Review

Clinical and methodological aspects of endothelial function in patients with systemic autoimmune diseases

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ABSTRACT

The endothelium is not merely a barrier but it plays a key role in the maintenance of vascular homeostasis. An alteration of the endothelial function (EF) is an early marker of atherosclerosis, also contributing to the development of atherosclerotic lesions and later clinical complications. Systemic autoimmune diseases are characterized by the occurrence of premature atherosclerosis and cardiovascular disease. In view of the prognostic significance of EF for the development of atherosclerotic disease, many studies have evaluated the endothelium in systemic autoimmune diseases, using different techniques. The aim of the present paper is to review the different available techniques to study EF, their advantages and limitations and the data available on the study of EF in systemic autoimmune diseases. Vascular reactivity tests represent the most widely used methods in the clinical assessment of endothelial function. Several techniques were developed to study microcirculation (resistance arteries and arterioles) and macrocirculation (conduit arteries). Studies assessing microvasculature in systemic autoimmune diseases have shown the presence of reduced endothelium dependent vasodilation, while no agreement exists on the presence of endothelium independent alterations. Flow mediated dilation (FMD) has been widely used to evaluate endothelium-dependent vasodilation in peripheral macrocirculation. The majority of studies in systemic autoimmune diseases have shown a decreased brachial artery FMD, whereas endothelium-independent response appears unaffected by the disease in this district. These data strongly underline the different information that could be obtained by different techniques and suggests their combined use in prospective cohorts. Circulating markers of EF include direct products of endothelial cells that change when the endothelium is activated, such as measures of NO biology, inflammatory cytokines, adhesion molecules, as well as markers of endothelial damage and repair. Many of these circulating markers are difficult to measure and quite expensive, and currently are only used in research settings. In view of the complexities in the evaluation of EF, results represent the interaction of several endothelial pathways. No single test currently available is specific for the vascular district tested or the risk factor/diseases considered, and a panel of several tests is therefore needed to characterize the multiple aspects of endothelial biology.

Introduction

The endothelium is not merely a barrier but it plays a key role in the maintenance of vascular homeostasis as well as in the control of vascular function and structure by the production of nitric oxide (NO). Beyond its vasodilating properties, NO maintains the vascular wall in a quiescent state by inhibition of inflammation, cellular proliferation, atherosclerosis and thrombosis (1). In pathological conditions, such as aging, diabetes, dyslipidemia, hypertension, characterized by reduced NO availability, the endothelium produces, as a compensatory mechanism, an EDHF (endothelium derived hyperpolarizing factor), whose vasodilating effect is mediated by hyperpolarization of vascular smooth muscle cell (2). The reduction of NO availability furthermore, interferes with the mobilization of circulating endothelial progenitor cells, an alternative mechanism for maintenance and repair of the endothelium (3), therefore this mechanisms may be impaired in patients with cardiovascular (CV) risk factors (4).

Appreciation of the central role of the endothelium in the atherosclerotic disease process has led to the development of a range of methods to assess different aspects of endothelial function (EF). These have provided not only novel insights into pathophysiology, but also a clinical opportunity to detect early disease, quantify risk, judge response to interventions designed to prevent progression of early disease, and reduce later adverse events in patients (5, 6). It should be noticed that EF, unlike measures of vessel wall morphology, has intrinsic biological variability, and thus a single measurement, may give only a snapshot and limited information. Nevertheless, several longitudinal studies have shown that an EF has a prognostic value in patients with established coronary disease and at high CV risk (7).

Systemic autoimmune diseases are characterized by the occurrence of premature atherosclerosis and CV disease (8-14). In view of the prognostic significance of EF for the development of atherosclerotic disease, many studies have evaluated the endothelium in systemic autoimmune diseases, using different techniques. The aim of the present paper is to offer a review of different techniques available to study EF, their advantages and limitations and to review the data available on the study of EF in systemic autoimmune diseases.

Clinical assessment of endothelial function in patients with autoimmune diseases

Vascular reactivity tests

Vascular reactivity tests represent the most widely used methods of clinical assessment of EF (5, 6). Their principle is to activate or block endothelial cell function while measuring the consequent changes in vascular tone in selected vascular districts. Tests have been developed to study macro- (conduit arteries) and microcirculation (resistance arteries and arterioles), in which different endothelial pathways might active.

