Assessment of endothelial damage and dysfunction: observations in relation to heart failure

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Introduction

More than 150 years ago, Virchow proposed that abnormalities in blood flow, vessel wall and blood components predispose to thrombosis, constituting what is now known as ‘Virchow’s triad’ for thrombogenesis.1 This rather simplistic view has been continually modified by new discoveries and concepts, as we now know that the process of thrombus formation requires complex interactions involving injury to the vascular endothelium, platelet adherence, aggregation and release, and clotting factor activation, eventually leading to thrombin generation and fibrin formation.2

The endothelium has many vital and diverse (depending on the particular vascular bed) physiological roles, such as regulation of blood vessel tone, permeability, metabolism and haemostasis. Impairment of endothelial function manifests clinically as oedema, hypertension, abnormal vasoconstriction and hypercoagulability. Indeed, it is a widely held view that impaired endothelial function is also the initial step in atherogenesis, which is largely responsible for ischaemic heart disease and thrombotic strokes decades later. Impaired endothelial function is also associated with hypertension, diabetes mellitus and heart failure (regardless of aetiology), although whether as a cause or a consequence is undetermined. Hence, understanding endothelial function is likely to be a key to modifying risk factors of cardiovascular disorders and their sequelae.

Nevertheless, the ideal method(s) of assessing endothelial physiology (and, therefore, pathology) remains uncertain. Various indices have been used to assess endothelial activation, dysfunction and damage: the ideal index would not only be specific to the endothelium but would also be stable and easily measurable—the ‘gold standard’ remains uncertain, as available indices quantify different aspects of endothelial physiology. In addition, words such as damage, injury, dysfunction and activation are currently freely used in the study of endothelial cell biology without a clear definition, or even a consensus, of their meaning. Certainly, a continuum is likely to exist between endothelial activation (e.g. by cytokines), endothelial dysfunction (resulting in thrombogenesis and atherogenesis) and endothelial damage (resulting in overt vascular damage and atherosclerosis).

Normal physiology of the endothelium

The inner lining (intima) of all blood vessels consists of a monolayer of flattened, orthogonal cells referred to as the endothelium, positioned on the internal elastic lamina. We now recognize that the vascular endothelium is not just a cell lining, but plays an active role via various mediators in the equilibrium of haemostasis and fibrinolysis, and regulation of vessel tone and permeability, as well as synthesis of growth factors.2 In that respect, the endothelium can be regarded as an endocrine organ in its own right. Indeed, the endothelium is
estimated to have a mass equivalent to five normal hearts, and area equivalent to half a dozen tennis courts, in an average 70 kg man. Various stimuli will cause the endothelium to secrete or release biologically active molecules such as nitric oxide, endothelin, tissue factor and tissue plasminogen activator (Table 1). The surface of the cells also has metabolically active structures (such as ecto-enzymes and thrombomodulin), as well as an array of adhesion and recognition structures (such as, respectively, vascular cell adhesion molecule (VCAM) and human leukocyte antigen (HLA)). Furthermore, the presence, expression and release of many of these molecules are influenced by metabolic and immunological signals, such as those provided by cytokines and hormones.

The loss of appropriate control of the expression and/or release of these products may result in pathological changes. These changes include leukocyte adherence to and infiltration of the vessel wall (due to increased expression of adhesion molecules, hypertension, peripheral vasoconstriction, and abnormal vascular compliance from imbalance of nitric oxide, endothelin and prostaglandin synthesis) and oedema from loss of correct permeability functioning. Furthermore, thrombosis may arise from the loss of the normally anticoagulant nature of the endothelium. The anticoagulant nature is maintained by synthesis of prostacyclin, nitric oxide and tissue plasminogen activator, as well as protein C activation by the thrombin/thrombomodulin complex. Conversely, the expression of tissue factor and binding sites for factors IX and X makes it more prothrombotic. In heart failure, it is likely that the balance is tipped in favour of the latter, as there is some excess in thromboembolic phenomena.

Endothelial dysfunction and damage can be detected by various methods: a measure of its ability to respond appropriately to simulated increased shear force (i.e. flow-mediated dilatation) or the concentrations of various molecules that it produces may each give an indication of abnormality.

### Plasma markers associated with endothelial damage/dysfunction

**von Willebrand factor**

von Willebrand factor (vWF) is a multimeric glycoprotein that is synthesized exclusively in endothelial cells and megakaryocytes. Indeed, Northern blot experiments have failed to demonstrate messenger RNA (mRNA) for vWF in fibroblasts, HeLa cells (a leukaemia cell line), kidney cells and other tissues.

