Q J Med 2003; **96**:253–267
doi:10.1093/qjmed/hcg037

Review

QJM

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

A.-Y. CHONG, A.D. BLANN and G.Y.H. LIP

From the Haemostasis Thrombosis and Vascular Biology Unit, University Department of Medicine, City Hospital, Birmingham, UK

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

Address correspondence to Professor GYH Lip, Haemostasis Thrombosis and Vascular Biology Unit, University Department of Medicine, City Hospital, Birmingham B18 7QH. e-mail: g.y.h.lip@bham.ac.uk

Table 1 Normal physiology of the endothelium

Anticoagulant
Prostacyclin
Nitric oxide
Thrombomodulin
Tissue plasminogen activator
Procoagulant
Tissue factor
Binding sites for factors IX and X
Plasminogen activator inhibitor-1
Vasodilator
Nitric oxide
Prostacyclin
Endothelin (acting on endothelial ET_B receptors)
Vasoconstrictor
Endothelin (acting on vascular smooth muscle ET_B receptors; also expressed in adrenal cortex, myocardium, vascular smooth muscle cells, renal tubular epithelial cells, glomerular mesangial cells, glial cells, macrophages, mast cells and pituitary cells[87,88])
Thromboxane A_2
Prostaglandin H_2

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.[3]

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.[4,5] 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.[2] 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.[6]

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.[7]

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.[2] Other binding sites for collagen and vitronectin mediate binding to the subendothelium,[8] 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.[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.[7] Constitutively secreted vWF is found in the basement membrane and free in the plasma.[2] An additional pool is present in the storage granules of platelets (α granules) and endothelial cells (Weibel-Palade bodies), which can be released in a regulated fashion in response to vascular injury.[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.[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.[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 α granule.[9] However, plasma vWF levels do not correlate with established platelet markers such as beta thromboglobulin.[8] Aspirin, which is an inhibitor of platelet activity, reduces beta thromboglobulin but has no effect on vWF levels.[10] It is also said that platelet vWF tends to remain bound to the platelet surface after release from α granules[2] and that this does not exchange with the plasma pool.[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.[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.[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,[13,14] and as such, may be elevated even in the absence of definite endothelial damage.[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.[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.[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.[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.[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.[24] In addition, high vWF predicts the development of thromboembolic events and poor prognosis in patients with rheumatoid arthritis and systemic sclerosis.[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.[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.[31] 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.[32] 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.[33]

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.[2,34] Levels of vWF are also positively correlated with New York Heart Association class in chronic heart failure.[33] 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 (PGI_2), 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).[35]

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.[35–37] 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,[36] and in a non-functional form, from neutrophils. In addition, thrombomodulin has been isolated in smooth muscle, keratinocytes, epithelial cells and syncytiotrophoblast of placenta.[8]

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.[12] 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.[8,35,38]

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.[35] TNF-α *in vitro* leads to internalization and lysosomal degradation of thrombomodulin,[39] and inhibition of thrombomodulin transcription and translation.[40] 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, [13] although sTM increases *in vivo* with various diseases with elevated systemic or local levels of inflammatory cytokines, including TNF-α,[41] which is somewhat contradictory.

Levels of sTM are elevated in diabetes mellitus and atheromatous arterial disease, and are higher with increased vascular complications.[42,43] 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.[44] Levels of sTM may also be altered by treatment with ACE inhibitors,[45] 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.[46] In earlier studies, sTM was unrelated to conventional cardiovascular risk factors such as blood pressure, lipids or even electrocardiographic evidence of ischaemic heart disease.[47,48] However, there are also reports of elevated sTM in the presence of peripheral vascular disease and coronary artery disease.[49–51] It may be that sTM is not

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[41] 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).[52–54]

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.[55] 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 prothrombotic. 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.[56,57]

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[58] rather than damage. It is not expressed by normal resting endothelial cells.[12,59,60]

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.[12,15,59,61–68] 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.[12]

In vitro experiments suggest that soluble E-selectin may have a regulatory role in leukocyte interactions with the cell surface forms of the molecules.[65,69] 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.[60] 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.[65,68,70] Although both soluble E-selectin and vWF are elevated in hypertension, controlling blood pressure reduces vWF but not E-selectin.[66] In addition, vWF, but not E-selectin, is elevated in hypercholestrolaemia.[68] All three markers, (vWF, E-selectin and sTM) are increased in ischaemic heart disease.[15,24]

E-selectin is also elevated in CHF, regardless of aetiology, and its level normalizes following cardiac transplantation.[71] 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,[15] 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.[72] 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.[65]

Nitric oxide ('endothelial-derived relaxing factor')

It is more than 20 years since Furchgott and Zawadzki[73] 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 NOS 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 expression[78] 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]

Table 2 Nitric oxide and heart failure

Beneficial effects in heart failure
Vasodilator, therefore, reduces afterload and preload
Inhibits platelet aggregation
Detrimental effect in heart failure
Negative inotrope
Potentially lethal to cardiac myocytes (however, shown to improve survival in combination with hydralazine)

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 stress[80] 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 neurohumoural 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 counteracting 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,[85] 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.[86–88] Using electron microscopy and immunoreactivity, the isoform ET-1 has been localized to endothelial cytoplasm, rather than specific organelles.[89]

