REVIEW ARTICLE

Targeting vascular (endothelial) dysfunction

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Cardiovascular diseases are major contributors to global deaths and disability-adjusted life years, with hypertension a significant risk factor for all causes of death. The endothelium that lines the inner wall of the vasculature regulates essential haemostatic functions, such as vascular tone, circulation of blood cells, inflammation and platelet activity. Endothelial dysfunction is an early predictor of atherosclerosis and future cardiovascular events. We review the prognostic value of obtaining measurements of endothelial function, the clinical techniques for its determination, the mechanisms leading to endothelial dysfunction and the therapeutic treatment of endothelial dysfunction. Since vascular oxidative stress and inflammation are major determinants of endothelial function, we have also addressed current antioxidant and anti-inflammatory therapies. In the light of recent data that dispute the prognostic value of endothelial function in healthy human cohorts, we also discuss alternative diagnostic parameters such as vascular stiffness index and intima/media thickness ratio. We also suggest that assessing vascular function, including that of smooth muscle and even perivascular adipose tissue, may be an appropriate parameter for clinical investigations.

LINKED ARTICLES

This article is part of a themed section on Redox Biology and Oxidative Stress in Health and Disease. To view the other articles in this section visit http://onlinelibrary.wiley.com/doi/10.1111/bph.v174.12/issuetoc

Abbreviations

ACE, angiotensin-converting enzyme; ADMA, asymmetric dimethylarginine; AGEs, advanced glycation end products; AOE, antioxidant enzyme; BH_4, tetrahydrobiopterin; CRP, C-reactive protein; eNOS, endothelial nitric oxide synthase; EPCs, endothelial progenitor cells; ET-1, endothelin-1; FMD, flow-mediated dilation; GCH-1, GTP cyclohydrolase-1; HDAC, histone deacetylase; HDL, high density lipoprotein; ICAM, intercellular cell adhesion molecule; miRNA, microRNA; Nox, NADPH oxidase catalytic subunit (isoforms 1, 2 and 4); PECAM, platelet endothelial cell adhesion molecule; PEG, polyethylene glycol; PGIS, prostacyclin synthase; PVAT, perivascular adipose tissue; PWV, pulse wave velocity; RAGE, receptor for advanced glycation end products; ROS, reactive oxygen species (in the present review mostly represents superoxide and hydrogen peroxide); sGC, soluble guanylyl cyclase; SOD, superoxide dismutase

Tables of Links

TARGETS		
GPCRs[a]	Angiotensin-converting enzyme	Serine/threonine kinase 11
AT$_1$ receptor	Arginase	Sirtuin 1
Bradykinin receptors	DNA (cytosine-5-)-methyltransferase 1	Soluble guanylyl cyclase
Catalytic receptors[b]	Endothelial nitric oxide synthase	**Other protein targets**[d]
Type IV RTKs	PDE5A	Advanced glycosylation end product-specific receptor
Enzymes[c]	Phosphodiesterases	
AMPK subfamily	Prostacyclin synthase	

LIGANDS		
Acetylcholine	Entinostat	Methotrexate
Adiponectin	Ethanol	Nitric oxide
α-linolenic acid	Gemfibrozil	Nitroglycerin
Angiotensin II	ICAM-1	Prostacyclin
Atrial natriuretic peptide	IL-1β	Resveratrol
Azacitidine	IL-17A	Sapropterin
Bradykinin	Iloprost	Scriptaid
Brain natriuretic peptide	Isosorbide dinitrate	Sildenafil
Butyric acid	Isosorbide mononitrate	Sodium hydrogen sulfide
Canakinumab	L-arginine	Trichostatin A
Cinaciguat	L-ascorbic acid	Vardenafil
cGMP	LPS	Vascular cell adhesion protein 1
Endothelin-1	Metformin	Vasopressin

These Tables list key protein targets and ligands in this article that are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 ([a,b,c,d]Alexander *et al.*, 2015a,b,c,d).

Introduction

Risk factors, disease burden and life span

The contribution of different risk factors to the global disease burden and their impact on life expectancy has shifted over the last 20 years from risks for communicable childhood diseases towards those for non-communicable adulthood diseases that are more frequently observed in the elderly (Lim *et al.*, 2012; Murray *et al.*, 2012). This shift is largely due to demographic changes, improved clinical prevention of childhood mortality, reductions in several preventable causes of death and a lower exposure to some risk factors. Socio-economic and scientific progress have led to improvements in water quality and sanitation, significant reductions in vitamin A and zinc deficiencies, and to lower exposures to particulate matter in households and the environment. There are large regional differences in the extent of these epidemiological and socio-economic changes and their impact on the importance of different risk factors, disease burden and mortality (e.g. poverty and childhood diseases continue to be the highest risk factors in sub-Saharan Africa).

A comparison of estimated deaths and disability-adjusted life years (= sum of years lived with a disability) between 1990 and 2010 was based on a new calculation model using 67 risk factors for 21 regions (Lim *et al.*, 2012; Murray *et al.*, 2012). In 2010, the global disease burden was largely affected by three leading risk factors, with high blood pressure accounting for 7.0%, tobacco (active and passive) smoking being responsible

for 6.3%, and exposure to particulate matter in households accounting for 4.3% of disability-adjusted life years. In 1990, the primary risk factors for the global disease burden were being underweight during childhood (7.9%), followed by household air pollution from solid fuels (6.8%), and tobacco smoking (6.1%). In 2010, malnutrition (mostly related to diets low in fresh fruits and vegetables or high in sodium) and physical inactivity collectively accounted for 10.0% of global disability-adjusted life years. As shown in Figure 1, the leading risk factors and diseases accounting for global deaths and disability-adjusted life years are hypertension (approx. 18 and 7%), ischaemic heart disease (approx. 14 and 5%), tobacco smoking (approx. 12 and 6%) and cerebrovascular disease (approx. 11 and 4%). It is important to note that these are all cardiovascular risk factors and diseases (tobacco smoking, independent of its carcinogenic effects, is a well-accepted cardiovascular risk factor), thus stressing their significance as the leading modifiable causes of death, particularly in Western societies. The impact of traditional risk factors on the extent and severity of coronary atherosclerosis was identified more than 15 years ago (Wilson *et al.*, 1999). The striking importance of hypertension as a risk factor for cardiovascular and all-cause mortality was recently demonstrated by the results of the SPRINT trial (Group *et al.*, 2015). These results show that aggressively lowering blood pressure to below 120 mmHg in patients with a high risk of cardiovascular events significantly decreased the incidence of major cardiovascular events and death from any cause despite significantly higher rates of other adverse side effects.
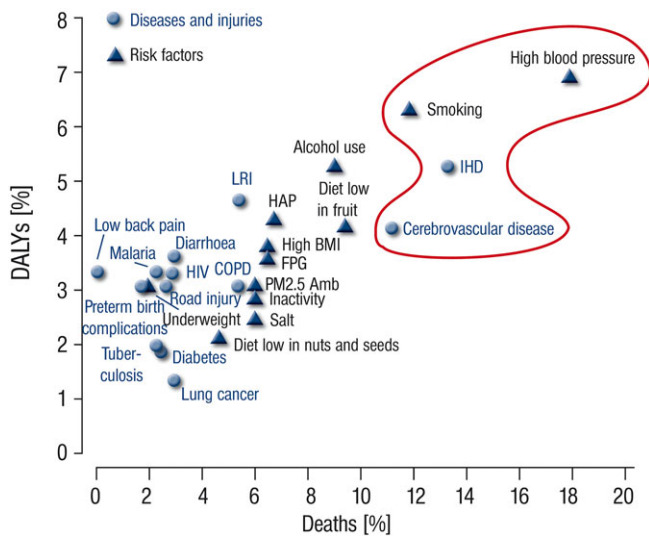
Figure 1

Contribution of the major diseases/injuries and risk factors to global deaths and global disability-adjusted life years in the year 2010. The top 25 causes of diseases, injuries, and risk factors with respect to their contribution to disability-adjusted life years and deaths are shown. DALYs = disability-adjusted life years. IHD = ischaemic heart disease. LRI = lower respiratory infections. COPD = chronic obstructive pulmonary disease. HAP = household air pollution from solid fuels. BMI = body-mass index. FPG = fasting plasma glucose. PM2.5 Amb = ambient particulate matter pollution. *Tobacco smoking, including second-hand smoke. †Physical inactivity and low physical activity. Adopted from (Murray et al., 2012). With permission of Elsevier. Copyright © 2012 Elsevier Ltd. All rights reserved.

Cardiovascular risk and endothelial function

Based on the aforementioned data on a major role of cardiovascular disease and risk factors for global deaths and disability-adjusted life years, physicians and pharmacologists have for several decades searched for a reliable, early predictor of cardiovascular mortality. One of the most promising candidates is the measurement of endothelial function (encompassing production of the different endothelium-derived messengers that help to control vascular tone, blood flow, immune cell and platelet activity/adhesion, thereby regulating perfusion and/or blood pressure), which also correlates with classical markers of inflammation, obesity and cardiovascular risk such as C-reactive protein (CRP), adiponectin and brain natriuretic peptide (BNP) (Gonzalez and Selwyn, 2003; Okui et al., 2008; Pauriah et al., 2012). Classic cardiovascular risk factors such as arterial hypertension (Panza et al., 1990), hypercholesterolaemia (Vita et al., 1990), diabetes mellitus (Calver et al., 1992) and chronic smoking (Celermajer et al., 1993) are all associated with endothelial dysfunction. The presence of several risk factors produces synergistic effects on endothelial function as well as the associated cardiovascular prognosis (Munzel et al., 2008). Previous studies confirmed that hypercholesterolaemia or chronic smoking lead to a moderate impairment of endothelial function (reduction in the maximal acetylcholine-dependent vasodilatation by ~ 30%), whereas the presence of both risk factors caused severe endothelial dysfunction (reduction

in the maximal acetylcholine-dependent vasodilatation by ~ 60%) (Heitzer et al., 1996b).

Endothelial dysfunction is the first clinical correlate of atherosclerosis identified (Panza et al., 1990; Vita et al., 1990). Since most cardiovascular diseases are either related to or are a direct consequence of atherosclerosis, endothelial dysfunction is an early predictor of subsequent cardiovascular events or mortality (Gokce et al., 2002; Gokce et al., 2003) (Figure 2). Patients with peripheral arterial occlusive disease (Anderson et al., 1995; Boger et al., 1998), coronary artery disease (Schachinger et al., 2000; Suwaidi et al., 2000; Heitzer et al., 2001) or heart failure (Heitzer et al., 2005) demonstrate impaired endothelial function. Endothelial dysfunction is also clearly associated with oxidative stress (Heitzer et al., 2001), which is another feature in the development of atherosclerosis (Miller et al., 1998; Harrison et al., 2003; Laufs et al., 2005). Levels of oxidized thiols were also negatively correlated with cumulative survival in a large scale clinical trial of patients undergoing coronary angiography, thus providing strong evidence for a role of oxidative stress in cardiovascular disease-associated mortality (Patel et al., 2016).

