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# New assessment of endothelium dependent flow-mediated vasodilation to characterize endothelium dysfunction

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## Abstract

**T**he vascular endothelium plays an important role in the regulation of vascular tone, cell growth, inflammation and thrombogenicity. Endothelium dysfunction is then considered to promote several disorders that initiate the atherosclerosis process. The vascular tone dysfunction can be determined by high resolution ultrasonographic imaging of the brachial artery enabling to assess endothelium-dependent flow-mediated dilation (FMD). It is based on the principle that an increase in blood flow, specifically in shear stress, provokes the release of nitric oxide, and then a vasodilation that can be quantified. In this study, the brachial artery diameter evolution is continuously followed during baseline and hyperemia after forearm occlusion, using a custom designed software. Some techniques employed to measure FMD are limited by operator-dependence. We present a new automated and versatile method of flow-mediated vasodilation quantification based on B-mode echographic images and edge detection algorithms. Edges for each image in the acquired sequences are recognized as interfaces, based on the grey-level profiles of the averaged pixel values. Within-reading and within-subject FMD% coefficients of variation attained 7% and 10% respectively. This technique largely improves manual measurements and shows to be appropriate for wide clinical use.

**Key words:** Flow mediated vasodilation, Ultrasonography, Endothelium, Age

## Introduction

**T**he endothelium is a single-cell layer that plays a mechanical and biological barrier role between the blood stream and the vascular wall. This interface regulates the permeability between vascular tissue and blood cells, platelet aggregation, thrombogenicity, cellular proliferation and ultimately modulates the arterial vasomotor activity due to the release of vasoactive substances i.e. nitric oxide (NO) [1]. The later induce arterial vasodilatation that is the most analyzed function in clinical studies. Endothelial dysfunction can be understood as a state where this role is altered, inducing vascular constriction, leukocyte adherence, platelet activation, thrombosis, vascular inflammation and ultimately atherosclerosis [2].

Normally, blood vessels dilate as a response to a flow rise. More specifically, a flow increase causes higher levels of friction between blood and endothelium cells, i.e. shear stress, that release NO, synthesized from L-arginine under the influence of the enzyme NO synthase (NOS) [3]. This endothelium-derived relaxing factor is mainly responsible for vascular tone modulation, and its bioavailability reduction characterized endothelium dysfunction.

Endothelial function in humans can be assessed with several invasive [1] and non-invasive [4-7] methods. Invasively, coronary arteries can be studied under angiography or using Doppler flow measurements, to quantify the vasomotor responses of epicardial arteries to different concentrations of acetylcholine or oth-

er agonists. This invasive method, adopted as the gold standard, is restricted to patients undergoing clinically indicated cardiac catheterization. Non-invasive results in brachial arteries showed similar atherogenic risk factors than impair coronary endothelial function [7].

In this work, a complete non-invasive technique developed in our laboratory will be described. Briefly, the measure allows the assessment of endothelium-dependent flow-mediated dilation of the brachial artery caused by a reactive hyperemia induced by forearm occlusion. The complete computerized system is based on high-resolution ultrasound images and aims to reduce the operator dependence.

### Subjects

Twenty-four subjects aged  $40 \pm 10$  (12 selected from members of our staff and 12 consecutively referred to our department for cardiovascular risk evaluation) entered into the study. They were free of any treatment and free of any cardiovascular disease. After giving informed consent they were examined the morning, after 12 hours fasting, in a quiet and temperature-controlled ( $22 \pm 1^\circ$ ) room, and in the supine position for at least 10 minutes with the right arm in extension inside a cushion designed to avoid lateral movements. Blood pressure was monitored in the opposite arm by automatic device (Omron). The brachial artery was visualized longitudinally and continuously by high-resolution ultrasound (ATL 5000, Philips) with a 7-12Mhz transducer probe positioned above the elbow and fixed in a robotic arm allowing maintaining its stability [6].

### Measurements

Two consecutive maneuvers were performed for each patient. To evaluate endothelium-dependent vasodilation, and after 10 minutes baseline conditions, occlusion of flow to the forearm was provoked and maintained for 5 minutes by a cuff inflated to 250 mmHg around the upper forearm, and reactive hyperaemia was induced by sudden cuff deflation [5]. Additionally, peak flow velocity was measured during baseline and in the first 15 seconds after the occlusion release using the echograph Doppler capability.

To study a vasodilation effect independent from endothelium effects, a second measure was carried on. After 10 minutes of rest, a sublingual spray of NTG (0.3mg) was dosed to each patient, inducing an arterial vasodilation independent from the endothelium action. Images were captured during 4/5 minutes to register the diameter evolution. Additionally, and for the apparent healthy group, a second examination was repeated with one-week interval.