The human genome has a single vWF gene on chromosome 12, coding for a mRNA molecule of approximately 9000 nucleotides with at least 50 exons, which makes up 0.3% of the total mRNA pool of endothelial cells. It encodes an initial prepropolypeptide of 2813 amino acid residues. It is then cleaved in the endoplasmic reticulum to yield a distinct protein with a molecular mass of 360 kDa designated vWF antigen II, the function of which is unknown. This then is further cleaved to yield a protein with a molecular mass of approximately 260 kDa, which is the mature vWF. vWF and its propolypeptide are stored in intracellular Weibel-Palade bodies, which are specific to endothelial cells; these organelles are enclosed by a unit membrane and are typically 0.1 μm wide and up to 4 μm long.

Each vWF subunit has binding sites for collagen, vitronectin, heparin, glycoprotein Ib (GPIB), glycoprotein IIb/IIIa (GPIIb/IIIa) and factor VIII. Platelets need to be activated before receptors for glycoprotein IIb/IIIa become available for binding. Following that, by virtue of its affinity for glycoprotein Ib and glycoprotein IIb/IIIa, vWF is able to crosslink platelets, allowing a plug to form. Other binding sites for collagen and vitronectin mediate binding to the subendothelium, thus stabilizing the platelet plug at the site of vascular injury. Under normal circumstances, vWF is normally bound to factor VIII, stabilizing the latter in plasma. By serving as a carrier to factor VIII, one could surmise that...
vWF also coordinates formation of the fibrin-rich thrombus that follows.\textsuperscript{2} The secretion of vWF is via constitutive and regulated pathways. In cultured endothelial cells, 95\% of synthesized vWF is secreted constitutively, while the remainder is packaged into Weibel-Palade bodies. The constitutive pathway can be blocked by protein synthesis inhibitors and runs directly from the endoplasmic reticulum via the Golgi apparatus to the extracellular surface.\textsuperscript{7} Constitutively secreted vWF is found in the basement membrane and free in the plasma.\textsuperscript{2} An additional pool is present in the storage granules of platelets (\(\alpha\) granules) and endothelial cells (Weibel-Palade bodies), which can be released in a regulated fashion in response to vascular injury.\textsuperscript{2}

The constitutive form of vWF is predominantly composed of dimers and small multimers, while that from the storage compartment in platelets and endothelial cells is only of high molecular mass. Since the largest multimers are the most active in platelet adhesion assays, it seems reasonable that it is this pool of protein that is actively released at the time of vascular injury.\textsuperscript{7} In vivo, the release of vWF from storage pools in the vascular endothelium is stimulated by the administration of adrenaline, vasopressin and nicotinic acid as well as interleukin-1 and TNF, resulting in elevated levels of plasma vWF. In experimental circumstances, vWF is released from storage granules by thrombin, fibrin, histamine and complement proteins C5a-9; indeed, thrombin and fibrin are found at sites of vascular injury or damage, while histamine release and complement activation occur at sites of inflammation or injury. As a result of these stimuli, vWF is released from endothelial storage granules, resulting in a rapid response to vascular injury and endothelial damage.\textsuperscript{2,8}

The notion that plasma vWF originates from platelets as well as endothelial cells is controversial. It is known that platelets contain vWF mRNA and that vWF is a constituent of the platelet \(\alpha\) granule.\textsuperscript{9} However, plasma vWF levels do not correlate with established platelet markers such as beta thromboglobulin.\textsuperscript{8} Aspirin, which is an inhibitor of platelet activity, reduces beta thromboglobulin but has no effect on vWF levels.\textsuperscript{10} It is also said that platelet vWF tends to remain bound to the platelet surface after release from \(\alpha\) granules\textsuperscript{2} and that this does not exchange with the plasma pool.\textsuperscript{7} Transplantation of von Willebrand disease bone marrow into a haemostatically normal human recipient results in normal plasma vWF levels, but low levels of platelet vWF from the donor megakaryocytes.\textsuperscript{11}

If all of the above were true, then one could conclude that most, if not all, circulating plasma vWF is derived from the endothelium.\textsuperscript{12} If so, plasma levels of vWF should reflect endothelial function, and abnormal levels would indicate endothelial dysfunction and damage. The one caveat is that vWF is also known to be an acute phase reactant affected by inflammatory cytokines,\textsuperscript{13,14} and as such, may be elevated even in the absence of definite endothelial damage.\textsuperscript{15} Furthermore, no consistent correlation between vWF and other endothelial markers (discussed further below) has been shown.

However, vWF levels are also influenced by non-pathological conditions. For example, vWF increases with exercise and in pregnancy.\textsuperscript{16–19} Its production is also stimulated by oestrogen, although levels do not seem to fluctuate in women, in either the luteal or follicular phases of the menstrual cycle. Increased levels are also associated with drugs such as adrenaline (epinephrine), vasopressin and cyclosporin. Perhaps most intriguingly, mean vWF levels in patients with blood group O are lower than in non-O patients, in keeping with the reported excess of non-O people having myocardial infarctions, chronic heart disease and other forms of atherosclerosis.\textsuperscript{7} There is also evidence that haemophiliacs experience less ischaemic heart disease than expected. Bearing in mind that there are ethnic differences in the distribution of ABO blood grouping, it is conceivable that different vWF levels could partly be responsible for the differences in incidence of those diseases. However, the Northwick Park Heart Study found no evidence that the association between vWF with ischaemic heart disease is determined by ABO group.\textsuperscript{20} In that study, the effect of ABO blood group on ischaemic heart disease was independent of vWF.