Prognostic value of endothelial function measurement

At present, the most reliable parameters with prognostic value are circulating markers such as BNP or CRP as well as risk scores that are based on calculations that consider different risk factors/markers such as Framingham Risk Score, Synergy Between PCI With Taxus and Cardiac Surgery Score (Verma et al., 2004; Matsuzawa et al., 2013). During the last 15 years, data from a number of clinical studies support the prognostic importance of coronary and peripheral measurements of endothelial dysfunction not only in patients with coronary artery disease (Schachinger et al., 2000), peripheral arterial occlusive disease (Anderson et al., 1995), arterial hypertension (Perticone et al., 2001), postmenopausal women (Sharma et al., 2011) and heart failure (Heitzer et al., 2005), but also in healthy subjects (Maruhashi et al., 2013; Shechter et al., 2014) (Table 1). Of particular interest is a study that assessed endothelial function (measured by flow-mediated dilation, FMD) for the prediction of future cardiovascular events in patients undergoing coronary bypass surgery: the results were expressed in tertaies representing the lowest event rate (normal FMD > 8%), an intermediate event rate (FMD = 4-8%) and the highest event rate (FMD < 4%) (Gokce et al., 2002). Another large cohort study identified the hyperaemic velocity, a stimulus for FMD and also a marker of microvascular function, but not FMD itself, as a prognostic marker for future cardiovascular events (Anderson et al., 2011). Results of the general population-based Gutenberg Health Study (5,000 individuals) revealed a strong correlation between the biomarker of cardiovascular disease, pro- atrial natriuretic peptide (ANP), and non-invasive measurement of conduit artery and peripheral arterial performance (Schnabel et al., 2012).

Large-scale clinical trials conducted recently suggest that endothelial function has no significant prognostic value in cohort studies of mostly healthy subjects (Suessenbacher et al., 2013) and is not an independent predictor of
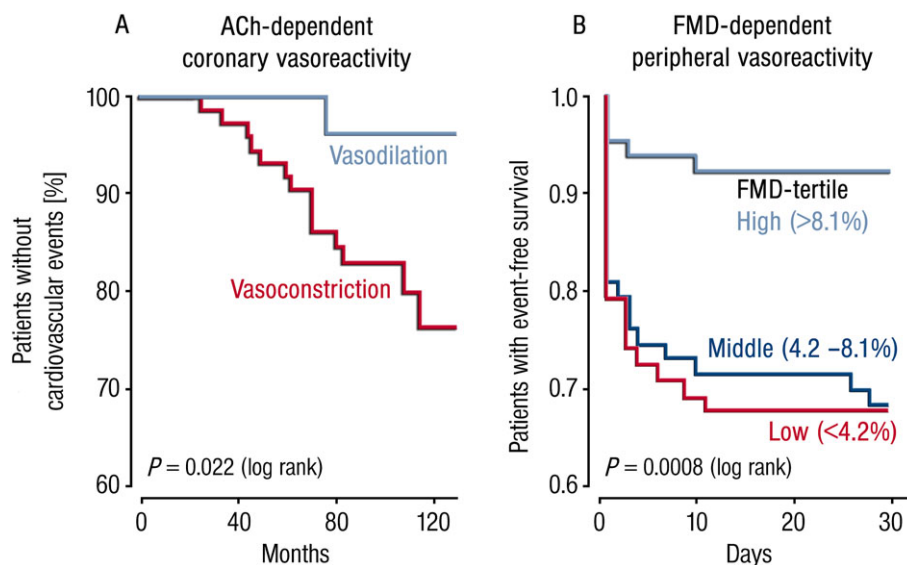
Figure 2

Endothelial function as a prognostic parameter for cardiovascular events and mortality. (A) Kaplan–Meier-curves for the correlation between acetylcholine (ACh)-induced coronary vasoreactivity and prognosis of patients with coronary heart disease (n = 147). Patients displaying ACh-induced vasodilatation had a better prognosis than those with ACh-dependent vasoconstriction. Cardiovascular events included lethal and non-lethal myocardial infarction, stroke, coronary and peripheral revascularization and symptoms of unstable *Angina pectoris*. Redrawn from (Schachinger *et al.*, 2000). With permission of Wolters Kluwer Health, Inc. Copyright © 2000 Wolters Kluwer Health, Inc. All rights reserved. (B) Kaplan–Meier-curves illustrating the correlation between flow-mediated dilation (FMD) of capacitance vessels in the forearm and event-free survival of patients after coronary or peripheral bypass surgery (n = 187). Patients displaying higher FMD values (indicator of endothelial function) had a better prognosis than those with lower FMD values. Cardiovascular events included lethal and non-lethal myocardial infarction, symptoms of unstable *Angina pectoris*, atrial fibrillation and increased troponin-I. Redrawn from (Gokce *et al.*, 2002). With permission of Wolters Kluwer Health, Inc. Copyright © 2002 Wolters Kluwer Health, Inc. All rights reserved.

cardiovascular events in individuals with intermediate cardiovascular risk (Yeboah *et al.*, 2012). Similarly, results of the Gutenberg Health Study revealed that non-invasive measurement of vascular function is unlikely to improve the prognostic value of the European Society of Cardiology risk score (Schnabel *et al.*, 2011). However, in patients at high risk of cardiovascular disease, peripheral endothelial dysfunction was significantly correlated with impending cardiovascular events (Matsuzawa *et al.*, 2013). A systematic review of 15 cohort studies indicates a significant correlation of endothelial function measurements using FMD with diagnosed atherosclerosis in patients (Garcia *et al.*, 2012). There are several possible reasons that may account for the large discrepancies reported in endothelial function in various studies: in addition to the nature of the cohorts (healthy versus unwell patients), other variables such as differences in methods (tonometry, plethysmography, FMD), procedures, devices, vascular beds, operator-dependency of the complex methods should be considered along with the effects of physical exercise, age, body mass index (BMI), blood pressure, gender, and baseline brachial diameter in the case of forearm-based measurements on endothelial function (Benjamin *et al.*, 2004). Therefore, measurements of intima-media thickness (Suessenbacher *et al.*, 2013) and stiffness index (Mitchell, 2015) could well be considered better parameters for determining endothelial or more importantly vascular function. Importantly, intima-media thickness also positively correlates with the levels of oxidized thiols and

early atherosclerosis, so providing a link between vascular dysfunction and oxidative stress (Ashfaq *et al.*, 2006).

Physiological role of endothelial (vascular) function

The central role of the cardiovascular system is the transportation of nutrients, biomolecules and signalling molecules, and importantly, transport of gasses to and from all organs, tissues and cells (Pries and Kuebler, 2006; Mikhed *et al.*, 2015a). In addition, the cardiovascular system is an important regulator of host defence by the immune system (Libby *et al.*, 2006; Pries and Kuebler, 2006; Karbach *et al.*, 2014a) and blood haemostasis/coagulation (Arnout *et al.*, 2006; Pries and Kuebler, 2006; Steven *et al.*, 2015). The endothelium, a mono-layer of cells lining the inner /luminal surface of blood vessels, acts as a barrier to control the exchange of nutrients, biomolecules and messengers with surrounding tissues. The endothelium also prevents the adhesion of immune cells and infiltration of monocytes into the sub-endothelial space of lesion-prone areas, an essential step in the development of atherosclerotic plaques (Cheng *et al.*, 2005; Lau and Baldus, 2006). The endothelium controls vascular tone by releasing vasoconstrictors such as endothelin-1 and vasodilators such as nitric oxide ($^{\bullet}NO$) (formerly known as endothelium-derived relaxing factor, EDRF), endothelium-derived hyperpolarizing factor (EDHF), prostacyclin or

Table 1

Selected studies on the prognostic value of endothelial dysfunction in cardiovascular disease

Reference	Number of patients	Population*	Method and target vessel	Follow-up period (months)	ED as (independent) predictor of events
(Suwaidi et al., 2000)	157	Patients w/o significant coronary stenosis	ACh i.c.Coronary resistance vessels	28	No statement
(Schachinger et al., 2000)	147	Patients with coronary heart disease	ACh i.c.Epicardiac capacity vessels	80	Yes
(Perticone et al., 2001)	225	Patients with untreated arterial hypertension	ACh i.a.Resistance vessels of the forearm	32	Yes
(Heitzer et al., 2001)	281	Patients with coronary heart disease	ACh i.a.Resistance vessels of the forearm	54	Yes
(Halcox et al., 2002)	308	Patients with and without coronary heart disease	ACh i.c.Epicardiac capacity vessels and coronary resistance vessels	46	Yes
(Gokce et al., 2002)	187	Patients with future peripheral and coronary bypass surgery	FMDCapacity vessels of the forearm	1	Yes
(Targonski et al., 2003)	503	Patients with no significant coronary stenosis	ACh i.c.Coronary resistance vessels	16	Yes
(Gokce et al., 2003)	199	Patients with peripheral arterial occlusive disease	FMDCapacity vessels of the forearm	14	Yes
(Brevetti et al., 2003)	131	Patients with peripheral arterial occlusive disease	FMDCapacity vessels of the forearm	23	Yes
(Fichtlscherer et al., 2004)	198	Patients with acute coronary syndrome	ACh i.a.Resistance vessels of the forearm	48	Yes
(Heitzer et al., 2005)	289	Patients with mild heart failure (EF 35–50%)	ACh i.a.Resistance vessels of the forearm	36	Yes
(Anderson et al., 2011)	1574	Healthy men	FMDCapacity vessels of the forearm	60	Yes (Hyperaemic velocity, a marker of microvascular function)
(Yeboah et al., 2012)	1330	Individuals with intermediate cardiovascular risk	FMDCapacity vessels of the forearm	120–144	No, not independent
(Matsuzawa et al., 2013)	528	Patients at high-risk for cardiovascular events	RH-PATResistance vessels of the fingertip	60	Yes
(Maruhashi et al., 2013)	5314	Healthy subjects	FMDCapacity vessels of the forearm	28	Yes
(Suessenbacher et al., 2013)	396	Patients with chest pain	FMDCapacity vessels of the forearm	144	No
(Shechter et al., 2014)	618	Healthy subjects	FMDCapacity vessels of the forearm	34–77	Yes

*ACh, acetylcholine; i.a., intra-arterial; i.c., intra-coronary; EF, ejection fraction; FMD, flow-mediated dilation; RH-PAT, reactive hyperaemia-peripheral arterial tonometry.

natriuretic peptides (Mombouli and Vanhoutte, 1999; Busse and Fleming, 2006; Moncada and Higgs, 2006; Spieker et al., 2006). The endothelium-derived vasodilators also possess anti-aggregatory properties, suppress thrombus formation, vascular stenosis (Willerson et al., 1989) and, in the case of the $^{\bullet}$NO/ cGMP axis, also retard cardiac hypertrophy (Ritchie et al., 2009). More recently, hydrogen sulphide (H_2S) was identified as another gaseous endothelium-derived vasodilator (Yang et al., 2008) that acts in concert with $^{\bullet}$NO (Cortese-Krott et al., 2015; Yuan et al., 2015). Endothelium-derived vasoactive messengers act together with other regulatory systems, which consist of vasoconstrictors such as catecholamines and other vasoactive peptides (e.g. angiotensin II, vasopressin). An imbalance in the formation of these vasoactive messengers is an important determinant in the development of endothelial dysfunction that is often further aggravated by oxidative stress (Munzel et al., 1999; Kahler et al., 2000). There is also evidence that the glycocalyx, which is located on the luminal surface of vascular endothelial cells and consists of proteoglycans, glycosaminoglycans, glycoproteins and glycolipids, plays an essential role in the adhesion of leukocytes and platelets, and accordingly in endothelial function (van den Berg et al., 2006). Importantly, areas of dysfunctional endothelium can be repaired by circulating endothelial progenitor cells (EPCs) and the number as well as quality of EPCs largely affect cardiovascular outcomes (Werner et al., 2005) as well as endothelial function (Hill et al., 2003).