### B-mode scan-analysis

A custom automated system (Hemodyn 4M, Dinap SRL, Buenos Aires, Argentina) was used to acquire and process the captured images. Two sequences of artery imaging, the first during 10 seconds in base-

line conditions and the other during hyperaemia from the 15th to the 180th second following cuff deflation, were acquired for the endothelium dependent maneuver. After 10 minutes of rest, a 10 seconds and 4 minutes sequences were registered during baseline and NTG administration respectively. A personal computer (Intel 1GHz, 120Gb HD, 512Mb RAM) was equipped with a frame grabber (Data Translation 3130 series) and connected to the robotic system to integrate the measuring system. All sequences were analyzed off-line by a reader using an automated step by step algorithm applied to each image. Similar to IMT measuring systems, an interface detection algorithm was implemented to contour arterial anterior and posterior walls based on grey-level profiles [11]. The detection of wall interfaces defining artery diameter was done automatically for each image in the sequences. The edge detection algorithm worked fast, 40 ms per image, and provided automated diameter detection at each time of the cardiac cycle, resulting in continuous measurements of diameter waveform.

### Dilatation response

The beat to beat change in diastolic diameter measured at baseline and during reactive hyperaemia allowed obtaining the total brachial artery vasodilation curve response to hyperaemia (Fig. 1A). Baseline diastolic diameter was averaged on successive cardiac beats during 10 seconds. Maximum diastolic diameter during reactive hyperaemia was automatically derived from the curve. The maximum change in diameter between hyperaemia and baseline was the difference between maximum diameter and baseline diameter. FMD was calculated as the ratio between maximum change in diameter and baseline diameter, and expressed in percent change of baseline diameter. In the same way, the independent muscle relaxation, achieved after NTG, produced the equivalent endothelium-independent dilation (Fig. 1B) and was quantified with an analog procedure.

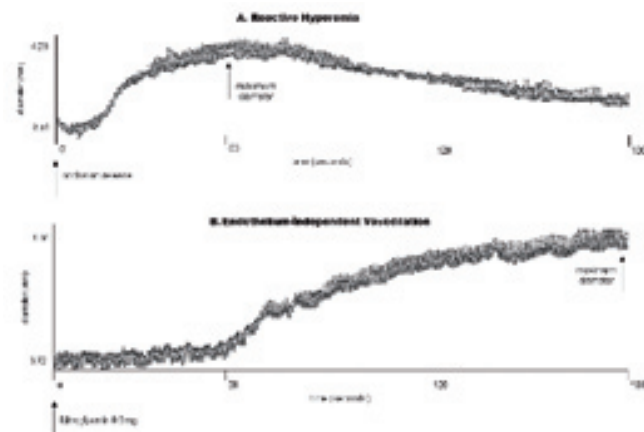


FIGURE 1 Continuous diameter detection. Baseline and post-occlusion dilations for reactive hyperemia (A) and after 0.3mg nitroglycerine (NTG) administration (B). Note that during NTG the diameter rise was slower and generally more intense with respect to reactive hyperemia.

### Statistical and variability analysis

Data are expressed as the mean value  $\pm$  SD and coefficient of variation as  $(CV=[SD/mean] \times 100\%)$ . Linear regression analysis were performed using least square method. Group differences were determined by Student t test. Diameters were measured in millimeters and FMD in percentage. Two independent readers measured the control group sequences to achieve the within-reading variability. One-week variability was calculated for the control and patient group respectively.

In the overall population FMD was  $5.9 \pm 3.0\%$ . A negative linear relationship was found between FMD and age ( $r = 0.48$ ;  $p < 0.01$ ) (Fig 2). Using the median age as a threshold (42 years) the subjects were divided into two groups. No differences in gender, body mass index and in baseline brachial artery diameter were observed in younger as compared to older subjects (Table 1). Slight higher systolic and diastolic blood pressure were shown in older than in younger subjects ( $p < 0.05$ ). Flow-mediated-vasodilation was higher in younger compared to older group ( $7.6 \pm 3.0\%$  vs.  $4.4 \pm 2.3\%$ ;  $p < 0.01$ ). The FMD differences between younger and older subjects persisted after adjustment for blood pressure. Nitroglycerin administration induced brachial artery dilations  $>10\%$  in all cases and differences were no significant between groups.

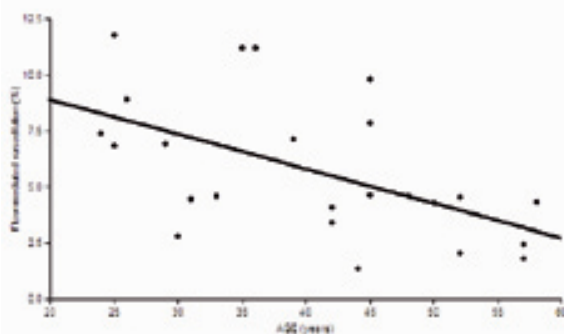


FIGURE 2 Correlation of flow-mediated vasodilation (FMD%) with age in 24 subjects included in the study. ( $r = -0.48$ ;  $p < 0.01$ ).