Endothelin has many roles, including regulation of cellular proliferation and apoptosis, activation of monocytes and cellular matrix production.[90,91] There is also some evidence that in the failing heart, endothelin is synthesized predominantly from vascular endothelial cells and macrophages.[92] Indeed, various studies have shown that ET-1 level is an excellent prognostic marker in CHF,[93–98] 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.[86] 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.[99,100] Nevertheless, it is unclear which cell type(s) may be responsible for the increased expression of endothelin and endothelin receptors in the failing myocardium.[86] However, it is widely believed that the isoform ET-1 is primarily produced by endothelial cells abluminally[101] and acts on the underlying smooth muscle cells.[102,103]

Endothelin has positive inotropic, and positive chronotropic, mitogenic and pro-inflammatory properties, as well as its vasocontrictor effects. Its release is stimulated by many factors, such as shear stress, hypoxia, epinephrine, angiotensin II, cortisol, thrombin, pro-inflammatory cytokines (TNF-α, IL-1 and IL-2) and transforming growth factor β.[103–107] The different actions of ET-1 are mediated through the two receptor subtypes and their locations.[108] ET-1 acting on ET_A receptors on vascular smooth muscle cells results in vasoconstriction and proliferation of smooth muscle cells.[102] 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.[109]

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.[86,110,111] 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.[110–112] ET-1 and vWF levels are also directly correlated in both congestive heart failure and after heart transplantation in idiopathic dilated cardiomyopathy.[113] 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.[114,115] There is also experimental evidence that ET-1 inhibits synthesis of NO in smooth muscle cells via ET_A receptors.[116] 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.[117] By FMD, we mean the high-frequency ultrasound assessment of arterial

diameter rather than plethysmography; where plethysmography is used, it has been qualified.

Celermajer *et al*. first described FMD to detect endothelial dysfunction in children and adults at risk of atherosclerosis in 1992,[118] in which the underlying mechanism was nitric oxide (NO) release.[119] They measured FMD in the superficial femoral and brachial arteries by comparing the diameter of the aforementioned arteries using high frequency ultrasound at rest, and comparing with measurements during reactive hyperaemia induced by an inflated pneumatic cuff at a pressure of 300 mmHg for 4.5 min, and after sublingual glyceryl trinitrate.[118] 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*.[120] 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 brachial artery.[120] 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.[121] 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.[121]

FMD has also been shown to be dependent upon the anatomical vessel size[122,123] but independent of body mass index.[123] The effect of age on FMD is unclear, as some studies have shown a correlation,[122,124] whereas others have not.[123] That FMD is greater in smaller arteries may be explained by greater hyperaemic wall shear stress in response to the same stimulus.[125] Variations in technique have resulted in recent guidelines for FMD,[126] in order for results in different studies to be satisfactorily compared.

Reduced FMD has been demonstrated in children with familial hypercholesterolaemia and adult smokers at risk of atherosclerosis or with established coronary artery disease.[118] Others have demonstrated impaired FMD in patients with hypertension,[124] and shown it to be a marker of future cardiovascular events in patients with essential hypertension.[127] Moreover, endothelium-dependent vasodilatation by plethysmography has also been shown to be impaired in type 2 diabetics,[128] as well as in human and animal models of congestive heart failure.[129–132] 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.[133] 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.[133] 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.[129] 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,[134] 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).[135–137] That impaired endothelial-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.[138] While FMD is impaired in CHF whatever the aetiology, it is reversed by cardiac transplantation only in patients with antecedent non-ischaemic cardiomyopathy.[139] 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.[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.[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.[130] In various studies involving the use of ACE inhibitors, however, including heart failure trials[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, suggesting an effective adaptive response.[145] Nevertheless, it remains hypothetical if patients with congestive heart failure adapt in a similar fashion. If we were to extrapolate this, we would expect aspirin to reduce FMD by abolishing the effect of prostanoids that then worsens vasoconstriction, the hallmark of congestive heart failure.

FMD is a measure of endothelial function/dysfunction, and the method indirectly measures NO release in response to shear stress due to laminar blood flow. The method requires specialized and expensive equipment, as well as highly trained technicians, to produce valid, reproducible data.[88,126] Its attraction is that it is non-invasive and allows repeated measurements.[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.[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.[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.[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.[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.[147,148] Thus, expression of

Table 3 Endothelial markers and their significance

Marker	Endothelial activation	Endothelial dysfunction	Endothelial damage/injury	Prognostic
vWF	Yes	Yes	Yes	Yes
sTM	No	No	Yes	Yes
E-selectin	Yes	No	No	? (May be prognostic in CAD)
FMD	No	Yes	No	? (Prognostic in hypertension)
CEC	No	No	Yes	?
Endothelin	No	Yes	Yes	Yes

these markers may signify a 'pro-adhesive' and 'pro-coagulant' endothelium.[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.

Acknowledgements

We acknowledge the support of the City Hospital Research and Development programme for the Haemostasis Thrombosis and Vascular Biology Unit.

References

1. Virchow R. *Gesammelte Abhandlungen zur wissenschaftlichen Medizin*. Frankfurt, Medinger Sohn and Co., 1856:219–732.
2. Lip GYH, Blann A. von Willebrand factor: a marker of endothelial dysfunction in vascular disorders? *Cardiovasc Res* 1997; **34**:255–65.
3. Henderson AH. Endothelium in control. *Br Heart J* 1991; **65**:116–25.
4. Pearson JD. Endothelial cell function and thrombosis. *Baillieres Best Pract Res Clin Haematol* 1999; **12**:329–41.
5. Pearson JD. Normal endothelial cell function. *Lupus* 2000; **9**:183–8.
6. Ginsburg D, Handin RI, Bonthron DT, Donlon TA, Bruns GAP, Latt SA, Orkin SH. Human von Willebrand Factor (vWF): isolation of complementary DNA (cDNA) clones and chromosomal localisation. *Science* 1985; **228**:1401–6.
7. Blann A. von Willebrand factor and the endothelium in vascular disease. *Br J Biomed Sci* 1993; **50**:125–34.
8. Blann A, Seigneur M. Soluble markers of endothelial cell function. *Clin Hemorheol Microcirc* 1997; **17**:3–11.
9. Ginsburg D, Konkle BA, Gill JC, Montgomery RR, Bockenstedt JL, Johnson TA, Yang AY. Molecular basis of human von Willebrand disease: analysis of platelet von Willebrand factor mRNA. *Proc Natl Acad Sci USA* 1989; **86**:3723–7.
10. Green D, Kucuk O, Haring O, Dyer A. The factor VIII complex in atherosclerosis: effects of aspirin. *J Chronic Dis* 1981; **34**:21–6.