According to more recent data, evaluation of endothelial function in itself may not be sufficient as vascular function is also affected by processes within the smooth muscle (e.g. activity of soluble guanylyl cyclase [sGC]) (Stasch et al., 2006). It is also now recognized that perivascular adipose tissue (PVAT) contributes to vascular homeostasis by producing vasoactive compounds such as adipokines, reactive oxygen species (ROS) and $^{\bullet}$NO (Brown et al., 2014; Jankovic et al., 2017). It is beyond the scope of this review to address all of these tissues and processes involved in detail and we will mainly focus our discussion on pathways related to endothelial function in health and disease. An excellent overview on open biological and physiological questions in endothelial (dys)function can be found in the editorial "Perspectives in pharmacology of endothelium: from bench to bedside" in the respective special issue (Chlopicki, 2015).

Mechanisms of development of endothelial (vascular) dysfunction

Endothelial (vascular) dysfunction implies impaired production of the different endothelium (smooth muscle, PVAT)-derived messengers that results in a vasoconstrictor, pro-inflammatory and pro-atherothrombotic phenotype leading to an impaired regulation of perfusion and/or vascular tone. Since the development of endothelial (vascular) dysfunction is a multi-factorial process, we will concentrate on some of its most important components in the following sections (Figure 3). Among the most important vasoactive compounds produced by endothelial cells are $^{\bullet}$NO, EDHF, prostacyclin and endothelin-1 since they not only affect vascular tone but also the activity and adhesion of platelets

and immune cells to the endothelium (Pries and Kuebler, 2006; Selivanova et al., 2007; Karbach et al., 2014a; Steven et al., 2015). The redox regulation of their production was previously reviewed in detail (Kahler et al., 2000; 2001; Bachschmid et al., 2005; Daiber et al., 2014; Schulz et al., 2014) and is discussed extensively in this virtual collection (Daiber et al., 2017). In brief, endothelial nitric oxide synthase (eNOS) uncoupling, sGC desensitization, nitration and inactivation of prostacyclin synthase (CYP8A1), oxidative activation of the endothelin-1 system and direct inactivation of $^{\bullet}$NO by superoxide play important roles in the development of endothelial dysfunction (for review see Forstermann and Munzel, 2006; Daiber et al., 2014, 2017). According to more recent reports, there is also a strong interaction and cross-regulatory mechanism between these important vasodilators (Hink et al., 2003; Bachschmid et al., 2005) and newly identified mediators such as H_2S (Cortese-Krott et al., 2015; Yuan et al., 2015) and other gasotransmitters such as carbon monoxide (Andreadou et al., 2015). Here, we will discuss new risk factors for endothelial dysfunction besides the classical ones (e.g. hypertension, hyperlipidaemia, diabetes, smoking, alcohol abuse) (Munzel et al., 2008; Ferdinandy et al., 2014).

Environmental factors: mental stress, noise exposure, air pollution and endothelial (vascular) dysfunction

Mental stress activates the immune system and leads to adverse cardiovascular effects (Marvar and Harrison, 2012a; Marvar et al., 2012b). Mental depression in humans is associated with higher cardiovascular risk and vice versa, as patients with cardiovascular events have more frequent depressive phases (Lippi et al., 2009). Recent studies also suggest that transportation (road, railway, aircraft) noise is a novel cardiovascular risk factor for stroke, myocardial infarction, chronic stable coronary artery disease and arterial hypertension (Sorensen et al., 2011; Raaschou-Nielsen et al., 2012; Munzel et al., 2014a); this was also supported by a recent meta-analysis for traffic noise exposure and incidence of ischaemic heart disease (Vienneau et al., 2015). Vascular function studies revealed that night-time aircraft noise exposure induced endothelial dysfunction, a deterioration of sleep quality, an increase in adrenaline levels and a trend for increased blood pressure in healthy subjects (Schmidt et al., 2013). Importantly, noise-induced vascular dysfunction was improved by oral administration of L-ascorbic acid (vitamin C) suggesting that ROS are involved in causing endothelial dysfunction (Schmidt et al., 2013). Another important finding of this study was that previous noise exposure sensitized the vasculature to damage by subsequent exposure to noise, strongly suggesting a lack of tolerance to the cardiovascular impact of noise. A recent study confirmed and extended these findings in patients with established coronary artery disease (Schmidt et al., 2015a). In these patients, night-time aircraft noise caused a marked degree of endothelial dysfunction, increased blood pressure and worsened sleep quality. Importantly, the deterioration of vascular function was independent of noise sensitivity and annoyance reactions of noise-exposed subjects.

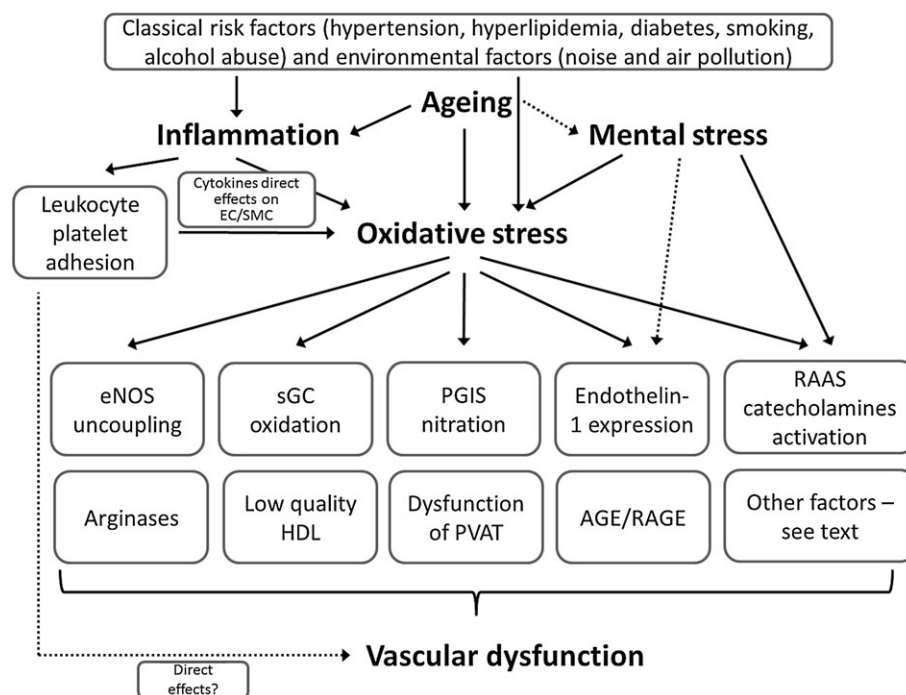Likewise, exposure to particulate matter causes endothelial dysfunction both in animals (Ying et al., 2015) and

Figure 3

Initiators of endothelial (vascular) dysfunction. Aging, inflammation, mental stress and oxidative stress are strong triggers of endothelial (vascular) dysfunction. It should be noted that to a certain extent the classical risk factors (see top of the scheme) converge at the level of inflammation and oxidative stress to further trigger the down-stream pathomechanisms. Inflammation can have detrimental effects by direct effects of cytokines on vascular cells leading to activation of secondary sources of ROS, direct leukocyte-derived ROS formation or effects of infiltrated immune cells on vascular structure and function. Aging and mental stress act via oxidative stress, inflammation but can also directly affect the major triggers of endothelial (vascular) dysfunction such as are eNOS uncoupling, sGC oxidation (maybe also imbalanced phosphodiesterase expression/activity), prostacyclin synthase (PGIS) nitration and inactivation, redox-triggered endothelin-1 (ET-1) signalling, activation of the renin-angiotensin-aldosterone system (RAAS) and other stress hormones (catecholamines), and finally, AGE/RAGE signalling. In addition, dysregulated arginase metabolism decreases levels of the eNOS substrate L-arginine. The quality of high density lipoprotein (HDL) changes under oxidative stress conditions and metabolic disease. Other factors that also contribute to endothelial (vascular) dysfunction are changes in fatty acid metabolism and release of adipokines by perivascular adipose tissue (PVAT) that occurs in some diseases.

humans (Krishnan *et al.*, 2012). The Heinz Nixdorf Recall Study indicates that environmental stress, e.g. air pollution and traffic noise, are independently associated with atherosclerosis (Arpornchayanon *et al.*, 2013). In summary, environmental factors affect endothelial (vascular) function and are now considered to be novel risk factors for cardiovascular disease (see Figure 1).

Inflammation and endothelial (vascular) dysfunction

According to recent clinical studies, cardiovascular risk is significantly increased in patients with chronic autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, and severe psoriasis (Hak *et al.*, 2009; Vena *et al.*, 2010; Soltesz *et al.*, 2011; Murdaca *et al.*, 2012). It is not surprising that psoriasis has been proposed as a risk factor for future cardiovascular events independently of the classical cardiovascular risk factors such as smoking, obesity and diabetes (Mehta *et al.*, 2010). The guidelines of the European League against Rheumatism strongly emphasize the increased cardiovascular risk associated with, for example, inflammatory arthritis (Peters *et al.*, 2010). Chronic inflammation causes a significant increase in the intima-media

thickness and is associated with impaired vascular function in patients with rheumatoid arthritis (Sodergren *et al.*, 2010). Infections (pathogens burden) are negatively correlated with endothelial function, show a positive correlation with the prevalence of coronary artery disease and predispose human subjects to atherosclerosis (Prasad *et al.*, 2002). Impaired endothelial function was observed by measuring FMD in patients with psoriasis (Balci *et al.*, 2009). More recently our group provided insight into the mechanisms of vascular dysfunction caused by inflammation of the skin in an experimental model of psoriasis (Karbach *et al.*, 2014b). These studies demonstrated that overexpression of IL-17A caused a psoriasis-like disease inducing infiltration of inflammatory cells and increased oxidative stress and vascular dysfunction (Karbach *et al.*, 2014b).

Further details of the interaction of inflammatory processes and endothelial and vascular dysfunction is extensively reviewed elsewhere (Harrison *et al.*, 2011; Karbach *et al.*, 2014a; Knorr *et al.*, 2014; Steven *et al.*, 2015). It is important to note that oxidative stress is a hallmark of all cardiovascular diseases and that it contributes to endothelial cell activation, priming it for adhesion and infiltration of immune cells as well as activation of these infiltrated immune

cells. Endothelial cell activation *per se* is not harmful since it represents a physiological adaptation to different stimuli and can be considered a response to injury; endothelial activation is also an early step in the development of endothelial (vascular) dysfunction as long as it is not counter-regulated and returns to a "normal" phenotype (e.g. as seen under chronic inflammatory conditions). Accordingly, there is a persistent low-grade inflammatory phenotype of the vasculature observed in most cardiovascular diseases.

Aging and endothelial (vascular) dysfunction

The aging process is associated with arterial stiffness and endothelial dysfunction, hallmarks of future cardiovascular events in humans (Ras *et al.*, 2013). The vasculature in the elderly is also more susceptible to atherosclerotic lesions, vascular injury, impaired angiogenesis and calcification (Herrera *et al.*, 2010). As a result, the incidence and frequency of cardiovascular complications such as coronary artery disease or stroke is higher in the elderly (Ras *et al.*, 2013; Savji *et al.*, 2013). Vascular oxidative stress and inflammation are increased during the aging process and may further stimulate each other in a positive feedback fashion (El Assar *et al.*, 2013; Mikhed *et al.*, 2015a). Activated and infiltrated immune cells induce secondary vascular sources of oxidative stress, with further endothelial cell activation that primes the endothelium for the adhesion of more leukocytes and platelets (Karbach *et al.*, 2014a). This immuno-redox crosstalk significantly contributes to aging-associated endothelial dysfunction (Mikhed *et al.*, 2015a). In addition to oxidative stress and inflammation, other causes of endothelial dysfunction in the aging vasculature include apoptosis and necrosis of various cell types, possibly including repair cells such as EPCs (Kujoth *et al.*, 2005).