High levels of vWF are a (poor) prognostic indicator for myocardial infarction, re-infarction and mortality.\textsuperscript{20–23} vWF is also a prognostic indicator of other cardiovascular events such as stroke and the requirement for arterial surgery in patients with hypertension, intermittent claudication, angina and ischaemic heart disease.\textsuperscript{24} In addition, high vWF predicts the development of thrombembolic events and poor prognosis in patients with rheumatoid arthritis and systemic sclerosis.\textsuperscript{25–30}

In unstable coronary artery disease, an early increase of vWF in 48 h is an independent predictor of adverse clinical outcome at 14 and 30 days.\textsuperscript{31} In a substudy of the ESSENCE (Efficacy and Safety of Subcutaneous Enoxaparin in Non Q Wave Coronary Events) trial, patients who were allocated to enoxaparin compared to unfractionated heparin had a smaller increase in vWF over 48 h, and this was associated with a lower composite end-point of
death, myocardial infarction, recurrent angina or revascularization.

In a further study of four different anticoagulant treatments in unstable coronary artery disease, enoxaparin, dalteparin, unfractionated heparin and PEG-hirudin (a direct thrombin inhibitor), the increase of vWF over 48 h was not observed in patients receiving enoxaparin or PEG-hirudin compared with the other two groups, and it was these two groups of patients who eventually had lower clinical events within 30 days of follow-up. Even within each treatment group, the mean change in vWF was always higher in patients with an event compared to those free of events at one month follow-up. Similar prognostic information is, however, lacking in CHF, although the introduction of ACE inhibitor therapy (but not beta blockers) has been shown to reduce vWF in patients with chronic heart failure in sinus rhythm.

In the setting of left ventricular dysfunction, levels of vWF have been shown to be abnormal, with the highest level associated with left ventricular aneurysms. Levels of vWF are also positively correlated with New York Heart Association class in chronic heart failure. This could be explained in two ways. Firstly, patients with the highest vWF levels may be at highest cardiovascular risk, resulting in the largest myocardial infarctions or recurrent infarctions, thus resulting in the most cardiac damage and subsequently, aneurysm formation. Alternatively, these patients may have the greatest endothelial dysfunction, leading to greater intravascular thrombogenesis.

**Soluble thrombomodulin (sTM)**

As previously mentioned, the endothelium is usually in a resting state and constitutes an anticoagulant surface. Under these conditions, the endothelium synthesizes thrombomodulin and secretes prostacyclin (PGI₂), nitric oxide (NO) and tissue type plasminogen activator (t-PA). With endothelial damage, the endothelium becomes activated and provides pro-coagulant activities at the surface, expressing tissue factor, adhesive molecules and binding sites for factors IX and X, and increasing secretion of plasminogen activator inhibitor (PAI-1).

Thrombomodulin is a transmembrane proteoglycan with a molecular mass of 75 kDa, located on the vascular and lymphatic endothelium surfaces, that functions as an anticoagulant. It has a high affinity for thrombin, forming a 1:1 thrombin-thrombomodulin complex that inhibits fibrin formation, platelet activation, and protein S inactivation by thrombin. The complex also activates protein C, which will inactivate factors Va and VIIIa of the intrinsic pathway. Moreover, formation of this complex directly inhibits the capacity of thrombin to clot fibrinogen and activate platelets.

Thrombomodulin has also been isolated in small amounts from human platelets where there are about 60 molecules per platelet compared with 50 000 to 100 000 per endothelial cell, and in a non-functional form, from neutrophils. In addition, thrombomodulin has been isolated in smooth muscle, keratinocytes, epithelial cells and syncytiotrophoblast of placenta.

Besides the transmembrane form, thrombomodulin also exists in a soluble form in the plasma. Indeed, the soluble forms may be a product of the cleaved transmembrane glycoprotein. Six soluble fragments of membrane thrombomodulin have been isolated, with various molecular masses ranging from 28 to 105 kDa, whilst seven have been isolated from urine. The levels of these soluble forms are influenced by liver and renal function.

In cultured cells, up-regulation of thrombomodulin is induced by cAMP analogues, and down-regulation by interleukin-1 (IL-1), tumour necrosis factor (TNF), lipopolysaccharide (LPS) and hypoxia. TNF-α in vitro leads to internalization and lysosomal degradation of thrombomodulin, and inhibition of thrombomodulin transcription and translation. The level of sTM in the supernatant when cultured endothelial cells are incubated with IL-1 or TNF-α is independent of IL-1 and TNF-α concentrations, although sTM increases in vivo with various diseases with elevated systemic or local levels of inflammatory cytokines, including TNF-α, which is somewhat contradictory.