11. Ware RE, Parker RI, McKeown LP, Graham ML. A human chimera of von Willebrand disease following bone marrow transplantation. *Am J Paed Haematol Oncol* 1993; **15**:338–42.
12. Blann AD, Taberner DA. A reliable marker of endothelial cell dysfunction: does it exist? *Br J Haematol* 1995; **90**:244–8.
13. Hirokawa K, Aoki N. Up-regulation of thrombomodulin in human umbilical vein endothelial cells *in vitro*. *J Biochem* 1990; **108**:839–45.
14. Pottinger BE, Read RC, Paleolog EG, Higgins PG, Pearson JD. von Willebrand factor is an acute phase reactant in man. *Thromb Res* 1989; **53**:387–94.
15. Blann AD, Amiral J, McCollum CN. Prognostic value of increased soluble thrombomodulin and increased soluble E-selectin in ischaemic heart disease. *Eur J Haematol* 1997; **59**:115–20.
16. Bloom AL. von Willebrand factor: clinical features of inherited and acquired disorders. *Mayo Clin Proc* 1991; **66**:743–51.
17. Chan SY, Chan PH, Ho PC, Yam A. Factor VIII-related antigen levels in normal pregnancy and puerperium. *Eur J Obstet Gynecol Reprod Biol* 1985; **19**:199–204.
18. Dahlman T, Hellgren M, Blomback M. Changes in blood coagulation and fibrinolysis in the normal puerperium. *Gynecol Obstet Invest* 1985; **20**:37–44.
19. Stirling Y, Woolf L, North WR, Seghatchian MJ, Meade TW. Haemostasis in normal pregnancy. *Thromb Haemost* 1984; **52**:176–82.
20. Meade TW, Cooper JA, Stirling Y, Howarth DJ, Ruddock V, Miller GJ. Factor VIII, ABO blood group and the incidence of ischaemic heart disease. *Br J Haematol* 1994; **88**:601–7.
21. Bickel C, Rupprecht HJ, Blankenberg S, Espiniola-Klein C, Schlitt A, Rippin G, *et al*. Relation of markers of inflammation (C-reactive protein, fibrinogen, von Willebrand factor, and leukocyte count) and statin therapy to long-term mortality in patients with angiographically proven coronary artery disease. *Am J Cardiol* 2002; **89**:901–8.
22. Haines AP, Howarth D, North WR, Goldenberg E, Stirling Y, Meade TW, *et al*. Haemostatic variables and the outcome of myocardial infarction. *Thromb Haemost* 1983; **50**:800–3.
23. Jansson J, Nilsson TK, Johnson O. von Willebrand factor in plasma: a novel risk factor for recurrent myocardial infarction and death. *Br Heart J* 1991; **66**:351–5.
24. Blann AD, McCollum CN. von Willebrand factor and soluble thrombomodulin as predictors of adverse events among subjects with peripheral or coronary atherosclerosis. *Blood Coagul Fibrinolysis* 1999; **10**:375–80.
25. Wallberg-Jonsson S, Dahlen GH, Nilsson TK, Ranby M, Rantapaa-Dahlqvist S. Tissue plasminogen activator, plasminogen activator inhibitor-1 and von Willebrand factor in rheumatoid arthritis. *Clin.Rheumatol* 1993; **12**:318–24.
26. Nusinow SR, Federici AB, Zimmerman TS, Curd JG. Increased von Willebrand factor antigen in the plasma of patients with vasculitis. *Arthritis Rheum* 1984; **27**:1405–10.
27. Ames PR, Lupoli S, Alves J, Atsumi T, Edwards C, Iannaccone L, *et al*. The coagulation/fibrinolysis balance in systemic sclerosis: evidence for a haematological stress syndrome. *Br J Rheumatol* 1997; **36**:1045–50.