Drug-induced endothelial (vascular) dysfunction

Multiple reports on drug-induced endothelial (vascular) dysfunction exist and it would be beyond the scope of this review to mention all of them. We briefly provide some important examples of drug (or radiotherapy)-induced endothelial dysfunction and refer to a recent review on this topic (Wojcik *et al.*, 2015).

Previous data reported that treatment with anticancer drugs and/or radiotherapy contributes to the development of endothelial dysfunction. Survivors of childhood acute lymphoblastic leukaemia receiving chemotherapy and/or chemotherapy combined with radiation displayed impaired FMD but not nitroglycerin-mediated dilation (NMD) as compared to healthy subjects (Dengel *et al.*, 2008). Of note, early damage to the endothelium can have prolonged (>20 years) effects in humans. A similar observation was made in lymphoma survivors treated with radiotherapy – this group had a higher burden of atherosclerotic lesions and greater peripheral endothelial dysfunction than the control group (Wethal *et al.*, 2014). Young adult survivors of Hodgkin Lymphoma following radiotherapy displayed impaired endothelial function as measured by peripheral arterial tonometry (PAT) (Zelcer *et al.*, 2010).

Although organic nitrates are among the nitric oxide replacement therapies, most of these drugs induce so-called nitrate tolerance with chronic administration, a phenomenon that is based on the loss of potency of the nitrovasodilators but also other severe side effects such as oxidative stress and endothelial dysfunction (cross-tolerance) (Munzel *et al.*, 2013; Daiber and Munzel, 2015). The development of endothelial dysfunction in humans was reported for nitroglycerin and isosorbide mononitrate (to a minor extent also isosorbide dinitrate) treatment, whereas pentaerithrityl tetranitrate may be the only organic nitrate in clinical use that is devoid of these side effects (Munzel *et al.*, 2011). The development of nitroglycerin-induced endothelial dysfunction is probably due to mechanisms similar to those produced by classical cardiovascular risk factors.

Other factors for endothelial (vascular) dysfunction

As already mentioned, the development of endothelial (vascular) dysfunction is a complex and multifactorial process and not all mechanisms can be adequately discussed here. Another important cause of endothelial dysfunction is the plasma concentration of high density lipoprotein (HDL), since low levels are associated with an increased burden of cardiovascular disease (Kontush, 2014). HDL levels also negatively correlate with markers of oxidative stress (Bencsik *et al.*, 2015). It may also be that the quality of HDL (e.g. content of oxidant and antioxidant proteins) regulates normal endothelial function and prevents cardiovascular diseases (Riwanto and Landmesser, 2013). Of note, the quality of HDL is subject to redox-driven modifications involving myeloperoxidase and paraoxonase (Huang *et al.*, 2013). HDL from healthy subjects has strong anti-oxidant effects and also inhibits the activation of NADPH oxidase in endothelial cells, all of which may explain the positive effects of HDL on the $^\bullet$NO/superoxide balance. Intra-arterial infusion of HDL alleviates endothelial dysfunction in hypercholesterolaemic men (Spieker *et al.*, 2002). Unfortunately, more recent studies clearly demonstrate that HDL from subjects with established coronary artery disease is dysfunctional, thus increasing rather than inhibiting oxidative stress in vascular tissue (Besler *et al.*, 2011).

The impact of alcohol (ethanol) on endothelial function is a dual-edged sword (Cahill and Redmond, 2012). Epidemiological studies demonstrate that frequent low-moderate alcohol intake is vasculoprotective, whereas acute binge drinking or chronic alcohol abuse is detrimental, as demonstrated by the development of alcohol-dependent cardiomyopathy and progression of atherosclerosis in chronic drinkers (Lucas *et al.*, 2005).

DNA damage (e.g. by oxidative stress or irradiation) results in the activation of poly(ADP-ribose) polymerase (PARP), which in turn leads to inhibition of glyceraldehyde-3-phosphate dehydrogenase, diabetic complications such as activation of NFκB, protein kinase C and generation of intracellular advanced glycation end products, all of which contribute to "glycaemic memory" and endothelial dysfunction (Szabo, 2009). Moreover, PARP activation contributes to the destabilization of atherosclerotic plaques, immune cell infiltration, sirtuin 1 inactivation and neointima formation, all of which promote endothelial (vascular) dysfunction (Xu *et al.*, 2014).

PVAT releases important vasoactive compounds, a function that can be dysregulated and so contribute to vascular dysfunction (Brown *et al.*, 2014). Importantly, redox processes play an important role in these alterations (Jankovic *et al.*, 2015). One of these PVAT-derived factors, adiponectin, is proposed to be a better predictor of endothelial function of coronary arteries than insulin resistance index (HOMA-R), body mass index (BMI), immuno-reactive insulin, or triglycerides (Okui *et al.*, 2008). Advanced glycosylation end products (AGEs) and their specific receptor (advanced glycosylation end product-specific receptor [RAGE]), play an important role in atherosclerosis and determine endothelial function in diabetic patients (Kajikawa *et al.*, 2015). AGE/RAGE signalling is subject to redox regulation and there is crosstalk with oxidative stress pathways (Wautier *et al.*, 2001; Coughlan *et al.*, 2009). The Janus face of bradykinin receptors in the control of vascular homeostasis underlies the clinical use of bradykinin receptor agonists (e.g. for the direct vasodilator effects and beneficial effects on components of the renin-angiotensin-aldosterone system (RAAS)) as well as antagonists (e.g. for the control of inflammatory and pain signalling) (Blaes and Girolami, 2013). Dysregulation of arginases represents another trigger for endothelial dysfunction, since arginases compete with eNOS for the substrate L-arginine and are upregulated under inflammatory conditions and released during haemolysis (Risbano and Gladwin, 2013). There is also evidence for endothelial dysfunction in oncological diseases; however, since most cancer patients receive anticancer therapy directly after diagnosis, it is challenging to distinguish between direct effects of cancer on vascular function and the indirect effects of the (mostly aggressive) anticancer therapies (e.g. chemotherapy, radiotherapy) (Wojcik *et al.*, 2015). Finally, oestrogen signalling has a significant impact on endothelial function as reflected by sex differences in the burden of cardiovascular disease as well as changes in the incidence of endothelial dysfunction in post-menopausal and pregnant women (Chakrabarti *et al.*, 2014).

Measurement of endothelial function and dysfunction

Besides general clinical signs of endothelial dysfunction, such as erectile dysfunction (Kaiser *et al.*, 2004; Kovacs *et al.*, 2008; Bhatia *et al.*, 2013), which is also a strong indicator of cardiovascular risk (Dong *et al.*, 2011; Vlachopoulos *et al.*, 2013), there are several established techniques to determine endothelial function *in vivo* (Figure 4). We mainly focus on those techniques that are routinely applied in clinical studies or emerging techniques that may be of relevance for human diagnostics in the future. Some more sophisticated methods, including biochemical markers and bioassays, measurement of endothelial-derived microparticles and progenitor cells, are discussed elsewhere (Lekakis *et al.*, 2011). A helpful correlation matrix of multiple parameters of endothelial function, arterial stiffness and end-points of atherosclerosis can be found elsewhere (Frolow *et al.*, 2015).

Acetylcholine binds to muscarinic receptors on endothelial cells leading to eNOS activation, elevation of vascular •NO production and vasodilatation of blood vessels (Ludmer *et al.*,

1986). The relaxation of coronary vessels upon infusion of acetylcholine can be detected by quantitative coronary angiography or measurement of coronary blood flow (Schachinger *et al.*, 2000). Similarly, acetylcholine infusion can be applied to any peripheral resistance vessel (e.g. in the forearm) and coupled with ultra-sound measurement of blood flow, which is termed forearm plethysmography (Heitzer *et al.*, 1996a). A non-invasive technique for measurement of endothelial function *in vivo* is quantification of flow mediated dilation (FMD) (Ramsey *et al.*, 1996). In this process, a baseline diameter of the brachial artery is first recorded by ultrasound; blood-flow is then interrupted by inflation of a blood pressure cuff for five minutes, and restoration of blood flow leads to a reactive hyperaemia that is associated with increased release of •NO. The subsequent vasodilation is again detected by ultrasound. Maximal dilation of the brachial artery is induced by sublingual application of nitroglycerin (NMD) and then used for normalization of FMD values. A newer non-invasive technique to detect endothelial function in humans is peripheral arterial tonometry (PAT), where a finger probe assesses digital volume changes and pulse waves that can be detected after induction of reactive hyperaemia (Bonetti *et al.*, 2003). Other advanced techniques for the diagnosis of endothelial dysfunction are low flow mediated constriction (Gori *et al.*, 2008; Gori *et al.*, 2012) and passive leg movement (PLM) as a potentially practical assessment of systemic vascular function via •NO-dependent mechanisms (Groot *et al.*, 2015; Hughes and Kruse, 2016). The most traditional technique for the ex vivo assessment of endothelial dysfunction is the isometric tension method as first described in animal tissues by the Nobel laureate Robert Furchgott (Furchgott and Zawadzki, 1980), or in human vessels from bypass surgery (Schulz *et al.*, 2002). An additional novel *ex vivo* technique to assess endothelial dysfunction is based on the identification of atherosclerotic lesions by Raman spectroscopy (Baranska *et al.*, 2015). The advantages and disadvantages of these methods were discussed in full detail elsewhere (Munzel *et al.*, 2008). Clearly the non-invasive techniques are easier to apply to a large study population and the risks of side effects (e.g. puncture-induced bleeding complications) are lower.

The early stages of atherosclerosis are characterized by endothelial dysfunction while the later stages result in arterial stiffness, both of which can be quantified by different techniques. Pulse wave velocity (PWV) has emerged as an established method to examine arterial stiffness and has a strong correlation with cardiovascular outcomes (Reference Values for Arterial Stiffness C, 2010). Applanation tonometry and Doppler ultrasound are also clinical methods available for evaluating global PWV (Lehmann *et al.*, 1993; Nelson *et al.*, 2010; Reference Values for Arterial Stiffness' Collaboration, 2010). However, the gold standard for measuring PWV remains flow meter or catheter-based pressure probes. Nevertheless, their invasiveness limits their use in clinical studies (Wentland *et al.*, 2014). In recent years magnetic resonance imaging (MRI) has become more accessible and is another minimally-invasive method to determine PWV (Boese *et al.*, 2000; Dogui *et al.*, 2011; Bar *et al.*, 2015).