1. Evaluation of macrocirculation

The most widely used technique for assessing EF is the so-called "flow-mediated dilation" (FMD). In this non invasive method, introduced in 1992 (15), brachial artery diameter is measured before and after an increase in shear stress induced by reactive hyperemia. When a sphygmomanometer cuff placed on the forearm distal to the brachial artery is inflated to 200 mmHg and subsequently released 5 minutes later, FMD occurs as a result of local endothelial release of NO (16, 17). Endothelium-independent dilator response can be tested by low dose sublingual nitroglycerin (18). FMD has been studied widely in clinical research as it enables serial evaluation of young subjects, including children. It also permits testing of lifestyle and pharmacological interventions on endothelial biology at an early preclinical stage, when the disease process is most likely to be reversible (6). However, some practical challenges need to be overcome before this technique could be suitable for use in routine clinical practice (5, 6, 19). These challenges include the need for highly trained operators, the expense of the equipment, and also the care required to minimize the effect of environmental or physiological influences. It is important to note that variations in technique, such as the position of the occluding cuff and duration of inflation, may produce results that are less representative of local NO activity, since FMD is also determined, in part, by the magnitude of post-ischemic forearm vasodilatation, which is a measure of microcirculatory function (6). Furthermore, due to the variability and degree of response, large study population are required for clinical studies (5, 6, 19).

In systemic autoimmune diseases EF was frequently assessed as brachial artery FMD (Tables I - III). In patients with rheumatoid arthritis (RA), the majority of studies have shown the presence of an altered EF as reduced FMD, which is partially restored after therapy. Vaudo *et al*. studied 32 young to mid-

Table I. Endothelial function and rheumatoid arthritis.

Author	n° patients	Technique	Vascular district	EDV	EIDV	Comments
Bergholm *et al*. (43)	10	perfused forearm tecnique	microcirculation	Impaired	Impaired	EDV improved after therapy
Hansel *et al*. (44)	8	perfused forearm tecnique	microcirculation	Impaired	Normal	No changes after switch to etanercept
Vaudo *et al*. (20)	32	FMD	macrocirculation	Impaired	Not assessed	Early onset patients, possible correlation with inflammation
Bilsborough *et al*. (21)	14	FMD	macrocirculation	Impaired	Not specified	EDV improved, EIDV unaffected after anti-TNF
Hurlimann *et al*. (23)	11	FMD	macrocirculation	Impaired	Not specified	EDV improved, EIDV unchanged after anti-TNF
Gonzalez-Juanatey *et al*. (26)	8	FMD	macrocirculation	Impaired	Normal	EDV improved after adalimumab
Van Doornum *et al*. (22)	25	FMD	macrocirculation	Normal	Normal	Significant reduction of large and small artery compliance

EDV: Endothelium-dependent vasodilation; EIDV: endothelium-independent vasodilation.

Table II. Endothelial function and systemic lupus erythematosus.

Author	n° patients	Technique	Vascular district	EDV	EIDV	Comments
El- Magadmi *et al*. (35)	62	FMD	macrocirculation	Impaired	Impaired	Increases in intima-media thickness associated with EDV
Lima *et al*. (34)	69	FMD	macrocirculation	Impaired	Impaired	EIDV reduced in patients with anticardiolipin antibodies
Kiss *et al*. (36)	61	FMD	macrocirculation	Impaired	Normal	EDV correlated with age, blood pressure
Piper *et al*. (37)	36	FMD	macrocirculation	Impaired	Normal	No correlation with development of CVD over 5 years follow-up

EDV: Endothelium-dependent vasodilation; EIDV: endothelium-independent vasodilation

Table III. Endothelial function and systemic vasculitis.