Levels of sTM are elevated in diabetes mellitus and atheromatous arterial disease, and are higher with increased vascular complications. Some argue that it is a marker of microvascular rather than macrovascular complications, as its levels are not affected by the presence of peripheral vascular disease in diabetics. Levels of sTM may also be altered by treatment with ACE inhibitors, which reduces albuminuria in diabetics, as well as preventing nephropathy, independently of blood pressure control. However, in the ARIC (Atherosclerosis Risk in Communities) study, low sTM was a predictor of future ischaemic heart disease at 6 years follow-up. In earlier studies, sTM was unrelated to conventional cardiovascular risk factors such as blood pressure, lipids or even electrocardiographic evidence of ischaemic heart disease. However, there are also reports of elevated sTM in the presence of peripheral vascular disease and coronary artery disease.

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elevated in the mere presence of cardiovascular risk factors and only becomes elevated in established significant atheromatous vascular disease.

One could argue that a low (subnormal) level of sTM in the plasma might reflect a dysfunctional endothelium resulting in a hypercoagulable state, making one susceptible to coronary artery thrombosis. However, in the presence of established disease process, elevated levels of sTM indicate endothelial injury. At present we are not aware of any published data on thrombomodulin in CHF.

It has been postulated that in vitro, sTM is released from endothelial cells following cell membrane injury, with the release of thrombomodulin directly related to duration and dose of hydrogen peroxide treatment. When endothelial cells are incubated either with TNF-α or neutrophils alone, no rise in sTM is seen; moreover, no morphological cell changes are visible on microscopy. In contrast, when endothelial cells are incubated with both TNF-α and neutrophils together, morphological cell changes are associated with elevated sTM. This suggests that sTM may be a marker of endothelial cell membrane injury  rather than endothelial cell activation. Another situation where vascular injury is present is in vasculitis, and sTM levels are significantly elevated in various vasculitides in their active phase, such as Wegener’s granulomatosis, polyarteritis nodosa, giant cell arteritis, Behçet’s disease and Takayasu’s arteritis (although the data on the last are less consistent).

Severe CHF is associated with a catabolic state, giving rise to the term ‘cardiac cachexia’. This is thought to be not a consequence of inadequate nutritional intake, but due to TNF-α. One might therefore expect severe CHF to be associated with elevated sTM as a marker of endothelial cell damage. In addition, the fact that TNF-α leads to a reduction in thrombomodulin expression by endothelial cells, as well as internalization and lysosomal degradation, might tip the overall balance in favour of thrombogenesis.

As mentioned above, TNF-α leads to a reduction in thrombomodulin, and consequently is pro-thrombotic. In theory at least, antagonizing TNF-α may therefore be beneficial. However, two trials, the RECOVER (Research into Etanercept: Cytokine Antagonism in Ventricular function) and RENAISSANCE (Randomized Etanercept North American Strategy to Study Antagonism of Cytokine) investigating the use of etanercept, a recombinant chimeric soluble TNF receptor type 2 in CHF, had to be discontinued prematurely due to a lack of benefit.

While TNF-α leads to a reduction of thrombomodulin and hence blocking its action may seem beneficial, TNF-α also induces iNOS, and the effect of this is uncertain. More work is clearly needed to ascertain the potential in targeting thrombomodulin as a therapeutic measure. Clearly, a more specific agent would be desirable.

E-selectin

E-selectin (CD62E) is a cell-surface-bound leukocyte adhesion molecule specific to endothelial cells. It mediates the interaction between leukocytes, platelets, and the endothelium. Increased surface expression of E-selectin is probably a reflection of endothelial activation rather than damage. It is not expressed by normal resting endothelial cells.

The soluble form of E-selectin can be detected in healthy controls, and is raised in patients with cancer, haematological disorders (myelodysplastic syndromes and thalassaemia), ischaemic heart disease, atherosclerosis, hypertension, diabetes and septic shock. It is however unclear how, or under which conditions, it is actively or passively shed from the cell membrane, or cleaved by a pathological process.

In vitro experiments suggest that soluble E-selectin may have a regulatory role in leukocyte interactions with the cell surface forms of the molecules. Soluble E-selectin may also be induced in vitro by inflammatory cytokines, such as IL-1 and TNF-α, suggesting a role in acute and/or chronic inflammation. Levels of soluble E-selectin and vWF also do not correlate with each other, in various conditions such as ischaemic heart disease, hypertension and hyperlipidaemia. Although both soluble E-selectin and vWF are elevated in hypertension, controlling blood pressure reduces vWF but not E-selectin. In addition, vWF, but not E-selectin, is elevated in hypercholesterolaemia. All three markers, vWF, E-selectin and sTM) are increased in ischaemic heart disease.