A new molecular imaging method for the detection of areas of dysfunctional endothelium is based on the infusion of compounds that release gaseous microbubbles that can be detected by ultrasound (reviewed by Villanueva *et al.*,
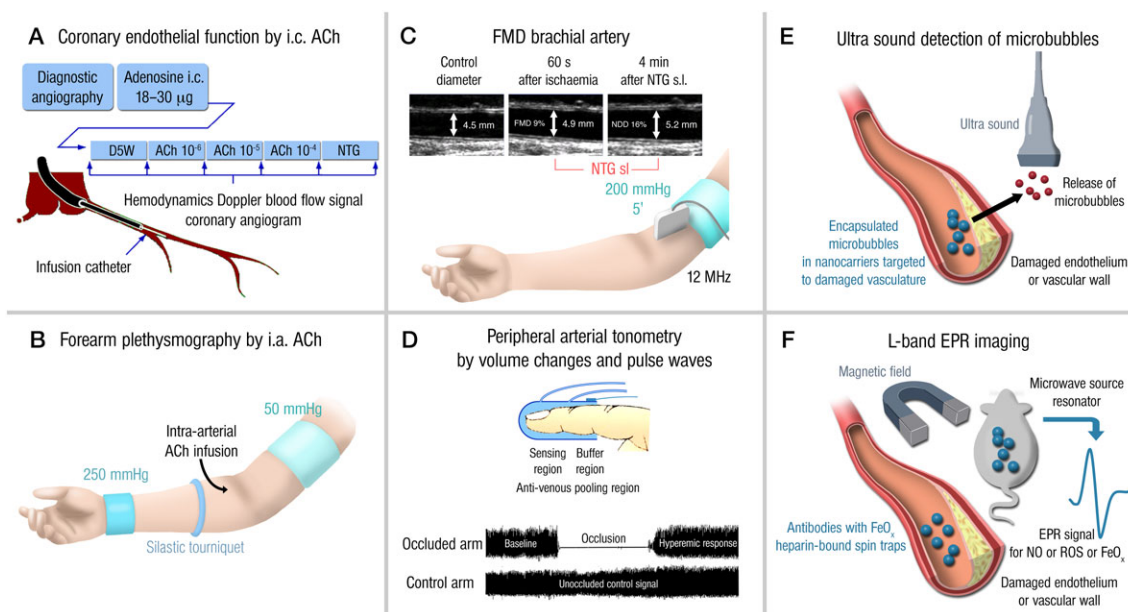
Figure 4

Invasive and non-invasive methods for the determination of endothelial function. (A) Acetylcholine (ACh)-dependent vasoreactivity of coronary vessels caused by intra-coronary (i.c.) ACh infusion. Vasodilatation and stenotic areas are monitored by angiographic imaging and Doppler ultrasound (blood flow). (B) ACh-dependent vasoreactivity of capacity vessels of the forearm upon intra-arterial ACh infusion. Vasodilatation is recorded by Doppler ultrasound (diameter and blood flow). (C) Flow-mediated dilation (FMD) of capacity vessels of the forearm (brachial artery) upon occlusion/ischaemia and reperfusion/hyperaemia. Vasodilatation is recorded by Doppler ultrasound (diameter and blood flow). Maximal vasoreactivity/dilation is determined by sublingual administration of nitroglycerin (NTG). Adapted from (Munzel, 2008). With permission of Georg Thieme Verlag KG Stuttgart. Copyright © 2008, Rights Managed by Georg Thieme Verlag KG Stuttgart • New York. All rights reserved. (D) Peripheral arterial tonometry measures volume changes and pulse waves by a finger probe. Additional details for the determination of pulse wave velocity (PWV) are explained in the text. (E) Ultrasound-based detection of microbubbles released from nanocarriers upon chemical reaction with ROS or upon destruction of the carriers by ultrasound. The nanocarriers are targeted to damaged endothelium (e.g. by bound antibodies). The released microbubbles are detected by ultrasound. (F) L-band electron paramagnetic resonance (EPR) spectroscopy can be used for the detection of paramagnetic compounds or particles (usually having an unpaired electron) in whole animals and tissues. Damaged vasculature can be labelled by antibodies linked to iron oxide (EPR active). Likewise, heparin-bound spin traps are bound to the endothelial surface and allow the detection of nitric oxide or ROS.

2002; Camici et al., 2012). Lipid-covered decafluorobutane microbubbles linked to specific antibodies [e.g. against P-selectin, vascular cell adhesion molecule 1 (VCAM-1)] were used to detect areas of vascular inflammation in obese primates upon ultrasound-triggered release of the microbubbles (Chadderdon et al., 2014). Another feature of the antibody-targeted microbubbles is specific drug targeting of inflamed, thrombotic or atherosclerotic endothelium (Tsutsui et al., 2004). In addition to using preformed microbubbles, *in situ* generated microbubbles from chemical reactions can also be used for ultrasound detection of damaged endothelium. A study in mice used ultrasound to measure the reaction of ROS with liposome-encapsulated allylhydrazine, a liquid compound, that yields nitrogen and propylene gas that is detected by ultrasound methods (Perng et al., 2012).

Another method that will probably be used in the future is L-band electron paramagnetic resonance (EPR) spectroscopy for the detection of areas of dysfunctional endothelium in intact animals. Previous studies used iron oxide particles bound to endothelium-specific antibodies (e.g. anti-E-selectin) for the detection of endothelial inflammation (Radermacher et al., 2009). Likewise, heparin-polynitroxide derivatives bind to the endothelium via heparin-binding sites and due to their superoxide scavenging potential, can serve as diagnostic and

therapeutic tools (Kleschyov et al., 2012; Kleschyov and Sen, 2013). Most of these probes can be used in L-band and MRI approaches. Combined with the respective spin trap, these techniques have the potential for the specific detection of vascular •NO or ROS formation in isolated tissues and whole animals (Fujii and Berliner, 2004).

Another new diagnostic technique to measure endothelial function could be based on the determination of the glycocalyx by intravital microscopy or by orthogonal polarization spectral imaging (Nieuwdorp et al., 2008). The distance between the endothelium and passing erythrocytes represents the thickness or dimension of the glycocalyx that correlates with levels of classical cardiovascular risk markers such as low density lipoprotein (LDL), HDL, blood glucose and BMI.

Targeting endothelial (vascular) dysfunction

The endothelium, which was initially regarded as a relatively inert cell layer, is now recognized as an important regulator (in concert with the smooth muscle and PVAT) of numerous physiological functions such as coagulation, fibrinolysis, arterial tone and vascular growth. As outlined above,

endothelial dysfunction is a key event in the development and progression of atherosclerosis, coronary artery diseases and myocardial ischaemia (Celermajer, 1997). The demonstration that endothelial dysfunction can be reversed raises the possibility of retarding or even preventing the progression of atherosclerosis, while also improving arterial function and decreasing the incidence of cardiovascular events (see Figure 5A for a summary of therapeutic options). Here we mainly focus on those treatment options that are routinely applied or are emerging therapies of likely relevance for patient care in the future.

Established drugs improving endothelial dysfunction

Angiotensin-converting enzyme (ACE) inhibitors, AT_1 receptor (angiotensin II type-1 receptor) antagonists, statins and many other cardiovascular drugs display pleiotropic indirect antioxidant properties (e.g., inhibition of Nox enzymes and secondary to this prevention of eNOS uncoupling) and anti-inflammatory effects leading to improved endothelial function (for review see Drexler and Hornig, 1999; Warnholtz and Munzel, 2000; Gori and Munzel, 2011; Steven et al., 2015). Similar beneficial effects were described
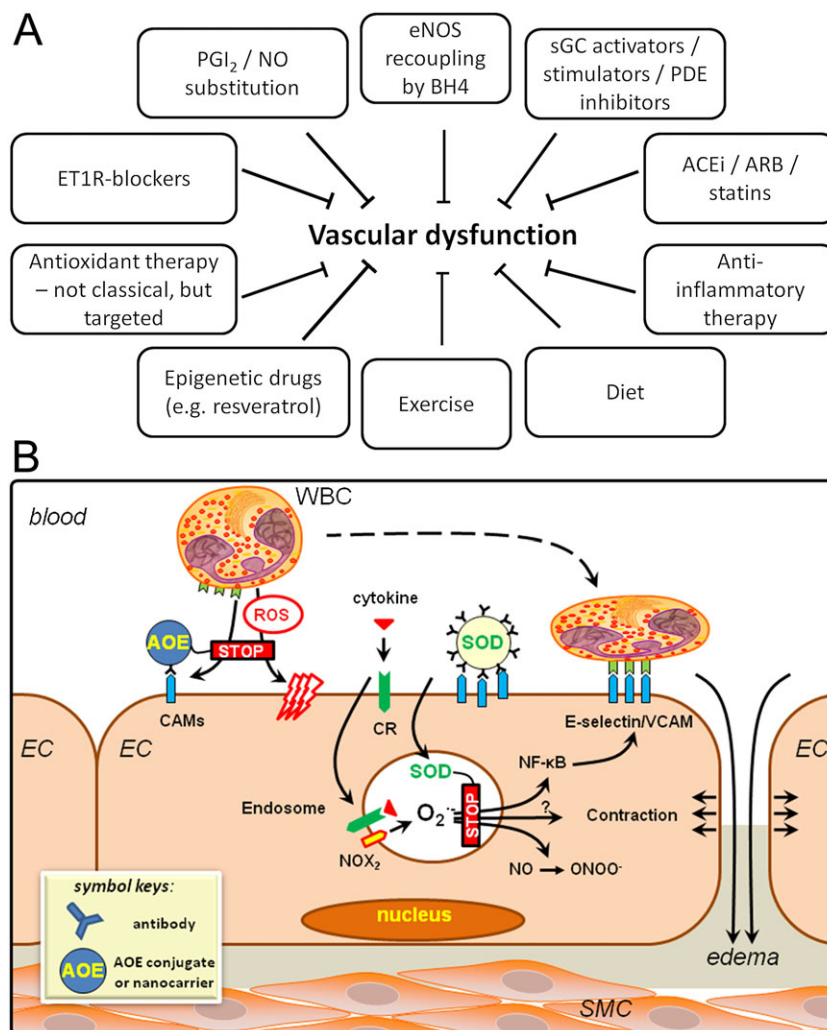

Figure 5

Overview of therapeutic options for the improvement of vascular dysfunction. (A) In addition to currently available cardiovascular drugs, several new therapies are currently in preclinical and clinical studies. Anti-inflammatory, "repair" and epigenetic therapy are promising candidates for the future. Exercise and dietary changes are non-pharmaceutical but highly potent "natural" alternatives. Classical antioxidant therapy failed in most large clinical trials and might require specific delivery of the antioxidants at sites of inflammation or increased oxidative stress. (B) Targeted antioxidant interventions to alleviate pro-inflammatory activation and oxidative stress in endothelial cells. Endothelial ROS from activated Nox2 enzyme in endosomes are formed in response to cytokine binding to the receptors and ignite signalling cascade of transcription factor NF-κB. Targeted delivery of antioxidants, antioxidant enzymes (AOE) and inhibitors of ROS production can be achieved using antibodies and other ligands of endothelial surface determinants including cell adhesion molecules PECAM and ICAM. Surface-bound targeted AOE intercept extracellular ROS, whereas targeted formulations using the same ligands configured in a way permitting internalization into the ROS-signalling endosomes allows interception of pro-inflammatory activation manifested among other characteristics by exposure of inducible cell adhesion molecules – E-selectin, VCAM-1 and ICAM-1 - that can be detected using imaging probes conjugated to the ligands of these molecules.

for endothelin-1 receptor antagonists (Clozel, 2003; Thorin and Clozel, 2010). Gemfibrozil is a lipid-lowering drug that also activates sGC in a nitric oxide- and haem-independent fashion, which may explain the more pronounced cardiovascular benefit observed with this drug as compared to other members of the fibrate group of drugs (Sharina et al., 2015).

Antioxidant therapy

Reactive oxygen species (ROS) from different sources play an important role in regulating vascular function (Karbach et al., 2014a; Steven et al., 2015). Superoxide reacts with $^\bullet$NO to form the highly reactive product peroxynitrite (ONOO$^-$), leading to not only reduced $^\bullet$NO bioavailability but also impaired endothelial enzymatic processes (Munzel et al., 2008). Several studies using vitamin C as an antioxidant therapy have been performed in an attempt to restore endothelial function and thereby reduce cardiovascular disease. Promising results were obtained in studies in which vitamin C was given acutely to patients suffering from classic risk factors for cardiovascular disease such as metabolic syndrome, smoking or hypertension (Takase et al., 2004; Teede et al., 2006; Cangemi et al., 2007; Plantinga et al., 2007). Even more intriguing are results showing a correlation between vitamin C deficiency and cardiovascular outcomes (Heitzer et al., 2001) (see Table 2 for an overview). Large-scale clinical trials performed to investigate the impact of antioxidants on clinical end-points and the progression of atherosclerosis have yielded rather disappointing findings, since almost no beneficial effects on cardiovascular outcome were detected (Salonen et al., 2003; Cook et al., 2007; Sesso et al., 2008). In fact, in a study on postmenopausal women with type 2 diabetes, administration of supplemental vitamin C correlated positively with mortality end-points (Lee et al., 2004).