Author	n° patients	Technique	Vascular district	EDV	EIDV	Comments
Booth *et al*. (45)	14 (ANCA+ vasculitis)	perfused forearm technique	microcirculation	Impaired	Normal	Correlation with disease activity Improved after anti-TNF
Raza *et al*. (28)	24 (SN vasculitis)	FMD	macrocirculation	Impaired	Normal	7/12 improved after therapy
Filer *et al*. (29)	54 (SN vasculitis)	FMD	macrocirculation	Impaired	Normal	EDV impaired in vascular beds clinically not involved by the process
Gonzalez-Juanatey *et al*. (30)	6 (Giant cell arteritis)	FMD	macrocirculation	Impaired	Normal	Improvement of EDV response 4 weeks after therapy onset
Chambers *et al*. (31)	19 (Behçet's disease)	FMD	macrocirculation	Impaired	Normal	Improvement of EDV with vitamin C
Oflaz *et al*. (32)	50 (Behçet's disease)	FMD	macrocirculation	Impaired	Normal	Impairment of EDV more prominent in patients with vascular involvement
Kayikcioglu *et al*. (33)	65 (Behçet's disease)	FMD	macrocirculation	Impaired	Impaired	No correlation with vascular involvement

EDV: Endothelium-dependent vasodilation; EIDV: endothelium-independent vasodilation; SN: systemic necrotizing.

dle aged patients with RA with low disease activity, free from cardiovascular risk factors and overt CV diseases and showed a significant reduction with respect to controls, suggesting a correlation with the disease associated chronic inflammatory condition (20). In 2006 Bilsborough *et al*. evaluated a group of 9 RA patients at baseline and after 36 months of anti TNF-α treatment compared with a control group of 5 RA patients on conventional therapy [21]. A significant improvement of FMD following anti TNF-α therapy was observed, while the endothelium-independent response was unaffected. Although Van Doornum *et al*. found a normal FMD, but large and small artery compliance significantly reduced in 25 young (ages ≤55 years) RA patients with active, longstanding disease (22), these findings were consistent with several previous observations. Hurlimann *et al*. observed a modest increase in FMD when infliximab was added to conventional therapy in 11 RA patients followed-up for 12 months (23). Infliximab therapy was also associated with decreased levels of adhesion molecules (24) and improved insulin resistance (25). More recently, Gonzalez-Juanatey *et al*. described an improvement of EF in 8 long standing RA patients after a short (12 weeks) period of treatment with adalimumab concomitantly with clinical and biochemical disease remission (26). Another therapeutic approach to improve endothelial dysfunction in RA patients includes the use of statins (27).

Studies conducted in systemic vasculitis have shown an impaired FMD, with no correlation with disease activity, or other variables such as disease duration, autoantibody profile, type of organ involvement. Raza *et al*. have observed an improvement of EF in 7 over 12 patients after therapy, however no changes were observed in the remaining five patients (28). In a study of 54 patients with different types of systemic vasculitis, Filer *et al*. have observed the presence of reduced FMD also in vascular beds clinically not involved by the inflammatory process (29). On this basis, they speculated that endothelial dysfunction could be the consequence of both an acute inflammatory state (reversible after therapy) and an established vascular damage (not reversible with treatment).

In 6 patients with active biopsy-proven giant cell arteritis, Gonzalez-Juanatey *et al*. serially tested FMD before and after starting steroid therapy (48 hrs, 4 weeks, 2 years) (30). At baseline, the authors found a significantly impaired endothelium-dependent vasodilation. An improvement of vascular response was observed in all patients 4 weeks after the onset of therapy concomitantly with a clinical and biochemical remission which was maintained 2 years later, after the end of the treatment.

FMD was found to be altered in patients with Behçet's disease. In 2001 Chambers *et al*. reported an impaired EF assessed by FMD in 19 patients with Behçet's syndrome and active disease (31). Similarly, in 2005 Oflaz *et al*. observed a reduction of the vascular response in 50 Behçet's patients compared to the healthy controls finding a significantly lower endothelium-dependent vasodilation in the group with documented previous or actual vascular involvement (arterial or venous) (32). The association between vascular involvement and FMD, however, was not confirmed in a cohort of 65 patients (33).

Data obtained in systemic lupus erythematosous (SLE) patients showed an altered FMD but normal endothelium-independent vasodilation (34-36). In a recent study assessing 62 SLE patients, El-Magadmi *et al*. described an inverse association between EF and intima-media thickness; each 0.01 cm increase in IMT being associated with a 0.92% decrease in FMD (35). This finding supports the concept that EF is associated with carotid IMT, an early marker of atherosclerosis. Recently Piper *et al*. have evaluated the correlation between EF and the development of CV damage and/or disease. EF was assessed in 36 female SLE patients, which were followed for the subsequent 5 years. Endothelium dependent response was altered in patients with respect to controls while no differences were observed relatively to endothelium independent vasodilation. Endothelial dysfunction was not correlated with the subsequent development of damage or occurrence of CV disease (37).