28. Blann AD, Sheeran TP, Emery P. von Willebrand factor: increased levels are related to poor prognosis in systemic sclerosis and not to tissue autoantibodies. *Br J Biomed Sci* 1997; **54**:5–9.
29. Mannucci PM, Lombardi R, Lattuada A, Perticucci E, Valsecchi R, Remuzzi G. Supranormal von Willebrand factor multimers in scleroderma. *Blood* 1989; **73**:1586–91.
30. Lee P, Norman CS, Sukenik S, Alderdice CA. The clinical significance of coagulation abnormalities in systemic sclerosis (scleroderma). *J Rheumatol* 1985; **12**:514–17.
31. Montalescot G, Philippe F, Ankri A, Vicaut E, Bearez E, Poulard JE, Carrie D, Flammang D, Dutoit A, Carayon A, Jardel C, Chevrot M, Bastard JP, Bigonzi F, Thomas D. Early increase of von Willebrand factor predicts adverse outcome in unstable coronary artery disease: beneficial effects of enoxaparin. *Circulation* 1998; **98**:294–9.
32. Montalescot G, Collet JP, Lison L, Choussat R, Ankri A, Vicaut E, Perlemuter K, Philippe F, Drobinski G, Thomas D. Effects of various anticoagulant treatments on von Willebrand factor release in unstable angina. *J Am Coll Cardiol* 2000; **36**:110–14.
33. Gibbs CR, Blann AD, Watson RD, Lip GY. Abnormalities of hemorheological, endothelial, and platelet function in patients with chronic heart failure in sinus rhythm: effects of angiotensin converting enzyme inhibitor and beta-blocker therapy. *Circulation* 2001; **103**:1746–51.
34. Lip GY, Lowe GD, Metcalfe MJ, Rumley A, Dunn FG. Effects of warfarin therapy on plasma fibrinogen, von Willebrand factor, and fibrin D-dimer in left ventricular dysfunction secondary to coronary artery disease with and without aneurysms. *Am J Cardiol* 1995; **76**:453–8.
35. Seigneur M, Dufourco P, Conri C, Constans J, Mercie P, Pruvost A, Boisseau M. Plasma thrombomodulin: new approach of endothelium damage. *Int Angiol* 1993; **12**:355–9.
36. Dittman WA, Majerus PW. Structure and function of thrombomodulin: a natural anticoagulant. *Blood* 1990; **75**:329–36.
37. Esmon CT. The roles of protein C and thrombomodulin in the regulation of blood coagulation. *J Biol Chem* 1989; **264**:4743–6.
38. Takano S, Kimura S, Ohdama S, Aoki N. Plasma thrombomodulin in health and diseases. *Blood* 1990; **76**:2024–9.
39. Moore KL, Esmon CT, Esmon NL. Tumor necrosis factor leads to the internalization and degradation of thrombomodulin from the surface of bovine aortic endothelial cells in culture. *Blood* 1989; **73**:159–65.
40. Lentz SR, Tsiang M, Sadler JE. Regulation of thrombomodulin by tumor necrosis factor-α: comparison of transcriptional and posttranscriptional mechanisms. *Blood* 1991; **77**:542–50.
41. Boehme MWJ, Deng Y, Raeth U, Bierhaus A, Ziegler R, Stremmel W, Nawroth PP. Release of thrombomodulin from endothelial cells by concerted action of TNF-α and neutrophils: *in vivo* and *in vitro* studies. *Immunology* 1996; **87**:134–40.
42. Gabat S, Keller C, Kempe HP, Amiral J, Ziegler R, Ritz E, *et al*. Plasma thrombomodulin: a marker for microvascular complications in diabetes mellitus. *Vasa* 1996; **25**:233–41.

