Proposal of a Cut-off Value to Identify by Non-invasive Ultrasound Measurement Patients With Endothelial Dysfunction

Short title: Non-invasive cut-off for endothelial dysfunction

Arturo PUJIA, MD, Roberto CERAVOLO, MD, Gaetano GORGONE, MD,

Giorgio VENTURA, MD, Vittorio EMANUELE, MD, Tiziana MONTALCINI, MD,

Giorgio SESTI, MD, Francesco PERTICONE, MD

Chair of Internal Medicine and Cardiovascular Diseases Unit

Department of Medicina Sperimentale e Clinica *"G. Salvatore"*,

University of Catanzaro *Magna Græcia*, Italy.

Address for correspondence: Francesco Perticone, MD

Department of Medicina Sperimentale e Clinica

Policlinico Mater Domini - Via Tommaso Campanella

88100 - Catanzaro (ITALY)

Phone: +39-0961-712264 Fax: +39-0961-775564

E-mail: perticone@unicz.it

Total words:4502

Search code: 95

Condensed abstract

Endothelial dysfunction, associated to cardiovascular risk factors, can predict an unfavourable prognosis in patients at risk for atherosclerotic manifestations. In 25 outpatients we compared two methods – strain gauge plethysmography and flow mediated dilation (FMD) – usually employed for the evaluation of endothelial function, to assess a possible relatioship among them and to find a FMD cutt-off. We found a good accuracy between tha above indicated methods; thus, the non-invasive FMD represents an useful tool in evaluating endothelial function.

ABSTRACT

Background: Impairment of endothelial function, found in presence of some cardiovascular risk factors represents the earlier vascular modification preceding the appearance of atherosclerotic lesions. In addition, on the basis of recent human studies, the presence of endothelial dysfunction appears to be able to predict future cardiovascular events. Assessment of endothelial function is based on invasive or non-invasive procedures. However, the last method was never used in clinical outcome trials. Thus, the aim of the present study was to compare the strain-gauge pletismography to the flow mediated dilation (FMD)

Methods and Results: We assessed endothelium-dependent and -independent vasodilation in 25 volunteers (aged 30-69 years) by strain-gauge pletismography an by ultrasound methods, applying the protocols described by Panza et al and by Celermajer et al respectively. We found a linear and significant relationship (r=0.06, p < 0.001) between the two methods. Besides a post-ischemic FMD of 7% (in males) and 10% (in females) was found to provide high sensibility, specificity, as well as a good predictive positive and negative value.

Conclusions: The proposed FMD cut-off allows to identify in a non-invasive manner patients with endothelial dysfunction who are at risk for future cardiovascular events.

Key words: Endothelium; ultrasonics; cardiovascular risk factors

Endothelial dysfunction, characterized by loss of endothelium-dependent vasodilation, occurs early in vascular disease and may be caused by a decreased production, an increased degradation or a less sensitivity to nitric oxide (NO) (1-4). Major risk factors for atherosclerotic vascular disease (e.g., hypertension, smoking, diabetes, obesity, and hypercholesterolemia) (5-9), and several other conditions (10-15) have been associated with endothelial cells dysfunction. Besides, more recently, it has been reported that both coronary (16,17) and forearm (18) endothelial dysfunction may predict long-term atherosclerotic disease progression and cardiovascular events rate. Thus, it might be clinically relevant to evaluate the endothelial function in individual patients. This indication may be supported by results of studies of lipid lowering and ACE-inhibitors treatment suggesting that improvement of endothelial dysfunction provides the link between experimental observations and early reduction in cardiovascular events obtained in secondary (19-21) and primary (22) prevention trials.