One single study conducted on patients with 25 Sjögren's syndrome (SS) has shown an impaired FMD and a significant correlation with the presence of Raynaud's phenomenon but not with other serological or clinical parameters (38).

Recently, Szucs *et al*. showed a significant FMD reduction, as compared to controls, in 29 patients with systemic sclerosis (SSc) (39). No change in endothelium-independent vasodilation was observed. Finally, an improvement of FMD in SSc patients after therapy with bosentan (40) and prostaglandin E1 (PGE1) (41), but not with ascorbic acid (42) has been described.

2. Evaluation of microcirculation

In microcirculation, EF can be evaluated in functionally isolated vascular districts, such as the forearm and coronary circulations (5, 6). The isolated and perfused forearm technique is a minimally invasive test, since it requires brachial artery cannulation for the administration of endothelial agonists (such as acetylcholine, ACh) for assessing changes in blood flow (measured by plethysmography), which are representative of modification in local vascular resistance (increase in flow = vasodilation). Endothelium-dependent vasodilation is commonly estimated by the dose-response curve to intra-arterial ACh. Endothelium-independent vasodilation is assessed by the dose-response curve to intra-arterial sodium nitroprusside (SNP), a direct smooth muscle relaxant. To evaluate NO availability, acetylcholine infusion can be repeated in the presence of the specific NO synthase inhibitor, NG-monomethyl-L-arginine (L-NMMA). The limitation of this test is mainly represented by its invasiveness, which limits the number of patients enrolled and the possibility to repeat testing frequently. Nonetheless, peripheral invasive tests are required to study the mechanisms of endothelial patho-physiology, since evaluation of the mere vasodilating response is not equivalent to the study of NO availability, which represents the crucial marker of EF. Thus, clinical studies should demonstrate not only the presence of a reduced (and/or improved after treatment) endothelium-dependent vasodilation but also blunted (and/or improved/restored) NO availability.

In coronary microcirculation, EF can be evaluated by coronary angiography and Doppler under the stimulation by local agonists infusion, induction of shear stress or mixed stimuli (cold pressor test, mental stress, exercise) (5, 6).

Few studies have evaluated EF in the peripheral microcirculation, particularly in patients with RA and systemic vasculitis (Tables I and III). In 2002 Bergholm *et al*. evaluated vascular function in a group of 10 newly diagnosed RA patients (disease duration ≤18 months) at baseline and after 6 months of therapy (43). Vasodilation to SNP and ACh were significantly lower in patients than in the control group. A significant restoration of response to ACh was observed after 6 months of therapy (NSAIDS, DMARDS, steroids) along with an improvement of the clinical and biochemical disease activity parameters. On the contrary the response to SNP was only slightly increased. These data suggested that the impairment of EF was selectively restored by therapy. In 2003, Hansel *et al*. prospectively investigated EF in 8 long-term RA patients, with low grade disease activity as measured by DAS 28 (44). EF was assessed during Methotrexate treatment and after switching to Etanercept. The authors found a significantly impaired endothelium-dependent vasodilatation to ACh in patients as compared to controls, while no alterations in endothelium-independent response were observed. Switching therapy did not result in an improvement of ACh response after 2 weeks of treatment.

One single study on 14 patients with active ANCA associated systemic vasculitis showed an inverse correlation between response to ACh and disease activity (45). EF was restored in 10 patients treated with anti TNF-α agents (in addition to steroids and/or traditional immunosuppressive drugs) concomitantly with a clinical and laboratory remission. Endothelium-independent responses were not reduced compared with controls.

Other vascular approaches

Alternative non-invasive approaches have been recently developed to study vascular biology in the peripheral circulation. These rely on the ability of the

β2 agonist salbutamol to reduce arterial stiffness and wave reflection measured by pulse wave analysis (radial artery tonometry), in an NO-dependent manner without significant reduction in blood pressure when given by inhaler at standard clinical doses (46, 47). Similarly, reactive hyperemia has been used to elicit changes in conduit artery pulse wave velocity and digital pulse volume that can be measured by oscillometry to identify limb arterial pulse pressure, wave form, timing, and also digital pulse amplitude tonometry (48, 49). However, when using these techniques, the relative contribution of structural alterations in the vessel wall and endothelial dependent biology remains uncertain. Further validation is therefore required, which should include a wider study of their reproducibility in different age groups and stages of disease, as well as clarification of their relationships with other established measures of EF. Moreover, these techniques are not jet applied in patients with systemic autoimmune diseases.