43. Seigneur M, Dufourcq P, Conri C, Constans J, Mercie P, Pruvost A, *et al*. Levels of plasma thrombomodulin are increased in atheromatous arterial disease. *Thromb Res* 1993; **71**:423–31.
44. Sernau T, Wilhelm C, Seyfert U, Gabath S, Henkels M, Amiral J, *et al*. Thrombomodulin is a marker of microvascular, but not for macrovascular endothelial cell damage. *Vasa* 1995; **24**:347–53.
45. Borcea V, Morcos M, Isermann B, Henkels M, Ziegler S, Zumbach M, *et al*. Influence of ramipril on the course of plasma thrombomodulin in patients with diabetes mellitus. *Vasa* 1999; **28**:172–80.
46. Salomaa V, Matei C, Aleksic N, Sansores-Garcia L, Folsom AR, Juneja H, *et al*. Soluble thrombomodulin as a predictor of incident coronary heart disease and symptomless carotid artery atherosclerosis in the Atherosclerosis Risk in Communities (ARIC) Study: a case-cohort study. *Lancet* 1999; **353**:1729–34.
47. Nilsson TK, Hellsten G, Amiral J. Plasma thrombomodulin concentrations in relation to cardiovascular risk factors in a population sample. *Blood Coagul Fibrinolysis* 1993; **4**:455–8.
48. Naruse M, Kawana M, Hifumi S, Naruse K, Yoshihara I, Oka T, *et al*. Plasma immunoreactive endothelin, but not thrombomodulin, is increased in patients with essential hypertension and ischemic heart disease. *J Cardiovasc Pharmacol* 1991; **17(Suppl. 7)**:S471–4.
49. Blann AD, Amiral J, McCollum CN. Circulating endothelial cell/leucocyte adhesion molecules in ischaemic heart disease. *Br J Haematol* 1996; **95**:263–5.
50. Blann AD, Amiral J, McCollum CN. Prognostic value of increased soluble thrombomodulin and increased soluble E-selectin in ischaemic heart disease. *Eur J Haematol* 1997; **59**:115–20.
51. Smith AP, Demoncheaux EA, Higenbottam TW. Nitric oxide gas decreases endothelin-1 mRNA in cultured pulmonary artery endothelial cells. *Nitric Oxide* 2002; **6**:153–9.
52. Akazawa H, Ikeda U, Kuroda T, Shimada K. Plasma endothelin-1 levels in Takayasu's arteritis. *Cardiology* 1996; **87**:303–5.
53. Hoffman GS, Ahmed AE. Surrogate markers of disease activity in patients with Takayasu arteritis. A preliminary report from The International Network for the Study of the Systemic Vasculitides (INSSYS). *Int J Cardiol* 1998; **66 (Suppl. 1)**:S191–4.
54. Boehme MW, Schmitt WH, Youinou P, Stremmel WR, Gross WL. Clinical relevance of elevated serum thrombomodulin and soluble E-selectin in patients with Wegener's granulomatosis and other systemic vasculitides. *Am J Med* 1996; **101**:387–94.
55. McMurray J, Abdullah I, Dargie HJ, Shapiro D. Increased concentrations of tumour necrosis factor in 'cachectic' patients with severe chronic heart failure. *Br Heart J* 1991; **66**:356–8.
56. Louis A, Cleland JG, Crabbe S, Ford S, Thackray S, Houghton T, *et al*. Clinical Trials Update: CAPRICORN, COPERNICUS, MIRACLE, STAF, RITZ-2, RECOVER and RENAISSANCE and cachexia and cholesterol in heart failure. Highlights of the Scientific Sessions of the American College of Cardiology, 2001. *Eur J Heart Fail* 2001; **3**:381–7.
57. Damas J, Gullestad L, Aukrust P. Cytokines as new treatment targets in chronic heart failure. *Curr Control Trials Cardiovasc Med* 2001; **2**:271–7.
58. Blann AD, Lip GYH. The endothelium in atherothrombotic disease: assessment of function, mechanisms and clinical implications. *Blood Coagul Fibrinolysis* 1998; **9**:297–306.
59. Gearing AJ, Newman W. Circulating adhesion molecules in disease. *Immunol Today* 1993; **14**:506–12.
60. Pigott R, Dillon LP, Hemingway IH, Gearing AJ. Soluble forms of E-selectin, ICAM-1 and VCAM-1 are present in the supernatants and cytokine activated cultured endothelial cells. *Biochem Biophys Res Commun* 1992; **187**:584–9.
61. Davies MJ, Gordon JL, Gearing AJ, Pigott R, Woolf N, Katz D, *et al*. The expression of the adhesion molecules ICAM-1, VCAM-1, PECAM, and E-selectin in human atherosclerosis. *J Pathol* 1993; **171**:223–9.
62. Sudhoff T, Germing U, Aul C. Levels of circulating endothelial adhesion molecules in patients with myelodysplastic syndromes. *Int J Oncol* 2002; **20**:167–72.
63. Alexiou D, Karayiannakis AJ, Syrigos KN, Zbar A, Kremmyda A, Bramis I, *et al*. Serum levels of E-selectin, ICAM-1 and VCAM-1 in colorectal cancer patients: correlations with clinicopathological features, patient survival and tumour surgery. *Eur J Cancer* 2001; **37**:2392–7.
64. Kyriakou DS, Alexandrakis MG, Kyriakou ES, Liapi D, Kourelis TV, Passam F, *et al*. Activated peripheral blood and endothelial cells in thalassemia patients. *Ann Hematol* 2001; **80**:577–83.
65. Blann AD, Amiral J, McCollum CN. Circulating endothelial cell/leucocyte adhesion molecules in ischaemic heart disease. *Br J Haematol* 1996; **95**:263–5.
66. Blann AD, Waite MA. von Willebrand factor and soluble E-selectin in hypertension: influence of treatment and value in predicting the progression of atherosclerosis. *Coron Artery Dis* 1996; **7**:143–7.
67. Blann AD, Miller JP, McCollum CN. von Willebrand factor and soluble E-selectin in the prediction of cardiovascular disease progression in hyperlipidaemia. *Atherosclerosis* 1997; **132**:151–6.
68. Blann AD, Davis A, Miller JP, McCollum CN. Von Willebrand factor and soluble E-selectin in hyperlipidaemia: relationship to lipids and vascular disease. *Am J Hematol* 1997; **55**:15–23.
69. Blann AD, McCollum CN. Circulating endothelial cell/leukocyte adhesion molecules in atherosclerosis. *Thromb Haemost* 1994; **72**:151–4.
70. Blann AD, Tse W, Maxwell SJ, Waite MA. Increased levels of the soluble adhesion molecule E-selectin in essential hypertension. *J Hypertens* 1994; **12**:925–8.
71. Andreassen AK, Nordoy I, Simonsen S, Ueland T, Muller F, Froland SS, *et al*. Levels of circulating adhesion molecules in congestive heart failure and after heart transplantation. *Am J Cardiol* 1998; **81**:604–8.
72. Blankenberg S, Rupprecht HJ, Bickel C, Peetz D, Hafner G, Tiret L, *et al*. Circulating cell adhesion molecules and death in patients with coronary artery disease. *Circulation* 2001; **104**:1336–42.
73. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 1980; **299**:373–6.