Initially, endothelial function in humans was assessed by intracoronary study (23) and by strain-gauge plethysmography (5) for direct measurement of forearm blood flow (FBF) during intra-arterial infusion of acetylcholine (ACh) or other vasodilating agents. Plethysmographic method, unless at low cost, has several limitations. As a matter of fact, it requires arterial catheterization at least for two hours. Furthermore, the patients should keep the arm bandaged for several hours to avoid local bleeding, even if there is a low risk for haematoma; after the procedure the patient may have some difficulties to drive and to repeat the examination. For these reasons this procedure is not well accepted by all patients.

To overcome these limitations Celermajer and coworkers proposed a non-invasive detection of endothelial dysfunction based on assessment of flow-mediated dilation (FMD) after reactive hyperaemia in the brachial or femoral artery using high resolution ultrasound (24). The major advantages of this methods are the non-invasive nature, the possibility to repeat multiple tests in the same patient or to evaluate large cohorts of subjects, the low cost, and the brief duration of the examination. Brachial artery FMD has been shown to correlate with measurement of coronary endothelial function (25) and to detect submillimeter differences in vessel diameter in phantom experiments. Finally, FMD shows a low variability and a good reproducibility (26). In addition, FMD has been successfully employed in human studies to evaluate patients with different cardiovascular risk factors such as hypercholesterolemia (24), diabetes mellitus (27) or smoking (28). However, this method was not compared to others and, thus, no cut-off value exists to simply discriminate between normal subjects and patients with endothelium dysfunction. Therefore, we have designed the present study to compare FMD with strain-gauge plethysmography and to assess a non-invasive cut-off value of normality.

METHODS

Study Population

For this study, we recruited 25 volunteers (12 men and 13 women) aged 32 to 69 years (mean ± SD = 49.8 ± 9.8), referred to Catanzaro University Hospital to evaluate their cardiovascular risk profile because of hypertensive status, hyperlipidemia or diabetes mellitus. All patients were Caucasian and underwent physical examination and review of their medical histories. At the time of vascular evaluation, none of the patients had peripheral vascular disease, coagulopathy, or any disease predisposing them to vasculitis or Raynaud's phenomenon. The following risk factors for atherosclerosis were assessed at the time of first evaluation: glucose, cholesterol, triglyceride, smoking, a positive family history for CAD. The body mass index ranged between 21 and 40 Kg/m^2. All participants had never been treated with vasoactive drugs.

The local ethics committee approved the study, and all participants gave written informed consent for all procedures.

Strain-gauge Plethysmography

All studies were performed in the morning after subjects had fasted overnight, with the subjects lying supine in a quiet, air-conditioned room (22 to 24°C); the protocol previously described by Panza et al. (5) and subsequently used by our group (8,13,14,18) was employed for the present study. Subjects were requested to refrain from smoking at least 4 hours before starting the examination. All patients underwent measurement of FBF and blood pressure during intra-arterial infusion of saline, ACh, and sodium nitroprusside (SNP) at increasing doses. All participants rested 30 minutes after artery cannulation to reach a stable baseline before data collection; measurements of FBF and vascular resistance (VR), expressed in units (U), were repeated every 5 minutes until stable.

Endothelium-dependent and endothelium-independent vasodilation were assessed by a dose-response curve to intra-arterial ACh infusions (7.5, 15, and 30 µg · mL^{-1} · min^{-1}, each for 5 minutes) and SNP infusions (0.8, 1.6, and 3.2 µg · mL^{-1} · min^{-1}, each for 5 minutes), respectively. According to findings previously reported by Panza et al. (5), we set the cut-point of the peak increase of ACh-stimulated FBF at 400% of increase. Therefore, we considered patients with normal endothelium-dependent vasodilation those showing a FBF increase >400%.

The sequence of administration of ACh and SNP was randomized to avoid any bias related to the order of drug infusion. The drug infusion rate, adjusted for forearm volume of each subject, was 1 mL/min.