On the contrary, arterial stiffness has been evaluated in systemic vasculitis, RA and SLE patients (50-52). In ANCA associated systemic vasculitis, carotid to femoral pulse wave velocity (PWV), an index of aortic stiffness, and augmentation index (AIx), which is a marker also of peripheral wave reflections, were related with markers of active inflammation (IL6 and CRP) and they were similar to controls in patients with inactive disease (50). In RA patients, increased PWV is associated with CV risk factors including age, blood pressure and the increase in trunk fat (53). AIx was also reduced in RA patients (51). In a large study including 220 SLE patients, traditional factors were primarily correlated with PWV among post-menopausal women, whereas SLE related factors and inflammation were associated with aortic stiffness among pre-menopausal women (52).

Circulating markers of endothelial function

Circulating markers of EF include direct products of endothelial cells that change when the endothelium is activated, such as measures of NO biology, inflammatory cytokines, adhesion molecules, as well as markers of endothelial damage and repair. These markers are difficult to measure and quite expensive, the biological and assay availability and variability limits their assessment in individual patients and currently used only in research settings.

Circulating levels of nitrites and nitrosylated proteins in part reflect endothelial generation of NO, but are difficult to measure and may not always represent endothelial NO production (54). Asymmetric dimethylarginine (ADMA) is an endogenously derived competitive antagonist of NO synthase, whose levels are elevated in subjects with CV risk factors and are associated with a reduction in NO bioavailability in both animal and clinical studies (55). Because ADMA levels have been linked to preclinical atherosclerotic disease and an adverse outcome, they may well prove to be a useful measure of endothelial status and a potential marker of risk in clinical practice (56). At present, however, the assay remains challenging and expensive.

Circulating endothelial cells (CEC) that detach in the context of endothelial activation and loss of integrity can be measured in the circulation by both flow cytometry and a combination of magnetic bead selection and fluorescent microscopy (57). Elevated circulating microparticles have been seen in a variety of conditions associated with endothelial activation or apoptosis (58). Their function is unclear, but it may not reflect only the state of the endothelium.

Circulating endothelial progenitor cells (CEPC) can be characterized by the expression of characteristic surface markers, which are detectable by flow cytometry, but the specificity of these measurements is controversial since a wide range of hematopoietic progenitor cells, which include abundantly present myeloid precursors, has the potential to adopt an endothelial phenotype (59). Further methods characterize CEPC biology (quantification of the differentiating potential into an endothelial cell phenotype, determination of functional characteristics, such as migration toward a chemical stimulus adhesion, formation of vascular tubules, and ability to attenuate ischemia in animal models) (60).

Measurement of CEC and CEPC levels provides a novel and exciting means to follow the determinants of endothelial injury and repair. Although the balance of these two cell populations has already been linked to other *in vivo* measures of EF, and has been shown to be associated with future cardiovascular events (61), these novel measures still remain far from clinical use. Circulating markers of EF have been tested in autoimmune diseases such as RA and SSc with controversial results. Del Papa *et al*. described significantly higher levels of CEPC and CEC in a cohort of patients with early SSc (62). By contrast, Kuwana *et al*. did not confirm these findings, describing a reduced number of CEPCs and an impaired capacity to differentiate into endothelial mature cells in SSc patients (63). More recently, Allanore *et al*. found higher CEPCs levels in SSc patients than in ostheaortritis patients but lower than in RA patients (64). CEC levels resulted also high both in SSc and RA patients but did not correlate with CEPCs levels (64). A decreased number of CEPCs was described in RA patients with higher activity disease (65) and in a small group of young RA patients with low disease activity and impaired EF, as assessed by forearm blood flow technique (66). Finally, it has been hypothesized that CEPCs depletion in RA patients could be related to the accumulation of the ADMA, suggesting a role of NO in CEPC mobilization and survival (67).

