aerobic physical exercise can improve endothelium-dependent vasodilation, not only in healthy middle-aged men[150], but also in patients with arterial hypertension[151], CAD[152] and chronic heart failure[153]. Endothelial function has also been improved by weight reduction either by diet[154, 155] or bariatric surgery[156], dietary interventions with foods rich in polyphenols (fruits, green tea and cocoa) [157, 158] as well as smoking cessation[159]. Beyond lifestyle interventions, the first finding that EC function measurements can be used to monitor pharmacological responses was obtained from controlled studies with statins[160, 161]. The mechanism is likely related to the documented anti-inflammatory and antioxidant properties of statins that result in improved availability of vascular NO[162]. Endothelial function can also be used to monitor responses to other drugs with an effect on cardiovascular risk factors[163] and some diabetes modulating drugs like metformin[164] or glitazones[164-166].

4.2 Integrating endothelial functional measurements with biomarkers of vascular function.

Genetic and epigenetic differences contribute to variation in endothelial function both in healthy individuals and in patients with CVD. Therefore, the ability to delineate patients at high cardiovascular risk and identify responders and non-responders to therapy can potentially be

enhanced by integrating endothelial function measurements with genomic and epigenomic datasets. Non-coding RNAs are epigenetic markers of potential clinical use due to their high plasma stability and advances in experimental techniques used in their assessment. For example, the detection of specific circular RNAs and microRNAs in plasma has been linked to CAD and ACS [167] and Sapp et al found that alterations in miR-126-5p correlated with endothelial function in response to exercise in healthy individuals[168]. There may also be value in quantitation of classical markers such as sICAM, sVCAM, IL-6, IL-8, IL-12, hsCRP and NO for integration with endothelial function assessment. There is also considerable interest in using circulating EMVs and ECFCs as a surrogate of endothelial health[169]. For example, anti-inflammatory treatment of patients with systemic lupus erythematosus simultaneously improved endothelial function and reduced EMV levels[170] suggesting that EMV levels may report on vascular function. Of particular note, a recent study from Zacharia et al found that endothelial function correlated with circulating microvesicles in patients with ACS[171]. Moreover, studies of circulating ECFCs revealed that they correlate with enhanced microvascular function and repair in patients with acute MI[172, 173]. These tools will significantly contribute to the field of precision medicine and identify patients at high risk of developing both micro- and macro-vascular complications[174].

4.3 Summary.

Measurement of endothelial function can be used to monitor responses to lifestyle changes and pharmacological intervention, and can identify patients that remain at residual risk despite optimal therapy. The prognostic value of endothelial function measurement may be enhanced by integration with patient-specific information from omics and epigenetic studies and/or from analysis of the physiology of EMVs and ECFCs. These data may be combined through an algorithm that will enhance risk stratification and improve patient management[175, 176].

CONSENSUS STATEMENTS

1. Endothelial dysfunction does not describe a single endothelial phenotype but is characterised by a spectrum of phenotypic states, exemplified by multiple EC subsets and plasticity in atherosclerosis. The vascular biology community should delineate the contribution of various EC dysfunctional states to CVD and develop new technologies to measure pathogenic EC subsets in the clinic.

2. FMD of the brachial arteries, the most commonly used measure of endothelial function, predicted cardiovascular risk in some large clinical trials but not others. Thus we recommend that a consensus, semi-automated methodology is adopted in future studies to

minimize technical variation, and that reference FMD values are established for different populations.

3. Newer techniques to measure endothelial dysfunction that are relatively easy to perform, such as finger plethysmography and the retinal flicker test, have the potential for increased clinical use provided a consensus is achieved on the measurement protocol used. In addition, larger clinical studies are needed to establish reference values and to assess their clinical utility.

4. Future work should determine whether the prognostic value of endothelial function measurement can be enhanced by integration with patient-specific information from omics and epigenetic studies and/or from analysis of patient-derived EMVs and ECFCs.

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CONFLICTS OF INTEREST.

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FIGURE LEGENDS

Figure 1. Endothelial dysfunction describes multiple phenotypic states.

Left Panel. In homeostatic conditions, the healthy endothelium regulates the physiological vascular function and structure through multiple beneficial effects of nitric oxide (NO), hydrogen sulphide (H_2S) and carbon monoxide (CO), as detailed in the text. Right Panel. Dysfunctional endothelium is characterised by decreased production of NO and chronic increase of reactive oxygen species (ROS) able to overwhelm the intracellular antioxidant defence leading to onset and progression of atherosclerosis. eNOS: endothelial nitric oxide synthase; (EndMT): endothelial-mesenchymal transition; AA: amino acids.

Figure 2. Schematic representation of the endothelial factors underlying cardiovascular risk.

ECFCs: Endothelial colony forming cells, EMVs: endothelial microvesicles, EndMT: -endothelial-mesenchymal transition, FMD: flow mediated dilatation, HSPG: heparan sulphate proteoglycans, IL: interleukin, MØ: macrophage, MR-proADM: Mid-regional pro-adrenomedullin, NO: nitric oxide, oxLDL: -oxidised low density lipoprotein, ROS: reactive oxygen species, TGFβ: transforming growth factor beta.