E-selectin is also elevated in CHF, regardless of aetiology, and its level normalizes following cardiac transplantation. This suggests that elevated E-selectin is a consequence of, rather than a precursor to, CHF. However, as E-selectin is also a marker of inflammation, this could be confounded by the use of immunosuppressive drugs following cardiac transplantation.

Although in earlier studies E-selectin did not predict outcome, in a recent report by Blakenberg et al., E-selectin was significantly related to future death from cardiovascular causes among patients with coronary artery disease. One suggested explanation was that as E-selectin is an leukocyte
adhesion molecule, some may be bound to its ligand in vivo, and be unavailable for measurement.85

**Nitric oxide (‘endothelial-derived relaxing factor’)**

It is more than 20 years since Furchgott and Zawadzki73 showed that the endothelium was essential for acetylcholine to induce relaxation in isolated rabbit aorta. This effect was not observed if the endothelium was removed; however, the aorta still dilated in response to glyceryl trinitrate. This led to the conclusion that a substance must exist which is derived from the endothelial cells that mediated the effect, hence its initial name, endothelial-derived relaxing factor (EDRF), before it was subsequently identified as nitric oxide (NO).

NO contributes to the control of basal and stimulated regional blood flow in man. Intra-arterial infusion of N-monomethyl-L-arginine (L-NMMA), a specific NO inhibitor, into arteries of healthy controls results in a significant fall in basal blood flow, and attenuates the dilator response to infused acetylcholine.74 The ability of blood vessels to vasodilate in response to increased shear force (i.e. the force exerted on the blood vessel wall as a result of laminar blood flow) also requires an intact endothelium. This was demonstrated in an earlier experiment on femoral arteries of dogs,75 in which dilatation of the vessels in response to local acetylcholine infusion and augmentation of femoral arterial flow (either by peripheral vasodilatation or arteriovenous shunt) was abolished by mechanical removal of the endothelial cells. This however, did not affect dilatation in response to norepinephrine and nitroglycerin. The same result was seen with hydrogen peroxide treatment of the arteries, which results in alteration of cellular function without signs of cellular decomposition.75

NO is a highly unstable molecule with a half-life of <6 s in vivo,76 being rapidly oxidized to nitrite, and subsequently nitrate. It is synthesized from L-arginine by NOS, and we now recognize that cardiac myocytes express two types of NO synthases, endothelial NO synthase (eNOS) and inducible NO synthase (iNOS). The production of NO is stimulated by shear stress via the eNOS, and by inflammatory cytokines such as TNF-α via the pro-inflammatory iNOS.77 TNF-α downregulates eNOS expression78 while at the same time inducing iNOS.

One of the hallmarks of advanced chronic CHF is systemic vasoconstriction.79 In one study, brachial artery diameter was progressively lower in patients with congestive cardiac failure compared to controls with increasing severity of cardiac failure.80

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<td><strong>Beneficial effects in heart failure</strong></td>
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<td>Vasodilator, therefore, reduces afterload and preload</td>
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<td>Inhibits platelet aggregation</td>
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<td><strong>Detrimental effect in heart failure</strong></td>
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<td>Negative inotrope</td>
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<td>Potentially lethal to cardiac myocytes (however, shown to improve survival in combination with hydralazine)</td>
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This is associated with reduced arterial compliance, resulting in increased pulse wave velocity of reflected pressure waves from the peripheral circulation, leading ultimately to increased left ventricular end-systolic stress80 and progressive heart failure.

While the release of NO on stimulation (via eNOS) is reduced in patients with CHF, the basal release of nitric oxide may actually be enhanced via iNOS, and play an important compensatory role by antagonizing neurohumoral vasoconstrictor forces in CHF—thus NO may have both beneficial and detrimental effects in CHF (Table 2). This was demonstrated by experiments in which the decrease in blood flow induced by L-NMMA, was exaggerated in patients with CHF.79 Furthermore, plasma nitrate, the stable end-product of nitric oxide production, was significantly increased in patients with CHF.81 Although patients who were receiving nitrate-containing medication had a higher plasma nitrate level as expected, it was not significant. Exclusion of these patients did not affect the highly significant difference in plasma nitrate between CHF patients and controls.81

In patients with CHF, increased production of NO may be counterproductive. There is some evidence that high levels of NO reduce myocardial contractility and induce myocyte injury.77,82 Experimental evidence suggests that NO depresses myocardial energy generation via an effect on mitochondria.83 On the other hand, others have shown that cardiac-specific overexpression of iNOS in rat models does not result in cardiac dysfunction.84 NO also inhibits platelet aggregation in vivo via an effect on cGMP and therefore plays a role in maintaining the antithrombotic property of the endothelium. Nevertheless, it remains to be seen if selective inhibition of iNOS can affect the prognosis of CHF. This seems to be at odds with the finding that the combination of hydralazine and nitrate reduces mortality in CHF, although not to the same extent as ACE inhibition. There must surely be a balance, as complete inhibition of iNOS will result in vasoconstriction, as shown in experiments using L-NMMA, which is detrimental in CHF.
Simultaneously, the inhibitory effect on platelet activation would also be lost.