Clinical significance of endothelial dysfunction

An alteration of EF precedes the development of atherosclerotic changes and can also contribute to the development of atherosclerotic lesions and later clinical complications. Thus, reduced EF is associated with most of the CV risk factors (aging, menopause, smoking, hypertension, dyslipidemia, diabetes) (68) and with the presence of early atherosclerosis (69). Moreover, in patients with established atherosclerosis, disturbed vasomotion associated with endothelial activation may contribute

to transient myocardial ischemia or changes in plaque composition and biology, which may influence plaque stability (7). These hypotheses are supported by the results of several longitudinal studies showing that a single measurement of EF in both the coronary and peripheral (macro- and micro-) circulation can be of prognostic value in a number of different clinical cohorts, which include patients with established coronary disease and at high CV risk (7). FMD may have a prognostic significance especially in low risk population, since recent meta-analysis showed that the relationship between brachial artery FMD and 10-year "Framingham risk score" was more evident in low risk (only one CV risk factor) as compared to moderate-high risk population (70).

Impaired EF can be restored or improved by non pharmacological and pharmacological treatments in patients with CV risk factors or diseases (71, 72). However, only one study performed in post-menopausal hypertensive women showed that EF improvement is associated with a better CV prognosis (73). In this study, FMD was evaluated before and 6 months after antihypertensive treatment. At the end of treatment two groups of patients were identified: those with no changes in FMD and those with an improvement of at least 10% from baseline After a mean follow-up of 67 months, cardiovascular event rate was significantly lower in the group of patients whose FMD was improved as compared to the other subgroup.

As far as systemic autoimmune diseases are concerned, the few studies assessing EF in the microvasculature have shown the presence of reduced endothelium-dependent vasodilation, while no agreement exists on the presence of an alteration in the endothelium-independent response. Unfortunately, these studies did not explore the presence of an altered NO availability. Moreover, the very limited number of the patients enrolled did not allow any clinical and serological correlations. On the other hand, in the macrocirculation the majority of studies have shown a selective impairment of endothelium-dependent vasodilation, while endothelium-independent response appears unaffected by the diseases. These data strongly underline the different information that could be obtained by different techniques and suggests their combined use in prospective cohorts. No correlations have been observed between FMD and clinical and serological variables, and no major differences were observed when comparing patients with active and inactive disease, although improvement has been frequently observed after therapy. As it might be expected FMD is inversely related with the presence of an increased carotid IMT, indicative or premature atherosclerosis.

Limitations of the available studies could still reside in the small number of enrolled patients, but also in the heterogeneity of patients included (active and inactive disease, different type of treatment and disease duration, pre-existent CV diseases). Thus, the prognostic significance of an altered EF in patients with autoimmune diseases still appears to be unclarified. Longitudinal studies could help in assessing the predictive significance of this test for the future occurrence of CV diseases.

Conclusions

Endothelium plays a central role in the maintenance of vascular homeostasis and many data suggest that alteration of its function may precede the development or aggravate atherosclerotic disease. Tests have been developed to study EF *in vivo*, able to assess its changes in relation with traditional CV risk factors as well as after therapy.

As far as the methodologies are concerned, in view of the complexities of endothelium, EF assessment is very difficult and results represent the interaction of several endothelial pathways. No single test currently available is specific for the vascular district tested or the risk factor/diseases considered, and a panel of several tests is therefore needed to characterize the multiple aspects of endothelial biology.

It is important to underline the concept that the study of vascular reactivity in humans needs rigorous experimental conditions, such as the population to be enrolled. Indeed, since it was clearly demonstrated that ageing is a major condition associated with endothelial dysfunction (74, 75), it is crucial that different populations selected are matched for age profile. In addition, it is important take into account all those CV risk factors, together with any concurrent pharmacological therapy, which could *per se* influence EF (68).

Studies in systemic autoimmune diseases have evaluated EF both in peripheral micro and macrocirculation. An altered EF has been observed, and a reduced NO bioavailability has been demonstrated in the microcirculation. Changes of FMD after therapy have also been described, however no clear correlation with clinical and serological variables have been obtained so far, nor the prognostic significance of endothelial dysfunction for the future development of atherosclerosis been clarified. Further longitudinal studies appear therefore necessary to clarify this issue.

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