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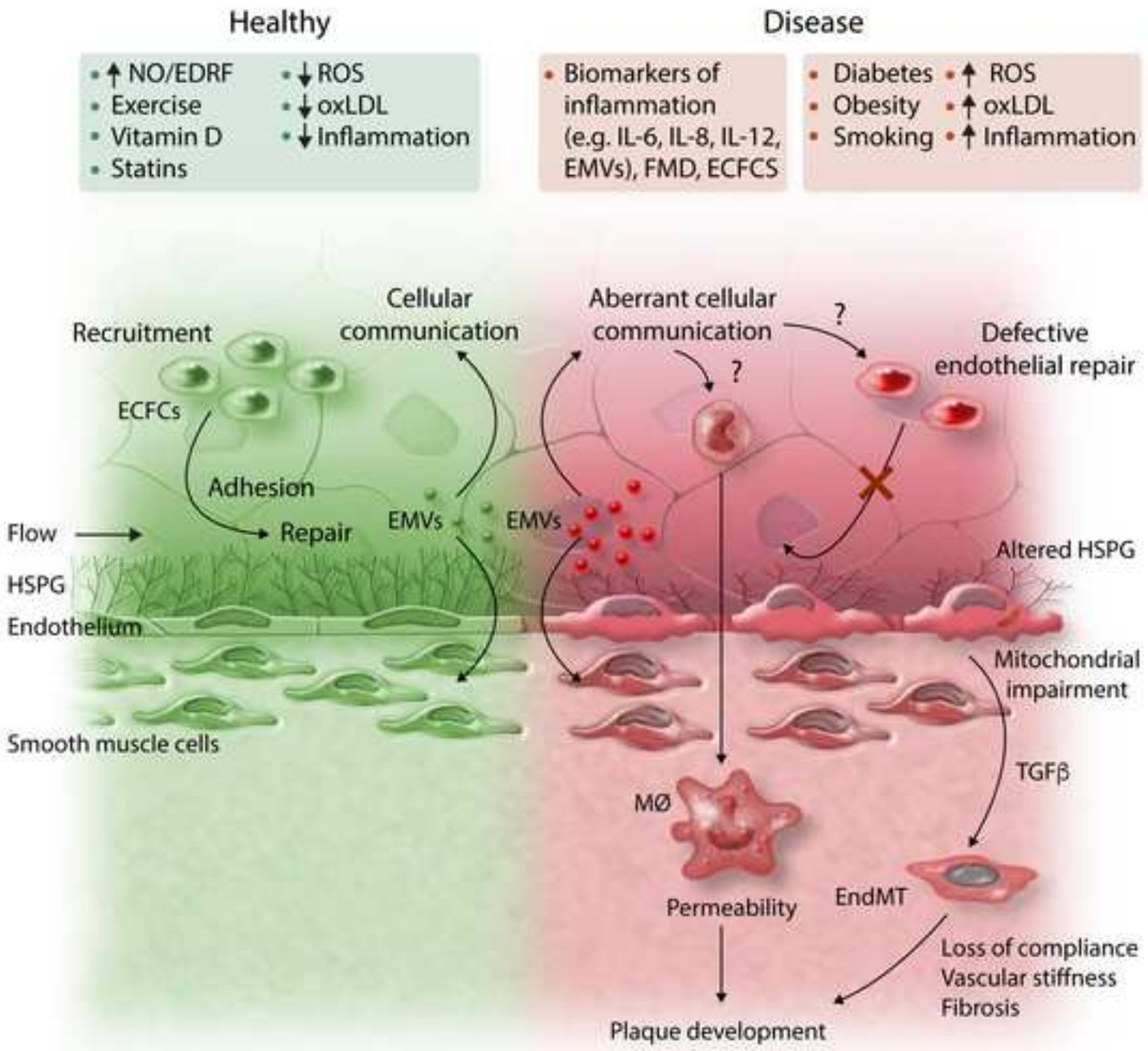
Table 1 Techniques used in to assess endothelial function.

Technique	Vascular bed	Advantages	Disadvantages
Coronary epicardial function	Epicardial macrovascular Conduit arteries	Assessment directly in the coronary vascular bed Gold standard	Invasive Expensive Time intensive Limited to those undergoing coronary angiography Impractical for serial measurements
Coronary microvascular function	Coronary microvascular Resistance arteries	Assessment in the coronary microvasculature	Invasive Expensive Time intensive Limited to those undergoing coronary angiography Impractical for serial measurements
Venous plethys-mography	Forearm vasculature Microvasculature	Easy access Correlation with invasive vascular function Possibility to infuse vascular active substances directly Contralateral arm as a control	Invasive (cannulation of the brachial artery) Time consuming
Flow-mediated vasodilation of brachial artery	Brachial artery Conduit artery	Easy access Correlation with invasive epicardial vascular function Many outcome studies Inexpensive Possibility to assess other important parameters (flow, baseline arterial diameters, flow-mediated constriction)	Iinter- and intraobserver variability Difficult to perform Need for standardization Different protocols
Finger plethysmogra phy	Finger Microvasculature	Easy to access and perform Low inter- and intraobserver variability	Dependent on different non endothelial factors lack of normal/ reference values and of randomized

		Correlation with invasive microvascular vascular function	clinical trials addressing prospective validation, incremental value and clinical outcome high costs per measurement system
Retinal endothelial function	Retinal arterioles	Easy access Partial correlation with invasive vascular function Sensitive to interventions	Inter- and intraobserver variability Training needed to perform Need for standardization Different protocols lack of normal/ reference values and of randomized clinical trials addressing prospective validation, incremental value and clinical outcome

Figure(s)



Figure(s)