Brachial Artery Ultrasound

Brachial ultrasound studies were performed at least two hours before the evaluation of vascular function by strain-gauge plethysmography. All ultrasound studies were conducted by the same experienced examiner (G.G.) using a high resolution ultrasound machine (ATL, HDI Ultramark 9) with a 7-to-10 MHz linear array transducer. For measuring vascular reactivity (endothelium-dependent and endothelium-independent arterial dilation) we used the ultrasound method previously reported by Celermajer et al. (24). Patients rested in the supine position for at least 10 min before the study and were kept in this position during the procedure. Blood pressure was monitored at 2-min intervals, and ECG leads were attached to the ultrasound recorder for on-line continuous heart rate monitoring. Longitudinal images of the brachial artery were obtained proximal to the antecubital fossa wherever the best ultrasound image could be obtained. Operating variables of the machine were kept constant during each study. Transmit focus zones were set approximately to the depths of the anterior and posterior vessel walls. Images were magnified, whereas depth and gain settings were set to optimize the image of the vessel wall, in particular, the media-adventitia interface *m* line, as previously described (24). When an adequate transducer position was obtained, the skin was marked and the arm kept in a constant position.

In all patients, longitudinal scans of the brachial artery were obtained before and during reactive hyperemia induced by inflation of a pneumatic tourniquet placed on the forearm to a pressure of 250 mm Hg for 4 min, followed by release inducing increased flow-producing endothelium-dependent dilation, and after 400 µg sublingual nitroglycerin (an endothelium-independent dilator). The flow-mediated vasodilator response to reactive hyperemia was then continuously recorded (from 30 s before to 5 min after cuff deflation). Maximal brachial artery diameter (BAD) after reactive hyperemia was assessed 45 to 90 s after cuff deflation in accordance with previously described methods. The end-diastolic diameter of the vessel, defined as the distance between near-wall and far-wall junctions of the media and adventitia was measured over four cardiac cycles with the use of digital calipers. The flow-mediated vasodilation (FMD) was expressed as percent change in BAD after reactive hyperemia.

Drugs

ACh (Sigma, Milan, Italy) was obtained from commercially available sources and diluted freshly to the desired concentration by the addition of saline. SNP (Malesci, Florence, Italy) was diluted in 5% glucose solution immediately before each infusion and protected from light with aluminum foil.

Statistical Analysis

Analysis of variance (ANOVA) was performed for clinical and biological data, and the differences between means were compared using unpaired and paired Student's *t*-test, as appropriate. Simple linear regression analysis was performed to assess the relationship between the peak percent increase in FBF in response to intra-arterial infusion of ACh and FMD. Receiver operating characteristic (ROC) curves were used to choose the best cut-off value for FMD to discriminate patients with normal and abnormal endothelium-dependent vasodilation. Significant differences were assumed to be present at $P < 0.05$. All group data are reported as mean ± SD. All the statistical analysis were performed by SPSS 10.0 for Windows.

RESULTS

All patients completed the protocol study. Ultrasound scans were of good quality in all patients. Demographic and clinical characteristics of the study population, divided by gender, are summarized in table 1. There were no significant differences between the groups in age, height, BMI, waist circumference, heart rate, systolic and diastolic BP, glucose, triglycerides, total cholesterol, HDL and LDL cholesterol.

Strain-gauge Plethysmography

Baseline FBF and VR were similar in males and females (Table 1). Intra-arterial infusions of ACh induced a dose-dependent and significant increase in FBF and decrease in forearm VR. The mean percent increase in FBF resulted 354.2 $\pm$ 142.8%, ranging between 109% and 610%. Therefore, according to previous defined cut-off of normality (maximum percent increase >400%), 8 patients had normal and 17 patients had impaired endothelium-dependent vasodilation. The differences between gender detected in ACh-stimulated FBF (301.7 $\pm$ 142.3% in males vs 402.6 $\pm$ 129.9% in females) did not reach statistical significance (p=0,07) and this may be attributed to the small sample size. VR significantly decreased from 31.9 $\pm$ 13.0 to 7.8 $\pm$ 3.1 U, without any significant differences in the gender groups. During SNP infusions, a significant increase in FBF and a decrease in forearm VR were observed, confirming the normality of the endothelium-independent vasodilation (data not shown).