74. Vallance P, Collier J, Moncada S. Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet* 1989; **2(8670)**:997–1000.
75. Pohl U, Holtz J, Busse R, Bassenge E. Crucial role of endothelium in the vasodilator response to increased flow in vivo. *Hypertension* 1986; **8**:37–44.
76. Griffith TM. Studies of endothelium-derived relaxant factor (EDRF), its nature and mode of action. *Eur Heart J* 1985; **6**:37–49.
77. Macdonald P, Schyvens C, Winlaw D. The role of nitric oxide in heart failure. Potential for pharmacological intervention. *Drugs Aging* 1996; **8**:452–8.
78. Agnoletti L, Curello S, Bachetti T, Malacarne F, Gaia G, Comini L, *et al*. Serum from patients with severe heart failure downregulates eNOS and is proapoptotic: role of tumor necrosis factor-alpha. *Circulation* 1999; **100**:1983–91.
79. Drexler H, Hayoz D, Munzel T, Hornig B, Just H, Brunner HR, Zelis R. Endothelial function in chronic congestive heart failure. *Am J Cardiol* 1992; **69**:1596–601.
80. Arnold JM, Marchiori GE, Imrie JR, Burton GL, Pflugfelder PW, Kostuk WJ. Large artery function in patients with chronic heart failure. Studies of brachial artery diameter and hemodynamics. *Circulation* 1991; **84**:2418–25.
81. Winlaw DS, Smythe GA, Keogh AM, Schyvens CG, Spratt PM, Macdonald PS. Increased nitric oxide production in heart failure. *Lancet* 1994; **344**:373–4.
82. Ikeda U, Shimada K. Nitric oxide and cardiac failure. *Clin Cardiol* 1997; **20**:837–41.
83. Kelm M, Schafer S, Dahmann R, Dolu B, Perings S, Decking UK, *et al*. Nitric oxide induced contractile dysfunction is related to a reduction in myocardial energy generation. *Cardiovasc Res* 1997; **36**:185–94.
84. Drexler H, Kastner S, Strobel A, Studer R, Brodde OE, Hasenfuss G. Expression, activity and functional significance of inducible nitric oxide synthase in the failing human heart. *J Am Coll Cardiol* 1998; **32**:955–63.
85. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Yazaki Y, Goto K, Masaki T. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 1988; **332**:411–15.
86. Colucci WS. Myocardial endothelin: does it play a role in myocardial failure? *Circulation* 1996; **93**:1069–72.
87. Rossi GP, Cavallin M, Nussdorfer GG, Pessina AC. The endothelin-aldosterone axis and cardiovascular diseases. *J Cardiovasc Pharmacol* 2001; **38(Suppl. 2)**:S49–52.
88. Ry SD, Andreassi MG, Clerico A, Biagini A, Giannessi D. Endothelin-1, endothelin-1 receptors and cardiac natriuretic peptides in failing human heart. *Life Sci* 2001; **68**:2715–30.
89. Properzi G, Terenghi G, Gu XH, Poccia G, Pasqua R, Francavilla S, *et al*. Early increase precedes a depletion of endothelin-1 but not of von Willebrand factor in cutaneous microvessels of diabetic patients. A quantitative immunohistochemical study. *J Pathol* 1995; **175**:243–52.
90. Best PJ, Lerman A. Endothelin in cardiovascular disease: from atherosclerosis to heart failure. *J Cardiovasc Pharmacol* 2000; **35(Suppl. 2)**:S61–3.
91. Ergul A. Endothelin-1 and endothelin receptor antagonists as potential cardiovascular therapeutic agents. *Pharmacotherapy* 2002; **22**:54–65.
92. Fukuchi M, Giaid A. Expression of endothelin-1 and endothelin-converting enzyme-1 mRNAs and proteins in failing human hearts. *J Cardiovasc Pharmacol* 1998; **31(Suppl. 1)**:S421–3.
93. Vidal B, Roig E, Perez-Villa F, Orus J, Perez J, Jimenez V, *et al*. Prognostic value of cytokines and neurohormones in severe heart failure. *Rev Esp Cardiol* 2002; **55**:481–6.
94. Selvais PL, Robert A, Ahn S, van Linden F, Ketelslegers JM, Pouleur H, *et al*. Direct comparison between endothelin-1, N-terminal proatrial natriuretic factor, and brain natriuretic peptide as prognostic markers of survival in congestive heart failure. *J Card Fail* 2000; **6**:201–7.
95. Frey B, Pacher R, Locker G, Bojic A, Hartter E, Woloszczuk W, *et al*. Prognostic value of hemodynamic vs big endothelin measurements during long-term IV therapy in advanced heart failure patients. *Chest* 2000; **117**:1713–19.
96. Pousset F, Isnard R, Lechat P, Kalotka H, Carayon A, Maistre G, *et al*. Prognostic value of plasma endothelin-1 in patients with chronic heart failure. *Eur Heart J* 1997; **18**:254–8.
97. Pacher R, Stanek B, Hulsmann M, Koller-Strametz J, Berger R, Schuller M, *et al*. Prognostic impact of big endothelin-1 plasma concentrations compared with invasive hemodynamic evaluation in severe heart failure. *J Am Coll Cardiol* 1996; **27**:633–41.
98. Hurlimann D, Enseleit F, Noll G, Luscher TF, Ruschitzka F. Endothelin antagonists and heart failure. *Curr Hypertens Rep* 2002; **4**:85–92.
99. Masaki T, Vane JR, Vanhoutte PM. International Union of Pharmacology nomenclature for endothelin receptors. *Pharmacol Rev* 1994; **46**:137–42.
100. Spieker LE, Noll G, Ruschitzka FT, Luscher TF. Endothelin receptor antagonists in congestive heart failure: a new therapeutic principle for the future? *J Am Coll Cardiol* 2001; **37**:1493–505.
101. d'Uscio LV, Barton M, Shaw S, Luscher TF. Endothelin in atherosclerosis: importance of risk factors and therapeutic implications. *J Cardiovasc Pharmacol* 2000; **35(Suppl. 2)**:S55–9.
102. Newby DE, Webb DJ. Advances in clinical pharmacology and therapeutics: endothelin. *Br J Hosp Med* 1996; **56**:360–4.
103. Haynes WG, Webb DJ. Endothelin as a regulator of cardiovascular function in health and disease. *J Hypertens* 1998; **16**:1081–98.
104. Yoshizumi M, Kurihara H, Morita T, Yamashita T, Oh-hashi Y, Sugiyama T, *et al*. Interleukin 1 increases the production of endothelin-1 by cultured endothelial cells. *Biochem Biophys Res Commun* 1990; **166**:324–9.
105. Rubanyi GM, Polokoff MA. Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. *Pharmacol Rev* 1994; **46**:325–415.
106. Yoshizumi M, Kurihara H, Sugiyama T, Takaku F, Yanagisawa M, Masaki T, *et al*. Hemodynamic shear stress stimulates endothelin production by cultured endothelial cells. *Biochem Biophys Res Commun* 1989; **161**:859–64.
107. Kourembanas S, Marsden PA, McQuillan LP, Faller DV. Hypoxia induces endothelin gene expression and secretion in cultured human endothelium. *J Clin Invest* 1991; **88**:1054–7.