The reasons for the failure of large clinical trials using oral antioxidant therapy are numerous and include, among others, the limited up-take of classic oral antioxidants in tissues undergoing oxidative stress, access to intracellular sites of ROS production, the limited reactivity towards specific ROS (e.g. hydrogen peroxide or superoxide) and most importantly, interference with essential physiological ROS signalling (for review see (Chen et al., 2012; Schmidt et al., 2015b)). Since most large-scale clinical trials on oral antioxidant therapy were either not properly controlled for compliance or for individual basal antioxidant serum levels [compare the different outcome of the EPIC Norfolk study

Table 2

Effects of anti-oxidant treatment on endothelial function

Inclusion criteria	Reference	Antioxidants used	Treatment	Improvement of endothelial function
Hypertension	(Solzbach et al., 1997)	Vitamin C	acute, i.v.	Yes
	(Teede et al., 2006)	Flavonoids	3 month, oral	No
	(Plantinga et al., 2007)	Vitamin C and E	8 weeks, oral	Yes
Smoking	(Neunteufl et al., 2000)	Vitamin E	4 weeks, oral	Yes
	(Mangoni et al., 2002)	Folic acid	4 weeks, oral	Yes
	(Antoniades et al., 2003)	Vitamin C and E	4 weeks, oral	Yes
	(Takase et al., 2004)	Vitamin C and E	4 weeks, oral	Yes
	(Heiss et al., 2005)	Flavonoids	2 hours, oral	Yes
	(Young et al., 2006)	Vitamin C	12 weeks, oral	Yes
	(Heitzer et al., 1996a)	Vitamin C	Acute, i.a.	Yes
Diabetes Type 1 and 2, Hyperglycaemia	(Skyrme-Jones et al., 2000)	Vitamin E	12 hours, oral	Yes
	(Regensteiner et al., 2003)	L-arginine, Vitamin C and E	1 week, oral and 1-week „wash out"	Yes
	(Pena et al., 2004)	Folic acid	8 weeks, oral	Yes
	(Economides et al., 2005)	Vitamin E	12 month, oral	No (Impairment of FMD)
	(Mangoni et al., 2005)	Folic acid	4 weeks, oral	Yes
	(Anderson et al., 2006)	Vitamin C	2 days, oral	Yes
Kidney failure	(Cross et al., 2003)	Vitamin C	Acute, i.a.	Yes
	(Ghiadoni et al., 2004)	Vitamin C	2 hours, oral	Yes
	(Nanayakkara et al., 2007)	Vitamin E	12 month, oral	Yes
Heart failure	(Ellis et al., 2001)	Vitamin C	2 hours, i.v.	Yes
Stable angina	(Tousoulis et al., 2005)	Vitamin C	Acute, i.v.	Yes
Healthy older men	(Jablonski et al., 2007)	Vitamin C	Acute, intravenously	Yes
Endotoxaemia in healthy subjects	(Aschauer et al., 2014)	Vitamin C	2 hours, intravenously	Yes

(Khaw *et al.*, 2001)], measuring the antioxidant status of each subject is necessary to determine if benefits occurred only in those with pre-existing antioxidant deficiencies (Schmidt *et al.*, 2015b).

Anti-inflammatory therapy

Inflammation is an independent risk factor for the development of cardiovascular disease (Kaptoge *et al.*, 2014). Levels of the acute phase protein CRP are elevated in various inflammatory diseases. The PROVE IT-TIMI 22 study demonstrated that high CRP levels in patients with acute coronary syndrome predicted death from myocardial infarction (Ridker *et al.*, 2005). There are numerous human and animal studies demonstrating a correlation between inflammatory processes and endothelial dysfunction (for review see (Karbach *et al.*, 2014a; Steven *et al.*, 2015)). Lipopolysaccharide (LPS) from the bacterial cell wall is a strong trigger for inflammation and endothelial (vascular) dysfunction in humans (Becker *et al.*, 2012). This occurs in severe inflammatory conditions, such as septic shock, and also in low-grade inflammatory diseases such as rheumatoid arthritis (Bergholm *et al.*, 2002; Hansel *et al.*, 2003) or type 2 diabetes (Henry *et al.*, 2004), causing endothelial (vascular) dysfunction and accelerated atherosclerosis. Furthermore, it is now generally agreed that in addition to atherosclerosis, arterial hypertension is also a low-grade inflammatory disease (Libby, 2002; Wenzel *et al.*, 2011).

Several clinical trials are currently underway to investigate whether anti-inflammatory treatment improves cardiovascular outcomes e.g. methotrexate therapy (TETHYS trial and CIRT trial) (Everett *et al.*, 2013; Moreira *et al.*, 2013) and blockade of the cytokine IL-1β with canakinumab for the management of cardiovascular disease (CANTOS trial) (Ridker *et al.*, 2011). Another new anti-inflammatory therapy could be based on the restoration of a disturbed glycocalyx, which is associated with higher susceptibility to triggers of atherosclerosis and leukocyte/platelet adhesion (Meuwese *et al.*, 2009; Drake-Holland and Noble, 2012; Tarbell and Cancel, 2016).

Repair therapy with pharmacological agents

Pharmacological targeting of oxidatively impaired eNOS and sGC. There is overwhelming evidence supporting an important pathophysiological role of eNOS dysregulation/uncoupling in several diseases, making targeting of this enzyme, in particular in its uncoupled state, an attractive therapeutic option. So far, eNOS-directed pharmaceutical attempts have included the development of so-called eNOS enhancers, compounds that up-regulate the expression of eNOS at the mRNA and protein level (Fraccarollo *et al.*, 2008; Wohlfart *et al.*, 2008; Frantz *et al.*, 2009). Despite quite favorable results in these preclinical studies an inherent problem may be that sole overexpression of eNOS without up-regulation of its co-factor sapropterin (BH$_4$) will ultimately lead to its uncoupling and so worsen disease conditions rather than improving them. However, some eNOS enhancers provide beneficial effects because of a simultaneous up-regulation of BH$_4$. Unfortunately to this date no clinical data are available to judge the efficacy of eNOS enhancers in patients with cardiovascular disease. In line with this, Channon and co-workers demonstrated that

eNOS overexpression leads to an increase in •NO formation only when the BH$_4$ synthase GTP-cyclohydrolase 1 (GCH-1) is also up-regulated (Crabtree *et al.*, 2009). Overexpression of GCH-1 or co-administration of BH$_4$ successfully prevented cardiac hypertrophy by pressure overload and also lowered blood pressure in the salt-sensitive low-renin hypertension model (Du *et al.*, 2008; Moens *et al.*, 2008). BH$_4$ supplementation improved cardiac and pulmonary function in a canine model of cardiopulmonary bypass (Szabo *et al.*, 2011). BH$_4$ treatment or treatment with analogues such as folic acid and sepiapterin improved endothelial dysfunction in e.g. chronic smokers and diabetes via recoupling eNOS (Heitzer *et al.*, 2000b; Heitzer *et al.*, 2000a) and in numerous *ex vivo* studies (Tiefenbacher *et al.*, 2000; Gori *et al.*, 2001) (for review see (Antoniades *et al.*, 2009)). The PHACeT Trial reported benefits of eNOS gene-enhanced progenitor cell therapy for pulmonary arterial hypertension (Granton *et al.*, 2015).

Recent studies have identified sGC as an important therapeutic target since oxidation of the enzyme at numerous sites impairs activation by •NO. The discovery of compounds such as haem-dependent sGC stimulators and haem-independent sGC activators that can stimulate the enzyme in a •NO-independent manner has led to the development of a clinical programme for the treatment of patients with pulmonary (arterial) hypertension (Ghofrani *et al.*, 2013a), chronic thromboembolic pulmonary hypertension (Ghofrani *et al.*, 2013b) and heart failure (Lapp *et al.*, 2009) (for review see (Evgenov *et al.*, 2006)). The sGC activator cinaciguat normalized vascular oxidative stress and coronary endothelial function in a rat model of myocardial infarction (Korkmaz *et al.*, 2009b), a canine model of cardiopulmonary bypass (Radovits *et al.*, 2011) and prevented endothelial dysfunction of isolated rat aorta when challenged with peroxynitrite (Korkmaz *et al.*, 2013). Inhibitors of phosphodiesterases (PDE) act down-stream of sGC to increase cGMP levels and are used for multiple indications (for review see (Boswell-Smith *et al.*, 2006)). A recent meta-analysis of subjects with type 2 diabetes mellitus (T2DM) suggests a beneficial effect of treatment with a PDE5 inhibitor (sildenafil) on endothelial function (Santi *et al.*, 2015). Likewise, therapy with the PDE5 inhibitor vardenafil prevented cardiovascular dysfunction in a rat model of T1DM (Radovits *et al.*, 2009) and a canine model of cardiopulmonary bypass with hypothermic cardiac arrest (Szabo *et al.*, 2009). Vardenafil also improved endothelial function of isolated rat aorta upon *ex vivo* challenges with peroxynitrite (Korkmaz *et al.*, 2009a) and hypochlorite (Radovits *et al.*, 2013).

Nitric oxide and other substitution therapies. Nitrovasodilators such as organic nitrates act in •NO substitution therapy and have been used for more than a century for the relief of anginal pain that is caused by stenosis or spasm of the coronary arteries associated with endothelial dysfunction (for review see (Daiber and Munzel, 2015). However, as is the case for eNOS enhancer therapy, chronic substitution of •NO alone does not produce beneficial effects since •NO under oxidative stress conditions rapidly yield the potent oxidant peroxynitrite (Warnholtz *et al.*, 2002) (for review see Gori and Parker, 2004). The target enzyme receptor for •NO, sGC, can also be inhibited under conditions of oxidative stress (Evgenov *et al.*, 2006). Therefore, a

combined antioxidant and •NO substitution therapy seems to be a more promising strategy, as shown for pentaerithrityl tetranitrate, the only organic nitrate that improves oxidative stress, nitrate tolerance and endothelial dysfunction (Munzel *et al.*, 2011; 2014b). Inorganic nitrite and nitrate are emerging options for •NO substitution therapy as they have beneficial effects in hypertension, myocardial infarction and acute heart failure (for review see (Bueno *et al.*, 2013; Bailey *et al.*, 2014; Rassaf *et al.*, 2014)). Accumulation of the endogenously produced eNOS inhibitor asymmetric dimethyl arginine (ADMA) under oxidative stress conditions is another trigger of endothelial dysfunction that could be remedied by treatment with high doses of L-arginine, which directly replaces ADMA at the level of eNOS enzyme (for review see (Sydow and Munzel, 2003)) or by prevention of endothelial ADMA accumulation through activation of the cationic amino acid extrusion transporter (Closs *et al.*, 2012). Another new therapeutic option is supplementation with an H_2S donor that not only confers direct antioxidant effects but which also acts synergistically with •NO via different signalling pathways (Cortese-Krott *et al.*, 2015; Yuan *et al.*, 2015). Prostacyclin substitution, e.g. by iloprost, is not generally used for the treatment of cardiovascular disease but more specifically for the therapy of severe pulmonary hypertension (Galie *et al.*, 2001).