In summary, basal production of NO is increased in CHF due to stimulation of iNOS. Experiments suggest that NO, while countering the systemic vasoconstriction in CHF, is potentially lethal to myocytes in high concentrations, in addition to being a negative inotrope. It remains to be seen whether selective inhibition of iNOS and stimulation of eNOS would affect prognosis favourably, or whether our current practice of prescribing nitrates that serve as NO donors could turn out to be harmful.

**Endothelin**

Endothelin, discovered in 1988, is an endogenous 21-amino-acid peptide and a powerful vasoconstrictor produced not only by vascular endothelial cells but also by other cell types, including adrenal cortex, myocardium, vascular smooth muscle cells, renal tubular epithelial cells, glomerular mesangial cells, glial cells, macrophages, mast cells and pituitary cells. Using electron microscopy and immunoreactivity, the isoform ET-1 has been localized to endothelial cytoplasm, rather than specific organelles.

Endothelin has many roles, including regulation of cellular proliferation and apoptosis, activation of monocytes and cellular matrix production. Indeed, various studies have shown that ET-1 level is an excellent prognostic marker in CHF, leading to the current interest in endothelin antagonists in the management of heart failure. While it has shown some promise in that it affects the haemodynamics favourably, it is not known whether these agents can reduce mortality in CHF.

Endothelin is synthesized as an approximately 200-amino acid pre-pro-hormone. Post-translational cleavage yields a 38–39 amino-acid pro-endothelin, which undergoes further cleavage to yield the final 21 amino-acid product. Four isoforms of endothelin have been identified to date, and designated ET-1, ET-2, ET-3 and ET-4, alongside two receptors for endothelin, termed ET$_A$ and ET$_B$, which are expressed on endothelial cells, vascular smooth muscle cells, cardiac myocytes and fibroblasts. Nevertheless, it is unclear which cell type(s) may be responsible for the increased expression of endothelin and endothelin receptors in the failing myocardium. However, it is widely believed that the isoform ET-1 is primarily produced by endothelial cells abluminally and acts on the underlying smooth muscle cells.

Endothelin has positive inotropic, and positive chronotropic, mitogenic and pro-inflammatory properties, as well as its vasoconstrictor effects. Its release is stimulated by many factors, such as shear stress, hypoxia, epinephrine, angiotensin II, cortisol, thrombin, pro-inflammatory cytokines (TNF-$\alpha$, IL-1 and IL-2) and transforming growth factor $\beta$. The different actions of ET-1 are mediated through the two receptor subtypes and their locations. ET-1 acting on ET$_A$ receptors on vascular smooth muscle cells results in vasoconstriction and proliferation of smooth muscle cells. On the other hand, ET-1 acting on endothelial ET$_B$ receptors causes vasodilation via release of NO and prostacyclin, but on vascular smooth muscle ET$_B$ receptors causes vasoconstriction.

Increased levels of plasma ET-1 have been observed in systemic hypertension, type 2 diabetes mellitus, dyslipidaemia, angina, cardiogenic shock, myocardial infarction, Raynaud’s phenomenon, cerebral vasospasm, atherosclerosis and heart failure. It has been suggested that elevated endothelin levels may reflect endothelial dysfunction and damage. Using vWF as a marker of endothelial dysfunction, ET-1 levels have been observed to be increased along with vWF in patients with type 2 diabetes mellitus and dyslipidaemia; synthesis of vWF and ET-1 are also increased following exposure of cultured endothelial cells exposed to tri-iodothyronine. ET-1 and vWF levels are also directly correlated in both congestive heart failure and after heart transplantation in idiopathic dilated cardiomyopathy.

However, in contrast to flow-mediated dilatation (see discussion below) which improves following heart transplantation for end-stage heart failure, there is evidence that ET-1 levels increase further, perhaps as a consequence of immunosuppressive therapy. There is also experimental evidence that ET-1 inhibits synthesis of NO in smooth muscle cells via ET$_A$ receptors. As ET-1 levels are elevated in CHF, this observation is entirely consistent with a concomitant decrease in NO.

**Flow-mediated dilatation/endothelium-dependent dilatation**

Flow-mediated dilatation (FMD) is now increasingly used as a research tool for the assessment of endothelial function, as it has been shown to be accurate and reproducible. By FMD, we mean the high-frequency ultrasound assessment of arterial...
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Glyceryl trinitrate. Reactive hyperaemia results in increased shear force (i.e. the force exerted by laminar blood flow on the vessel wall), which is already known to stimulate NO release. Sublingual glyceryl trinitrate, on the other hand, causes endothelial-independent vasodilation.