Both intra-arterial infusions of ACH and SNP caused no changes in BP or heart rate values.

Brachial Artery Ultrasounds

In baseline conditions, males had larger brachial artery diameter (BAD) than females (4.7 $\pm$ 0.4 mm vs 3.8 $\pm$ 0.4 mm, $P < 0.001$). In response to reactive hyperemia, the FMD mean value was 7.3 $\pm$ 2.8%. In particular, FMD was significantly higher in males than in females (5.6 $\pm$ 1.6% in males vs 8.9 $\pm$ 2.7% in females respectively). Therefore, for this reason we separately analysed the two gender groups.

No significant differences in BP and heart rate were observed during the studies

Correlational Analysis

FMD was in good accordance with ACh-stimulated FBF. Figure 1 shows the linear and significant relationship between the peak increase in FBF after ACh infusion and FMD after reactive hyperemia (r = 0.636, $P < 0.0001$), which accounts for 40.5% of the variation. The magnitude of the estimated correlation coefficient may be explained through the different mechanisms involved in vascular dilation evoked by reactive hyperemia or by muscarinic receptor stimulation.

In figure 2 we report an example of ultrasound image of brachial artery before and after reactive hyperemia and the corresponding strain-gauge plethysmographic tracings, obtained in a hypertensive patient.

The analysis based on the ROC curves demonstrated that a value of FMD less than 7% in males and 10% in females was able to discriminate between normal and abnormal endothelium-dependent vasodilation. Sensibility and specificity were 100% in males, and 83% and 86% in females. Predictive positive and negative values were 100% in males, and 86% and 83% in females. The overall accuracy of the ultrasound method was 92%.

DISCUSSION

Normal endothelium produces and releases a variety of autocrine, paracrine and endocrine substances that contribute to the regulation of vascular tone and its antiatherogenic properties (1-4). Furthermore, it is well established that major risk factors for atherosclerotic vascular diseases have been associated with an impairment of agonist-induced endothelium-dependent vasodilation (4-15). This condition, characterized by a decreased bioavailability of NO, is usually defined endothelial dysfunction.

In the last years, a variety of methods have been developed to assess endothelial function; some of these are based on invasive procedures, such as strain-gauge plethysmography (5), and other are based on non-invasive techniques, as vascular ultrasounds (24). The possibility to identify the endothelium impairment in patients at risk for adverse vascular events represents a crucial point, clinically relevant, because human studies have recently reported the prognostic significance of both coronary (16-17) and forearm (18) endothelial dysfunction. In fact, it is now well established that endothelial dysfunction represents the earlier modification, present in some cardiovascular risk factors, that precedes the physical appearance of the atherosclerotic lesions (29,30). So, taking into account these considerations, the evaluation of endothelial function might be proposed, with more caution, as a clinical index in the overall cardiovascular risk stratification of patients at risk for atherosclerotic manifestations. For measurement of endothelial function in large population, vascular ultrasound method appears more useful for its non-invasive nature, its brief duration, its repeatability and its low variability. However, contrary to strain-gauge plethysmography, the non-invasive method proposed by Celermajer et al. was never used in clinical outcome trials to establish its prognostic significance. For these reasons, we compared the two methods, using as golden standard the strain-gauge plethysmography, to achieve a FMD value able to discriminate patients with normal or abnormal vascular reactivity.

In the present study we have identified a FMD cut-off value different in males (<7%) and females (<10%) that identify patients with endothelial dysfunction. These cut-off values are characterized by a good sensibility and specificity being 100% in males, and 83% and 86% in females as well as their predictive positive and negative values being 100% in males, and 86% and 83% in females. The gender differences may be explained by different baseline BAD values that are in agreement with data previously reported by others (31). Most likely, the smaller vasodilating response to reactive hyperemia observed in males may be explained by a larger baseline BAD.