Inhibition of PARP to suppress down-stream effects of oxidative (or irradiation-induced) DNA damage. Pharmacological inhibition of PARP prevented irradiation-induced vascular dysfunction in isolated aortic tissue (Beller *et al.*, 2006), improved endothelial dysfunction in isolated vessels treated with hydrogen peroxide- (Radovits *et al.*, 2007a) or hypochlorite-induced oxidative stress (Radovits *et al.*, 2007c) and reversed cardiac and vascular dysfunction in aged rats (Radovits *et al.*, 2007b).

Epigenetic approaches

There is emerging evidence that epigenetic pathways are redox-regulated and play a causal role in cardiovascular diseases by directly affecting endothelial function (for reviews see Kim *et al.*, 2013; Mikhed *et al.*, 2015b). Due to their reversibility, epigenetic processes represent ideal targets for therapeutic approaches.

microRNAs. Since their discovery in *C. Elegans* in the last decade of the 20th century, microRNAs have been of increasing interest in biomedicine, with the cardiovascular field being no exception (Small and Olson, 2011). miRNAs were among the first of the small, non-coding RNA molecules (<200 nt) to be recognized in regulating gene expression; this ever-expanding family now includes another group of non-coding RNAs that are longer (>200 nt) and hence termed long non-coding RNAs (Uchida and Dimmeler, 2015). MicroRNAs (miRNAs) are short (20-24 nt) non-coding RNAs involved in the post-transcriptional regulation of gene expression by affecting both stability and translation of mRNA. Approximately 2600 miRNAs have been described in humans. More than half of all mRNAs are estimated to be targets of miRNAs, and each miRNA is thought to regulate hundreds of targets (Agarwal *et al.*, 2015). The role of miRNAs in vascular function and

atherogenesis has been comprehensively reviewed recently (Zampetaki and Mayr, 2012; Fernandez-Hernando and Baldan, 2013; Zampetaki *et al.*, 2013; Menghini *et al.*, 2014; Andreou *et al.*, 2015).

In the specific case of vascular/endothelial dysfunction, miRNAs have been grouped according to their roles in general programmes related to either endothelial senescence or endothelial inflammation (Menghini *et al.*, 2014). In the case of vascular aging, up-regulation of miR-29, miR-34, miR-217 and miR-146 have been linked to vascular wall alterations or endothelial senescence (Dimmeler and Nicotera, 2013). Targeting of Nox4 by miR-146a has been suggested to reduce ROS levels, potentially helping to mitigate endothelial dysfunction (Vasa-Nicotera *et al.*, 2011). In contrast miR-200 increases oxidative stress and endothelial cell senescence (Magenta *et al.*, 2011). The miRNA miR-126 is abundantly expressed in endothelial cells (Wei *et al.*, 2013) and is important for vascular integrity (Schober *et al.*, 2014).

Recent work shows promising avenues to explore specific miRNAs as potential therapeutic tools or targets to treat vascular and endothelial dysfunction. An interesting example is posed by miR-181b, which inhibits NF-κB activation, vascular inflammation and atherosclerosis in ApoE-deficient mice that were systemically treated with the miRNA (Sun *et al.*, 2014). In addition, circulating levels of miR-181b were reduced in patients with inflammatory conditions and in elderly people, suggesting a potential protective role for this miRNA. Similarly, *in vivo* inhibition of miR-92a that is associated with low shear stress and pro-atherogenic stimuli, reduced plaque size in LDL-receptor null mice (Loyer *et al.*, 2014) and produced beneficial effects in models of vascular injury (Daniel *et al.*, 2014). Nevertheless, other authors reported that perturbation of homeostatic levels of this miRNA alters redox-regulated responses and endothelial angiogenesis (Zhang *et al.*, 2014; Chen *et al.*, 2015). Overall it is fair to state that we are at the early stages of employing strategies directed towards regulating the levels of endogenous miRNAs for ameliorating vascular dysfunction, with rapid strides being made on an ongoing basis (van Rooij and Olson, 2012).

Histone acetylation/methylation and DNA methylation. Disturbed blood flow associated with atherosclerosis alters endothelial gene expression by inducing DNA (cytosine-5-)-methyltransferase 1 (DNMT1) mediated changes in genome-wide DNA methylation patterns (Dunn *et al.*, 2014). Treatment with the DNMT inhibitor azacitidine (5-aza-2′-deoxycytidine) restores normal methylation patterns and the expression of mechanosensitive master transcription factors including Homeobox protein A5 (HoxA5) and Kruppel-like Factor 3 (KLF3) to prevent endothelial dysfunction and atherosclerosis in animals. Likewise, endothelial function is improved by DNMT inhibitors (by abolishing the disturbed flow-induced silencing of the athero-protective transcription factor KLF4) in human aortic endothelial cells and adult swine aortas (Jiang *et al.*, 2014) and also by restoring the expression of KLF2in human umbilical vein endothelial cells (HUVECs), which is repressed in dysfunctional endothelium by LDL-mediated DNA methylation (Kumar *et al.*, 2013). Oxidized LDL (oxLDL) induces acetylation of histone H3 and H4 as well as phosphorylation of histone H3, resulting in the expression of

pro-inflammatory genes in endothelial cells, all of which was prevented by statin therapy (Dje N'Guessan *et al.*, 2009). Methyl-CpG-binding domain protein2 (MBD2) negatively regulates eNOS and vascular endothelial growth factor receptor 2 (VEGFR-2, Type IV RTKs) expression whereas knockdown of MBD2 in HUVECs enhanced angiogenesis, provided protection against H_2O_2-induced apoptosis and hind-limb ischaemic injury in Mbd2-KO mice (Rao *et al.*, 2011).

Histone acetyl transferase (HAT) p300 plays a role in diabetes and atherosclerosis by up-regulating the response to hyperglycaemia and shear stress, respectively (Chen *et al.*, 2010; Katsume *et al.*, 2011), with effects on eNOS expression (Chen *et al.*, 2008), NF-κB acetylation and endothelial inflammation (Zhang *et al.*, 2011). Likewise, the pan-histone deacetylase (HDAC) inhibitor scriptaid decreases Nox4 expression levels in human endothelial cells to reduce endothelial oxidative stress (Siuda *et al.*, 2012). Hyndman *et al.* propose HDAC1 inhibition as a method to prevent endothelial dysfunction, as HDAC1 up-regulation in primary bovine aortic endothelial cells significantly decreased •NO production through deacetylation of eNOS whereas knockdown of HDAC1 or its inhibition by the pan-HDAC inhibitor trichostatin A (TSA) increased eNOS activity and restored basal •NO production (Hyndman *et al.*, 2014). In contrast, Rössig *et al.* demonstrated that nonselective HDAC inhibition by trichostatin A, sodium butyrate, and the synthetic benzamide derivative entinostat (MS-275) reduced expression of eNOS in HUVECs and impaired •NO-dependent vasorelaxation and angiogenesis (Rossig *et al.*, 2002). HDAC2 indirectly regulates •NO production by suppressing the expression of arginase2 (Arg2), which competes for the eNOS substrate L-arginine (Ryoo *et al.*, 2008; Pandey *et al.*, 2014). In line with that, HDAC2 overexpression restored •NO production in oxLDL-treated human aortic endothelial cells and improved vascular relaxation in mouse aortic rings (Pandey *et al.*, 2014). Thus, therapeutic activation of HDAC2 has great promise as a new method for correcting endothelial dysfunction and atherosclerosis.

Resveratrol is a naturally occurring compound (e.g. in grapes) which acts as an activator of SIRT1, a class III HDAC. Several studies confirm that SIRT1 activity promotes eNOS expression/activity and improves endothelial cell survival and function (Lagouge *et al.*, 2006; Mattagajasingh *et al.*, 2007; Zhang *et al.*, 2008). Clinical trials are underway to examine the potential benefits of resveratrol administration in subjects with cardiovascular disorders (Schleithoff *et al.*, 2012; Xia *et al.*, 2017). However, there are still unsolved safety concerns regarding the chronic consumption of high resveratrol doses (Tome-Carneiro *et al.*, 2013).

Endothelium-specific antioxidant and anti-inflammatory drug-delivery

As outlined above, excessive ROS formation, especially superoxide generation, contributes to endothelial dysfunction, vascular contractility, oedema and a pro-inflammatory phenotype of vascular cells. Antioxidant therapies including N-acetyl cysteine (NAC) and antioxidant enzymes (AOE, i.e. catalase and SOD, quenching H_2O_2 and superoxide $O_2^{•-}$, respectively), in theory, can alleviate this pathological mechanism (Bowler *et al.*, 2001). Diverse approaches have

been designed to achieve specific delivery of antioxidants. For example, conjugation with polyethylene glycol (PEG), PEG-containing carriers or liposomes improve the bioavailability of antioxidants and their ability to detoxify ROS. However, encouraging data from *in vitro* and whole animal studies have not yet translated to clinical practice (Freeman *et al.*, 1985; Barnard *et al.*, 1993) (see also "Antioxidant therapy" section above). Current efforts to prevent oxidative stress have yielded rather mixed outcomes in clinical studies (Bowler *et al.*, 2001). This may be explained by the fact that endothelial ROS are marginally accessible to untargeted antioxidants (Bowler *et al.*, 2001). Most reactive and hence damaging ROS are short-lived species that act at angstrom to nanometre distances (Parthasarathy *et al.*, 2001), increasing the need for precise antioxidant delivery to quench endothelial ROS (Shuvaev *et al.*, 2011b).

Studies *in vitro* (Muzykantov *et al.*, 1987; Sakharov *et al.*, 1987) and *in vivo* established that drugs can be targeted to the endothelium using ligands binding to the angiotensin-converting enzyme (ACE) (Muzykantov *et al.*, 1996), and cell adhesion molecules such as ICAM-1 (Atochina *et al.*, 1998) and PECAM (Hood *et al.*, 2011). Further, by varying the configuration of these ligands in drug delivery systems, drugs may be either retained on the luminal surface or delivered into endosomes (Muro *et al.*, 2006; Simone *et al.*, 2009a). Endothelial ligands have been conjugated with antioxidant enzymes (Dziubla *et al.*, 2008; Shuvaev *et al.*, 2011a), as well as with antioxidant-loaded liposomes (Hood *et al.*, 2012; Howard *et al.*, 2014a), polymeric and non-polymeric nanocarriers (Dziubla *et al.*, 2005; Simone *et al.*, 2007) and magnetic nanocarriers (Hood *et al.*, 2014).

AOE conjugated to antibodies against ACE, ICAM and PECAM (Ab/AOE), but not untargeted IgG/AOE or PEG/AOE, binds to endothelial cells (Shuvaev *et al.*, 2007; 2011b), protecting them from the toxic effects of extracellular H_2O_2 (Sweitzer *et al.*, 2003) and $O_2^{•-}$ (Shuvaev *et al.*, 2007), as well as endothelial dysfunction caused by the accumulation of intracellular ROS (Shuvaev *et al.*, 2007). Ab/AOE but not IgG/AOE or PEG/AOE accumulates in endothelial cells after intravascular injection (Muzykantov *et al.*, 1996; Atochina *et al.*, 1998; Shuvaev *et al.*, 2011b). Ab/catalase targeted to PECAM or ACE injected into donor rats attenuated subsequent ischaemic injury in transplanted lungs (Kozower *et al.*, 2003; Nowak *et al.*, 2010), improved oxygenation and pulmonary microcirculation associated with warm lung ischaemia and transplantation in pigs (Preissler *et al.*, 2011) and alleviated lung ischaemia-reperfusion injury *in situ* in mice (Shuvaev *et al.*, 2009). Ab/SOD normalized vascular constriction induced by angiotensin II in mice (Shuvaev *et al.*, 2009), inhibited endothelial pro-inflammatory activation caused by cytokines and potentiated the anti-inflammatory effects of NO donors (Shuvaev *et al.*, 2011b; Shuvaev *et al.*, 2013). Ab/SOD enters endothelial endosomes and quenches superoxide anion produced in these vesicles by Nox enzymes, thereby inhibiting the NF-κB pathway (Shuvaev *et al.*, 2011b; 2013).