Others have modified and refined the method of ultrasound assessment of FMD. For example, the use of brachial artery vs. radial artery, and the location of the blood pressure cuff on the upper arm or forearm, has been studied by Agewall et al. They found that whilst FMD of the brachial artery was significantly higher after upper-arm occlusion compared to forearm occlusion, it seemed likely that local ischaemia played a part. They also found that FMD (measured as percentage change in diameter) of the radial artery was greater than that observed with forearm occlusion, despite a similar peak flow stimulus. Similarly, Doshi et al. assessed FMD before and during intra-arterial infusion of the NO synthase (NOS) inhibitor L-NMMA, and again found that dilatation following upper-arm occlusion was far greater than that observed with forearm occlusion, despite a similar peak flow stimulus. By using an infusion of L-NMMA, they concluded that FMD of the brachial artery following forearm, but not upper-arm occlusion, was mediated exclusively by NO. Indeed, following upper-arm occlusion, dilatation of the brachial artery was only partially attenuated by L-NMMA, suggesting that some other mechanism besides NO was at work, most probably local tissue ischaemia.

FMD has also been shown to be dependent upon the anatomical vessel size but independent of body mass index. The effect of age on FMD is unclear, as some studies have shown a correlation, whereas others have not. Reduced FMD has been demonstrated in children with familial hypercholesterolaemia and adult smokers at risk of atherosclerosis or with established coronary artery disease. Others have demonstrated impaired FMD in patients with hypertension, and shown it to be a marker of future cardiovascular events in patients with essential hypertension. Moreover, endothelium-dependent vasodilation by plethysmography has also been shown to be impaired in type 2 diabetics, as well as in human and animal models of congestive heart failure. This could perhaps be explained by the fact that stimulated release of NO is impaired, an end result of endothelial dysfunction.

Another study of 17 patients with idiopathic dilated cardiomyopathy found a positive correlation between serum TNF-α levels with forearm blood flow measured by plethysmography in response to acetylcholine and nitroglycerin. Although the number of patients studied was small, the authors concluded that the increase in TNF-α resulted in activation of the inducible form of NOS, which potentiated the vascular effects resulting from either stimulation of the constitutive form of NOS by acetylcholine or direct release of NO by nitroglycerin. This somewhat contradictory result suggests that reduced endothelium-dependent vasodilatation in heart failure may be a reflection of the underlying risk factors, rather than ventricular function or its sequelae. However, Kubo et al. reported no difference in the reduction of endothelium-dependent vasodilatation in patients with heart failure due to ischaemic heart disease or idiopathic dilated cardiomyopathy. Fichtlscherer et al. recently showed that using etanercept (a soluble TNF-α receptor which binds to and renders TNF-α ineffectual) improved endothelium-dependent and endothelium-independent forearm blood flow, as measured by venous occlusion plethysmography, providing further evidence that TNF-α seems to be linked to impaired endothelial function.

Long-term therapy with angiotensin converting enzyme (ACE) inhibitors improves FMD in congestive heart failure (CHF). That impaired endothelium-dependent vasodilatation is a consequence as well as a precursor of CHF, is supported by the fact that plethysmography shows that it improves following cardiac transplantation. While FMD is impaired in CHF whatever the aetiology, it is reversed by cardiac transplantation only in patients with antecedent non-ischaemic cardiomyopathy.

It may be that the predisposing cardiovascular risk factors in ischaemic cardiomyopathy such as hypertension, diabetes and hypercholesterolaemia, which remain post-transplantation, account for the impaired FMD.
The proposed mechanism by which ACE inhibitors improve FMD is interesting. ACE itself is virtually identical to kininase II, which degrades bradykinin in vivo. By inhibiting this enzyme, ACE inhibitors increase the availability of bradykinin. In experiments using icatibant, a bradykinin receptor antagonist, ACE inhibitors improved FMD via a bradykinin pathway. Indeed, bradykinin is a potent vasodilator that causes the release of NO, prostacyclin and endothelium-derived hyperpolarizing factor.\textsuperscript{140–142}

Aspirin could thus be expected to reduce prostacyclin by inhibiting the cyclo-oxygenase pathway, and thereby attenuate FMD. However, in hypertension, aspirin has been shown to increase FMD.\textsuperscript{143} In a rat model of CHF, Varin et al. also showed that diclofenac increased FMD in rats untreated with ACE inhibitors, and that this effect was lost when treated with ACE inhibitors, suggesting that the balance in untreated CHF was tipped towards an excess of vasoconstrictor prostanoids.\textsuperscript{130} In various studies involving the use of ACE inhibitors, however, including heart failure trials\textsuperscript{144} and the recent substudy of HOPE, investigating the effect of ramipril on secondary prevention of stroke, the survival benefit conferred by ACE inhibitors was attenuated in patients receiving aspirin. It therefore remains to be seen what effect aspirin has on FMD in other disease states, and if this is consistent with the observation that aspirin reduces the benefits conferred by ACE inhibitors. So far, the effect of NSAIDs on FMD seems inconsistent.