108. Seo B, Oemar BS, Siebenmann R, von Segesser L, Luscher TF. Both ETA and ETB receptors mediate contraction to endothelin-1 in human blood vessels. *Circulation* 1994; **89**:1203–8.

109. Verhaar MC, Strachan FE, Newby DE, Cruden NL, Koomans HA, Rabelink TJ, *et al*. Endothelin-A receptor antagonist-mediated vasodilatation is attenuated by inhibition of nitric oxide synthesis and by endothelin-B receptor blockade. *Circulation* 1998; **97**:752–6.

110. Morise T, Takeuchi Y, Kawano M, Koni I, Takeda R. Increased plasma levels of immunoreactive endothelin and von Willebrand factor in NIDDM patients. *Diabetes Care* 1995; **18**:87–9.

111. Seligman BG, Biolo A, Polanczyk CA, Gross JL, Clausell N. Increased plasma levels of endothelin 1 and von Willebrand factor in patients with type 2 diabetes and dyslipidemia. *Diabetes Care* 2000; **23**:1395–400.

112. Baumgartner-Parzer SM, Wagner L, Reining G, Sexl V, Nowotny P, Muller M, *et al*. Increase by tri-iodothyronine of endothelin-1, fibronectin and von Willebrand factor in cultured endothelial cells. *J Endocrinol* 1997; **154**:231–9.

113. Galatius S, Wroblewski H, Sorensen VB, Bie P, Parving HH, Kastrup J. Endothelin and von Willebrand factor as parameters of endothelial function in idiopathic dilated cardiomyopathy: different stimuli for release before and after heart transplantation? *Am Heart J* 1999; **137**:549–54.

114. Huang LQ, Whitworth JA, Chesterman CN. Effects of cyclosporin A and dexamethasone on haemostatic and vasoactive functions of vascular endothelial cells. *Blood Coagul Fibrinolysis* 1995; **6**:438–45.

115. Geny B, Piquard F, Follenius M, Thiranos JC, Charpentier A, Epailly E, *et al*. Endothelin participates in increased circulating atrial natriuretic peptide early after human heart transplantation. *J Heart Lung Transplant* 1998; **17**:167–75.

116. Ikeda U, Yamamoto K, Maeda Y, Shimpo M, Kanbe T, Shimada K. Endothelin-1 inhibits nitric oxide synthesis in vascular smooth muscle cells. *Hypertension* 1997; **29**:65–9.

117. Sorensen KE, Celermajer DS, Spiegelhalter DJ, Georgakopoulos D, Robinson J, Thomas O, Deanfield JE. Non-invasive measurement of human endothelium dependent arterial responses: accuracy and reproducibilty. *Br Heart J* 1995; **74**:247–53.

118. Celermajer DS, Sorensen KE, Gooch VM, Spiegelhalter DJ, Miller OI, Sullivan ID, Lloyd JK, Deanfield JE. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *Lancet* 1992; **340**:1111–15.

119. Joannides R, Haefeli WE, Linder L, Richard V, Bakkali EH, Thuillez C, *et al*. Nitric oxide is responsible for flow-dependent dilatation of human peripheral conduit arteries in vivo. *Circulation* 1995; **91**:1314–19.

120. Agewall S, Doughty RN, Bagg W, Whalley GA, Braatvedt G, Sharpe N. Comparison of ultrasound assessment of flow-mediated dilatation in the radial and brachial artery with upper and forearm cuff positions. *Clin Physiol* 2001; **21**:9–14.

121. Doshi SN, Naka KK, Payne N, Jones CJ, Ashton M, Lewis MJ, *et al*. Flow-mediated dilatation following wrist and upper arm occlusion in humans: the contribution of nitric oxide. *Clin Sci (Lond)* 2001; **101**:629–35.

122. Celermajer DS, Sorensen KE, Bull C, Robinson J, Deanfield JE. Endothelium-dependent dilation in the systemic arteries of asymptomatic subjects relates to coronary risk factors and their interaction. *J Am Coll Cardiol* 1994; **24**:1468–74.

123. Schroeder S, Enderle MD, Baumbach A, Ossen R, Herdeg C, Kuettner A, *et al*. Influence of vessel size, age and body mass index on the flow-mediated dilatation (FMD%) of the brachial artery. *Int J Cardiol* 2000; **76**:219–25.

124. Deng YB, Wang XF, Le GR, Zhang QP, Li CL, Zhang YG. Evaluation of endothelial function in hypertensive elderly patients by high-resolution ultrasonography. *Clin Cardiol* 1999; **22**:705–10.

125. Silber HA, Bluemke DA, Ouyang P, Du YP, Post WS, Lima JA. The relationship between vascular wall shear stress and flow-mediated dilation: endothelial function assessed by phase-contrast magnetic resonance angiography. *J Am Coll Cardiol* 2001; **38**:1859–65.

126. Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, *et al*. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *J Am Coll Cardiol* 2002; **39**:257–65.