Encapsulation into poly(ethylene glycol)-b-poly(lactic-co-glycolic acid) (PEG-PLGA) carriers protects AOE from proteases (Dziubla *et al.*, 2005; Dziubla *et al.*, 2008), and inhibits lysosomal proteolysis typical of protein conjugates such as Ab/AOE (Muro *et al.*, 2003; Dziubla *et al.*, 2005). Using

PEG-modified AOE and varying the polymeric content allows control of enzyme loading and shape of the nanocarriers (Simone *et al.*, 2007, 2009b,c; Hood *et al.*, 2011). Catalase loaded in PECAM-targeted Ab/PEG-PLGA carriers accumulated in the pulmonary vasculature, conferring sustained antioxidant protection (Dziubla *et al.*, 2008). A phospholipid derivative MJ33 (an indirect inhibitor of NADPH-oxidase) loaded in Ab/liposomes targeted to PECAM accumulated in the endothelial cells, inhibited ROS production and provided more potent protection than non-targeted counterparts against oxidative stress and inflammation in mice (Hood *et al.*, 2012). Ab/liposomes loaded with EUK-134, a superoxide dismutase/catalase mimetic, binds to endothelial cells and alleviates endotoxin-induced lung inflammation in mice (Howard *et al.*, 2014a). PECAM-targeted nanocarriers loaded with tocopherol and a SOD mimetic alleviated endothelial inflammatory activation (Howard *et al.*, 2014b). Magnetic nanoparticles loaded with AOE (Chorny *et al.*, 2010) targeted to PECAM accumulated in the pulmonary endothelium and alleviated oedema and inflammation in a mouse model of endotoxin-induced lung injury (Hood *et al.*, 2014).

The preferential targeting of the pulmonary circulation is based on several factors: the pulmonary vasculature is a privileged target tissue for endothelial delivery, because it contains about 20-25% of the total endothelial surface in the body, receives more than 50% of total cardiac output and is perfused at a relatively slow rate. Due to these factors, agents possessing an affinity for factors expressed on the surface of the endothelium throughout the body (i.e. pan endothelial determinants, such as CD31/PECAM-1), also accumulate in the lungs after systemic intravascular injections, especially via the intravenous routes that favour first pass through the pulmonary circulation (Han *et al.*, 2012).

In summary, endothelial targeting of antioxidants facilitates anti-inflammatory mechanisms based on the interception of endothelial ROS, as shown in Figure 5B. This drug delivery strategy may find utility in the management of acute vascular oxidative stress and inflammation.

Impact of dietary changes

Human dietary preferences are important influences on health in modern societies. An injudicious diet represents one of the leading causes of premature death and chronic disease (Katz and Meller, 2014). On the one hand, a single high-fat meal can transiently impair endothelial function in healthy subjects (Vogel *et al.*, 1997). On the other hand, optimal eating is associated with increased life expectancy and a reduction in lifetime risk of all chronic disease (Katz and Meller, 2014).

The health benefits of dietary patterns such as the Mediterranean, low glycaemic index, moderately low carbohydrate, and vegetarian diets have been widely promoted (Katz and Meller, 2014; Ley *et al.*, 2014). While it is not possible (perhaps also not necessary) to define which diet is best for health, it is nonetheless clear that healthy diet patterns have many common features: limited amounts of refined starches, added sugars, processed foods; reduced intake of certain fats; an emphasis on whole plant foods, with or without lean meats, poultry and seafood (Katz and Meller, 2014). These elements are reflected in the recommendations from major scientific associations (Ley *et al.*, 2014).

The Mediterranean diet, just as one example, reduces the incidence of major cardiovascular events in people with a high cardiovascular risk (Estruch *et al.*, 2013). In a randomized, single-blind trial involving 180 patients (99 men and 81 women) with metabolic syndrome, the Mediterranean diet (for 2 years) improved endothelial function and reduced the prevalence of metabolic syndrome (Esposito *et al.*, 2004). Interestingly, the health benefits of the Mediterranean diet are likely to persist for a long time. The original improvement in endothelial function from an 8-week Mediterranean diet intervention is still evident one year after the initial study (Klonizakis *et al.*, 2014).

The favourable effects of the Mediterranean diet on health are thought to result from a higher ratio of omega-6 and omega-3 fatty acids, higher intake of fibre, antioxidants and polyphenols (Zamora-Ros *et al.*, 2013). Both marine omega-3 fatty acids (Egert and Stehle, 2011) and plant-derived α-linolenic acid (Wang *et al.*, 2012; Egert *et al.*, 2014) improve vascular endothelial function in human subjects. Polyphenols are found in virtually all foods of plant origin (Landberg *et al.*, 2012). In particular, the red wine polyphenol resveratrol has received much attention. Indeed, resveratrol improves endothelial function in laboratory animals as well as in human subjects; at least part of the effect is attributable to stimulation of endothelial $^\bullet$NO production (see also chapter "Epigenetic approaches") (Xia *et al.*, 2014). Despite the relatively poor bioavailability of resveratrol, some beneficial effects can be achieved in humans *in vivo* with dietary doses of resveratrol (i.e. 5 mg day^{-1}) (Cai *et al.*, 2015).

In addition to the above cross-sectional trials, a recent longitudinal cohort study demonstrated that healthy diets attenuate endothelial dysfunction and reduce low-grade inflammation over a 7-year period in adults at risk of cardiovascular disease (van Bussel *et al.*, 2015). Food component analyses indicate that the consumption of more lean fish and raw vegetables, and fewer high-fat dairy products improves endothelial function (van Bussel *et al.*, 2015). A reduction in dietary salt intake (e.g. from 9 g salt day^{-1} to 6 g salt day^{-1}) improves endothelial function in normotensive overweight and obese subjects (Dickinson *et al.*, 2014). In summary, current data suggest that dietary changes positively affect endothelial function.

It is beyond the scope of this review to mention all dietary ingredients that affect endothelial function but it may be useful to mention some of particular interest. Flavanols from cacao have vasculoprotective effects (Balzer *et al.*, 2008; Rassaf *et al.*, 2016) and the polyphenol resveratrol from grapes/red wine markedly improves FMD in multiple clinical studies (Xia *et al.*, 2017). Likewise, nitrate from vegetables improves vascular dysfunction in the elderly (Rammos *et al.*, 2014) and hypercholesterolaemic patients (Velmurugan *et al.*, 2016). Importantly, all of these dietary ingredients improve eNOS function, reduce the burden of oxidative stress or directly generate $^\bullet$NO (see preceding chapters).

Impact of exercise

The landmark study by the Diabetes Prevention Program Research Group in 27 centres (3234 participants) used a randomized, double-blind, placebo-controlled design to compare metformin treatment with lifestyle intervention for the prevention or delay of type 2 diabetes in high risk

patients (Knowler *et al.*, 2002). Participants undergoing intensive lifestyle intervention (healthy diet, increased physical activity) had significantly improved outcomes (~60% reduction in cumulative incidence of diabetes) compared to those managed with metformin (Knowler *et al.*, 2002). A subsequent study in more than 8,000 same-sex twins revealed that leisure-time physical activity provided protection from type 2 diabetes independent of familial and genetic predisposition (Waller *et al.*, 2010). A related study by the same group using a monozygotic co-twin study revealed that leisure-time physical activity increased artery lumen diameters (Leskinen *et al.*, 2011). A more recent meta-analysis examined the effects of exercise (≥ 4 weeks) on FMD using data from 51 clinical trials consisting of 2260 participants with mixed comorbidities (Montero *et al.*, 2014). This meta-analysis confirmed that exercise (aerobic, resistance or combined) significantly improved FMD, with the intensity of aerobic exercise (but not resistance or combined exercise) related to improved endothelial function in a dose-related manner. Of note, physical exercise also improved cardiovascular parameters, restored glutathione and reduced CRP levels in patients with depression and subclinical vascular disease (Al Mheid *et al.*, 2016).

The study by Sinoway et al. was one of the first to report that exercise improves endothelial function (measured by changes in forearm blood flow) in the dominant forearm of tennis players (Sinoway *et al.*, 1986). Our current understanding of mechanisms contributing to the beneficial effects of exercise on endothelial function includes increased phosphorylation of eNOS, up-regulation of superoxide dismutase subtypes, down-regulation of NADPH oxidase subtypes, reduced uncoupling of eNOS, reduced levels of ADMA, increased circulating EPC, reduced endothelial senescence, improved endothelial repair, increased activation of AMP-activated protein kinase (AMPK) and its activator proteins such as SIRT1, serine/threonine kinase 11 (LKB1) and calmodulin kinases (regulators of eNOS) (Ross *et al.*, 2015). Plasma levels of ADMA, which normally increase with age, are reduced by aerobic exercise with simultaneous improvements in carotid arterial compliance in postmenopausal women (Tanahashi *et al.*, 2014). Physical activity below the accepted minimal international recommendations is sufficient to alleviate oxidative stress, reduce ADMA levels and improve small artery endothelial function in overweight/obese men and women (Merino *et al.*, 2015).

Efforts are underway to develop "exercise pills" that mimic the effects of physical exercise ostensibly by activating signalling pathways activated by exercise (Goodyear, 2008; Li and Laher, 2015). The currently described candidate exercise pills are unlikely to directly improve endothelial regulation of vascular tone as these agents do not appear to increase shear stress, a key factor for stimulating endothelial function (Johnson *et al.*, 2011).

Conclusions and clinical implications

The prognostic value of endothelial function measurements for risk stratification of patients for future cardiovascular events is under debate, although it is clear that endothelial dysfunction is a hall-mark of most cardiovascular risk factors and diseases. It is likely that parameters reflecting vascular function more globally (including smooth muscle and perivascular adipose tissue function), such as vascular stiffness index and intima/media thickness ratio, may be better predictors of future cardiovascular events. New diagnostic tools may improve the "imaging" of vascular (endothelial) dysfunction and so herald a new method to regularly predict cardiovascular events. Targeting vascular (endothelial) dysfunction still represents an attractive pharmacological approach due to its central involvement in the progression of most cardio- and cerebrovascular diseases as well as inflammatory processes. In addition to the classical cardiovascular drugs that have beneficial effects on vascular (endothelial) function, such as ACE inhibitors and statins, new therapeutic strategies based on site-specific drug delivery, anti-inflammatory as well as site- and source-specific antioxidant interventions are being developed. Moreover, physical exercise and healthy nutrition represent a main-stay in non-pharmacological cardiovascular therapy. Direct targeting of vascular homeostasis (e.g. •NO/cGMP signalling cascade by •NO donors, PDE inhibitors and other vasodilators) is still not fully explored, but it is emerging that compounds such as sGC activators and stimulators have therapeutic potential. As shown by the REPAIR-AMI study, the intracoronary administration of bone marrow-derived cells (containing EPC) with high migratory capacity increases event-free survival of patients with acute myocardial infarction (Assmus *et al.*, 2014). Finally, epigenetic therapy shows beneficial effects in animal models, but this requires more extensive investigation before it can be transferred to a clinical setting.

Conflict of interest

We certify that there is no conflict of interest with any financial organizations regarding the materials discussed in the manuscript.

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