In a study of eNOS knockout mice, Sun et al. showed that endothelium-dependent dilatation may also be due to endothelium-derived prostanoids in the absence of NO production by the endothelium as it is completely abolished by indomethacin. Compared to normal mice, endothelium-dependent dilatation was only reduced by 49\% by indomethacin. In their animal model, endothelium-dependent dilatation was close to normal, even in the absence of NO production by the endothelium as it is completely abolished by indomethacin. In their animal model, endothelium-dependent dilatation was only reduced by 49\% by indomethacin. The method requires specialized and expensive equipment, as well as highly trained technicians, to produce valid, reproducible data.\textsuperscript{88,126} Its attraction is that it is non-invasive and allows repeated measurements\textsuperscript{126} Although the technique appears deceptively simple, there are many pitfalls. As numerous factors affect flow-mediated vascular reactivity, including temperature, food, drugs and sympathetic stimuli, subjects should be studied in a quiet, temperature-controlled room, as well as being fasted for at least 8–12 h. Abstinence from any drug that can affect vascular reactivity, or even caffeine and cigarette smoking, should be observed whenever possible.\textsuperscript{126} Whilst an improvement in FMD is associated with improved prognosis, it is still unclear if the relationship is causal. In theory at least, FMD may have a role in assessing patient response to drug therapy or risk factor modification.\textsuperscript{126}

### Circulating endothelial cells

Perhaps the best proof of endothelial damage would be to observe desquamated, but not apoptotic endothelial cells in circulating blood. A method to capture these cells has been developed, and used to prove that endothelial injury occurs in acute myocardial infarction and unstable angina (but not stable angina), confirming a separate pathogenic mechanism.\textsuperscript{146} Mutin et al. used an immunomagnetic separation assay based on S-Endo 1 monoclonal antibody directed against the endothelial antigen CD146 to capture circulating endothelial cells in myocardial infarction and unstable angina. Captured cells were then counted and analysed further for DNA strand breaks as evidence of apoptosis. These investigators concluded that apoptosis could not have accounted for desquamation of the endothelial cells, as nuclear DNA fragmentation was observed in less than 10\% of cells, and may in fact reflect apoptotic changes occurring after cell detachment.

They also reported that circulating endothelial cells were not present in blood obtained from healthy controls and patients with stable angina, but were present in the blood of patients with myocardial infarction and unstable angina.\textsuperscript{146} This is consistent with the currently held theory that the pathogenesis of myocardial infarction and unstable angina involves atheromatous plaque rupture as the initial event, whereas a ‘fixed’ stenosis results in stable angina. The endothelial cells, however, did not express markers of endothelial activation, namely, intercellular adhesion molecule 1 (ICAM-1), vascular-cell adhesion molecule 1 (VCAM-1), and E-selectin. In contrast, similar methods for capturing circulating endothelial cells in sickle-cell disease, albeit using different endothelial-specific monoclonal antibody, produced cells which expressed these markers.\textsuperscript{147,148} Thus, expression of
these markers may signify a ‘pro-adhesive’ and ‘pro-coagulant’ endothelium.\(^\text{147}\)

The presence of circulating endothelial cells is therefore direct evidence of endothelial injury. However, the fact that endothelial cells express different markers in different disease states may reflect the different mechanisms of endothelial injury. In myocardial infarction and unstable angina, the pathogenesis of endothelial injury may be related to mechanical disruption of the endothelial layer, whereas in sickle cell anaemia, it may be the result of dysfunction. There are no published data on circulating endothelial cells in CHF, but such measurements may prove useful in the assessment of endothelial function.

**Conclusion**

The assessment of endothelial function is essential in cardiovascular disease. Modulation of endothelial function would have implications for the thrombus-related complications (including myocardial infarction, stroke and thromboembolism) that commonly occur in heart failure. As Table 3 shows, many endothelial markers are not mere bystanders in the disease process. Apart from FMD, which is an indirect measure of NO production, vWF, sTM and NO are all components involved in the complex process of thrombogenesis. Furthermore, the immune system may be more closely linked to the endothelial function than previously thought.

Not only is endothelial dysfunction thought to be the initial step in atherosclerosis, it may also play a role in the propagation of the disease process in conditions such as CHF. Whilst endothelial function has variously been described as activation, dysfunction, injury and damage, no one has precisely distinguished the aetiology of CHF relative to these changes. In ischaemic CHF, one assumes that atherosclerosis is the aetiology and hence endothelial dysfunction precedes it. In idiopathic dilated cardiomyopathy however, although endothelial dysfunction is associated with the condition, it has not been established whether it actually precedes it.

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**References**


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**Table 3**  Endothelial markers and their significance

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<tr>
<th>Marker</th>
<th>Endothelial activation</th>
<th>Endothelial dysfunction</th>
<th>Endothelial damage/injury</th>
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<td>vWF</td>
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<tr>
<td>sTM</td>
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<td>No</td>
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<tr>
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<td>No (Prognostic in hypertension)</td>
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