127. Perticone F, Ceravolo R, Pujia A, Ventura G, Iacopino S, Scozzafava A, Ferraro A, Chello M, Mastroroberto P, Verdecchia P, Schillaci G. Prognostic significance of endothelial dysfunction in hypertensive patients. *Circulation* 2001; **104**:191–6.

128. Watts GF, O'Brien SF, Silvester W, Millar JA. Impaired endothelium-dependent and independent dilatation of forearm resistance arteries in men with diet-treated non-insulin-dependent diabetes: role of dyslipidaemia. *Clin Sci (Colch.)* 1996; **91**:567–73.

129. Kubo SH, Rector TS, Bank AJ, Williams RE, Heifetz SM. Endothelium-dependent vasodilation is attenuated in patients with heart failure. *Circulation* 1991; **84**:1589–96.

130. Varin R, Mulder P, Tamion F, Richard V, Henry JP, Lallemand F, *et al*. Improvement of endothelial function by chronic angiotensin-converting enzyme inhibition in heart failure : role of nitric oxide, prostanoids, oxidant stress, and bradykinin. *Circulation* 2000; **102**:351–6.

131. Katz SD, Biasucci L, Sabba C, Strom JA, Jondeau G, Galvao M, *et al*. Impaired endothelium-mediated vasodilation in the peripheral vasculature of patients with congestive heart failure. *J Am Coll Cardiol* 1992; **19**:918–25.

132. Katz SD, Schwarz M, Yuen J, LeJemtel TH. Impaired acetylcholine-mediated vasodilation in patients with congestive heart failure. Role of endothelium-derived vasodilating and vasoconstricting factors. *Circulation* 1993; **88**:55–61.

133. Katz SD, Rao R, Berman JW, Schwarz M, Demopoulos L, Bijou R, *et al*. Pathophysiological correlates of increased serum tumor necrosis factor in patients with congestive heart failure. Relation to nitric oxide-dependent vasodilation in the forearm circulation. *Circulation* 1994; **90**:12–16.

134. Fichtlscherer S, Rossig L, Breuer S, Vasa M, Dimmeler S, Zeiher AM. Tumor necrosis factor antagonism with etanercept improves systemic endothelial vasoreactivity in patients with advanced heart failure. *Circulation* 2001; **104**:3023–5.

135. Vanhoutte PM. Endothelial dysfunction and inhibition of converting enzyme. *Eur Heart J* 1998; **19(Suppl. J)**:J7–15.

136. Joannides R, Bizet-Nafeh C, Costentin A, Iacob M, Derumeaux G, Cribier A, *et al*. Chronic ACE inhibition enhances the endothelial control of arterial mechanics and flow-dependent vasodilatation in heart failure. *Hypertension* 2001; **38**:1446–50.

137. Giannattasio C, Achilli F, Grappiolo A, Failla M, Meles E, Gentile G, *et al*. Radial artery flow-mediated dilatation in heart failure patients: effects of pharmacological and nonpharmacological treatment. *Hypertension* 2001; **38**:1451–5.

138. Kubo SH, Rector TS, Bank AJ, Tschumperlin LK, Raij L, Brunsvold N, *et al*. Effects of cardiac transplantation on endothelium-dependent dilation of the peripheral vasculature in congestive heart failure. *Am J Cardiol* 1993; **71**:88–93.

139. Patel AR, Kuvin JT, Pandian NG, Smith JJ, Udelson JE, Mendelsohn ME, *et al*. Heart failure etiology affects peripheral vascular endothelial function after cardiac transplantation. *J Am Coll Cardiol* 2001; **37**:195–200.

140. Hornig B, Arakawa N, Drexler H. Effect of ACE inhibition on endothelial dysfunction in patients with chronic heart failure. *Eur Heart J* 1998; **19(Suppl. G)**:G48–53.

141. Hornig B, Kohler C, Drexler H. Role of bradykinin in mediating vascular effects of angiotensin-converting enzyme inhibitors in humans. *Circulation* 1997; **95**:1115–18.

142. Hornig B, Drexler H. Endothelial function and bradykinin in humans. *Drugs* 1997; **54(Suppl. 5)**:42–7.

143. Monobe H, Yamanari H, Nakamura K, Ohe T. Effects of low-dose aspirin on endothelial function in hypertensive patients. *Clin Cardiol* 2001; **24**:705–9.

144. Stys T, Lawson W, Smaldone G, Stys A. Does aspirin attenuate the beneficial effects of angiotensin-converting enzyme inhibition in heart failure? *Arch Intern Med* 2000; **160**:1409–13.

145. Sun D, Huang A, Smith CJ, Stackpole CJ, Connetta JA, Shesely EG, *et al*. Enhanced release of prostaglandins contributes to flow-induced arteriolar dilation in eNOS knockout mice. *Circ Res* 1999; **85**:288–93.

146. Mutin M, Canavy I, Blann A, Bory M, Sampol J, Dignat-George F. Direct evidence of endothelial injury in acute myocardial infarction and unstable angina by demonstration of circulating endothelial cells. *Blood* 1999; **93**:2951–8.

147. Solovey A, Lin Y, Browne PV, Wayner E, Choong S, Hebbel RP. Phenotypic analysis of circulating endothelial cells reveals an activated endothelium in sickle cell anemia. *Blood* 1996; **88(Suppl. 1)**:648a abstract.

148. Solovey A, Lin Y, Browne P, Choong S, Wayner E, Hebbel RP. Circulating activated endothelial cells in sickle cell anemia. *N Engl J Med* 1997; **337**:1584–90.