Therefore, the definition of a FMD cut-off value of normality could allow to evaluate,

More feasibly, endothelial function in large population stratifying patients at increased risk for coronary atherosclerosis. In addition, improvement in endothelial dysfunction, induced by different therapeutic modalities (32-35), emphasizes the clinical utility to extensively measure vascular reactivity. In this way, the FMD in response to reactive hyperemia may represent an useful and validated non-invasive technique that may be used as widespread routine test. However, our data, even if interesting, are preliminary and need to be confirmed by further population study.

In summary, it is well established that vascular endothelium plays a pivotal role in the appearance and progression of atherosclerotic process. Furthermore, it is of clinical importance the adverse prognostic significance of endothelial dysfunction recently reported. We hope, thus, that endothelial dysfunction evaluation will assume a prominent role in the individuation and treatment of patients at risk for coronary atherosclerotic lesions. A non-invasive method, such as FMD after reactive hyperemia, and the definition of a cut-off value, useful in the identification of patients with endothelial dysfunction, might contribute to the extensive diffusion for the evaluation of endothelial function.

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Figures and Table

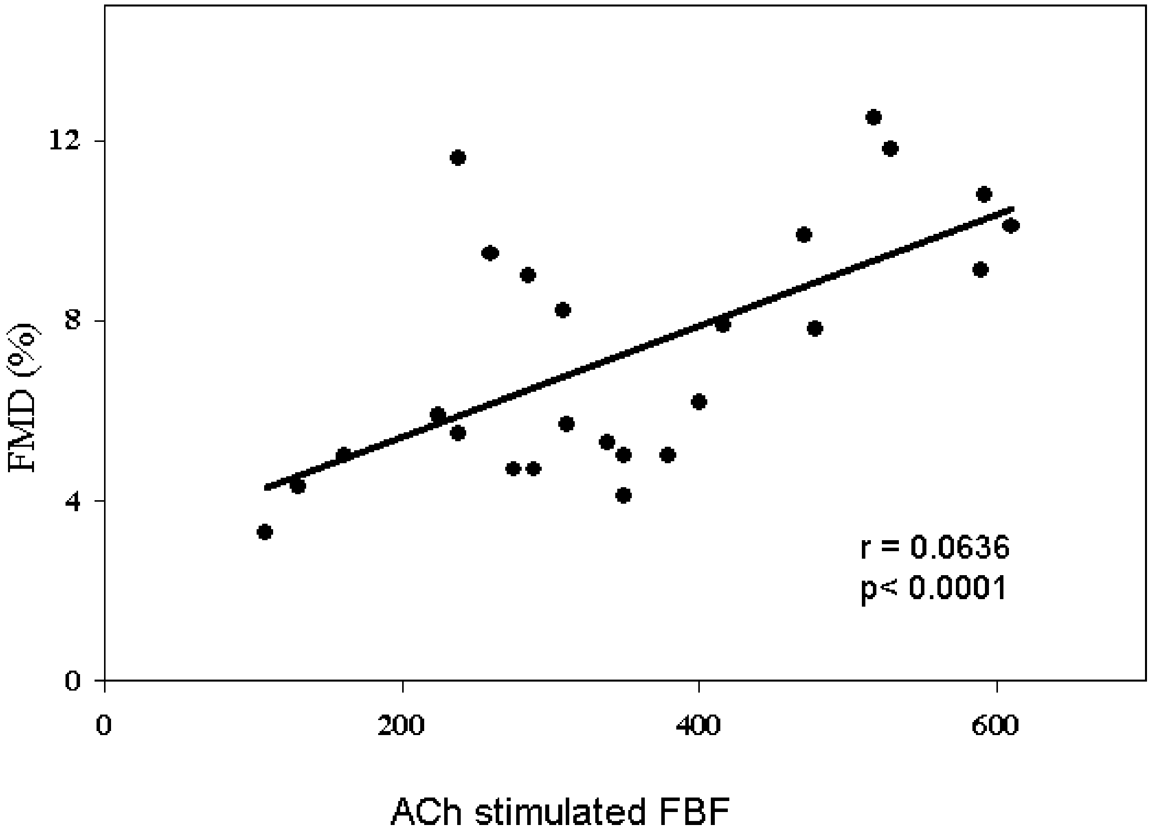

Figure 2

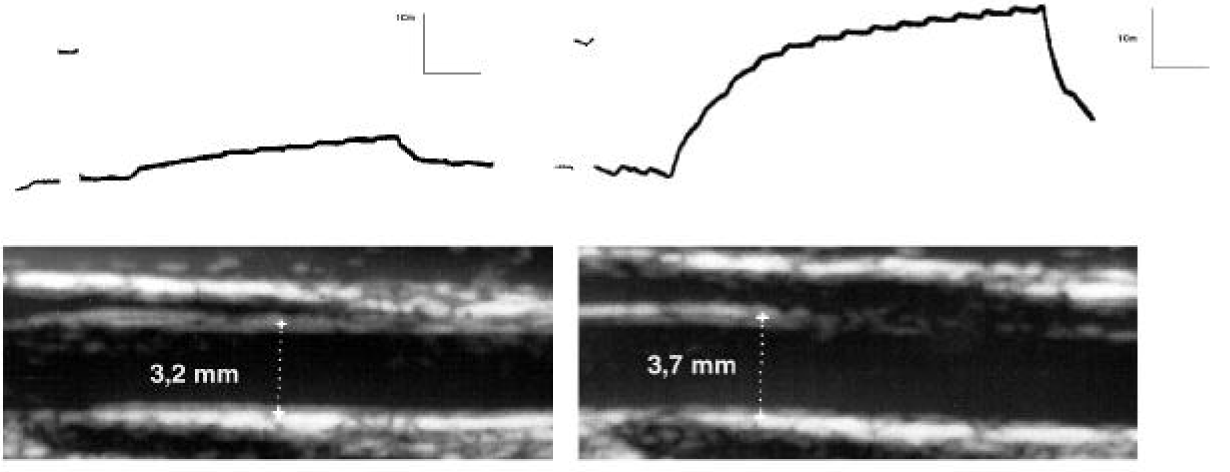

Figure 1

Table 1

Characteristics	**Total Population**		**Female (N=13)**		**Male (N=12)**		**P**
	Mean	***Standard deviation***	**Mean**	***Standard deviation***	**Mean**	***Standard deviation***	
Age (yy)	49,80	9,79	52,85	10,23	46,50	8,49	NS
Height (cm)	162,44	10,88	154,38	7,14	171,17	6,55	NS
BMI (Kg/m^2)	27,614	4,386	28,862	3,683	26,263	4,831	NS
SBP (mmHg)	128,0067	18,1779	132,0150	16,2696	123,9983	19,7776	NS
DBP (mmHg)	80,3896	9,3236	80,9842	5,9325	79,7950	12,0746	NS
Waist C (Cm)	95,78	11,74	97,92	10,49	93,45	13,07	NS
Gly (mg/dl)	106,94	33,89	101,19	29,19	113,17	38,66	NS
T Chol (mg/dl)	225,17	43,02	231,83	42,93	218,50	43,93	NS
HDL Chol (mg/dl)	49,46	14,23	54,50	11,85	43,86	15,19	NS
LDL Chol (mg/dl)	139,21	31,16	135,30	22,62	144,09	40,64	NS
Trig (mg/dl)	201,08	158,83	168,92	154,22	233,25	163,42	NS
BBD (mm)	4,2688	,6155	3,8392	,3899	4,7342	,4543	<0,001
FMD (%)	7,3134	2,7622	8,9170	2,6651	5,5763	1,6024	0,001
FBF (%)	354,16	142,76	402,62	129,95	301,67	142,35	NS