Table 1. Characteristics of the Study Groups

	Younger	Older
Number	11	13
Age (years)	$30 \pm 5$	$49 \pm 6^{**}$
Male gender (%)	55	54
BMI (Kg/m <sup>2</sup> )	$22 \pm 3$	$24 \pm 3$
SBP (mmHg)	$116 \pm 7$	$122 \pm 8^*$
DBP (mmHg)	$69 \pm 5$	$77 \pm 8^*$
BAD (mm)	$4.1 \pm 0.6$	$4.0 \pm 0.7$
FMD (%)	$7.6 \pm 3.0$	$4.2 \pm 2.3^{**}$

Data are mean  $\pm$  SD. SBP=systolic blood pressure, DBP=diastolic blood pressure, BAD=baseline brachial artery diameter, BMI=body mass index, FMD= flow-mediated vasodilation. \* $p < 0.05$ , \*\* $p < 0.01$

In the 12 selected members of our staff, two independent readers measured the same scan sequences to achieve the within-reading variability. They were 7.9% for FMD, 0.40% for SD% and 7.1% for the coefficients of variation. To evaluate reproducibility the examination were repeated two times at one-week interval. The FMD was 8.2%, the SD 0.7% and the coefficients of variation 9.8% respectively.

The objective of the present work is to present a complete automatic system to non-invasively quantify the flow mediated dilation of the brachial artery. The reproducibility of the methodology was analyzed using 2 independent readers that measured the same scans for the members of our staff. This within-reading variability of FMD was near 8%, a normal value for apparent healthy subjects reported in the literature [5]. The coefficient of variation (7.1%) was lower than other manual measurements [8] and similar to reported fully automatic systems [9]. Repeatability was evaluated within one-week interval in the same group demonstrating very good reproducibility rate. This can confirm the independence of the reader and the very small dispersion introduced by the operator in the whole manipulation.

It is well known that different factors interfere and affect vascular flow mediated dilation (FMD) including temperature, nutrition, drugs, exercise, smoking and menstrual conditions [5]. Accordingly, our population was carefully selected and all these circumstances taken into account. To ensure the reliability of the method other limiting factors should be considered, including the natural biological variability within the same patient, the study readers and operator dependence, the actual precision of the measurement system and the protocol development. Non-invasive methods to measure flow-mediated dilation normally rely on echographic B-mode images. The analysis of such images is critical depending on the reader and the technique. Most of the studies manually measure baseline diameter and maximum diameter after 1 minute of the occlusion release on static images using the cursor caliper provided by the echograph capability. Anterior and posterior walls are identified with 3-5 points and averaged to obtain an approximate diameter and to reducing the variability [7-8]. Maximum dilation is supposed to arrive around the minute of reactive hyperemia.

Errors attributed to cursor measurements, estimated to be near 1 mm [10], and maximum dilatation may occur before or later 1 minute.

The presented automated method shows several advantages. First, the complete diameter evolution, from baseline to the curve dilation, is automatically

identified and calculated. Second, the diameter detection is based on intima-media algorithms, which provide a reader independent wall contouring. Diameter can be achieved accurately with a precision less than 0.1mm and even to a sub-pixel level [11]. Third, distensibility is also available as the whole cardiac cycle, including systolic and diastolic diameter values, are calculated.

It is well accepted that endothelial dysfunction occurs in response to cardiovascular risk factors and precedes the development of atherosclerosis [4]. We observe that in our small sample population of subjects divided into two groups of age, the automated system is able to discriminate significant FMD differences between subjects. The age differences in FMD was not significantly related to systolic or diastolic blood pressure. This result is in line with several publications showing that FMD is prematurely altered by age as well as by traditional or emergent risk factors, helping to an early detection of future cardiovascular diseases. Indeed, endothelial function deteriorates with age, arterial hypertension, hypercholesterolemia, hypertriglyceridemia, low LDL, diabetes mellitus, insulin resistance, hyperglycemia, active and passive cigarette smoking, hyperhomocysteinemia, post menopause, family history of coronary disease and silent atherosclerotic pathologies [11,13-17].

More importantly, this whole endothelial dysfunction is a disorder that can be reversed [18]. The traditional strategies to reduce risk factors as the cholesterol level, hypertension, smoking, menopause treatments and exercise have proved to be efficient also to ameliorate endothelial function confirming its association with the mentioned risk factors [19]. The automated system described in the present work may be useful to reduce the number of subject to detect significant differences when responses to drugs are assessed in therapeutic protocols.

Concerning clinical applications, FMD studies should not be used to detect patients with cardiovascular risks. However, it could be useful in those patients with moderate risk levels and mostly within young population, where the reversion process can be early initiated with adequate therapeutics. The principal application of this FMD method is for clinical research studies to better understand the premature mechanisms that initiate atherosclerosis, and in clinical, epidemiological and therapeutic protocols.

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