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Dynamic Assessment of Cardiovascular Biomarkers

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Abstract

The behaviour of cardiovascular parameters during exercise remains unsettled. Arterial stiffness is one of the most promising and innovative vascular biomarkers; this vessel property is usually evaluated at rest, but, since it aims to describe a stress-strain vessel behaviour, it is not a static parameter. Hence, our aim was to develop a method able to evaluate carotid elasticity dynamically in order to investigate if this analysis can provide information regarding differences between populations (i.e. patients and healthy subjects). We developed an approach based on a contour tracking algorithm applied to ultrasound B-mode image sequences and used it in conjunction with a local pressure estimation to assess carotid distensibility. The method's reproducibility was evaluated by analyzing a group of healthy volunteers during two sessions 3 days apart. Repeatability was expressed as coefficient of variation and satisfactory results were obtained in exercise.

After testing the robustness of the technique, the approach was applied during graded bicycle semi-supine exercise session in patients with known or suspected coronary artery disease (CAD) and results were compared with a control group. 36 consecutive

patients (20 men, 61±8years), and 18 healthy volunteers (9 men, 34±3 years) were recruited. Right carotid diameter (D) and distension (ΔD) were estimated by the developed ultrasound B-mode image processing method, and central pulse pressure (PPa) by radial tonometry; then, carotid elasticity was expressed as cross-sectional distensibility coefficient (DC). Besides the vascular evaluation we introduced the estimation of left-ventricular elastance (ElvI) by echocardiography, in order to obtain a more integrated dynamic picture including arterial-ventricular coupling. All measurements were performed at rest, peak of age-dependent maximal heart rate and during recovery.

At rest, D and PPa were higher in patients than in controls, whereas no significant differences were observed in ΔD and mean blood pressure; DC and ElvI were lower in patients than in healthy volunteers. At peak mean blood pressure increased both in patients and controls; DC significantly decreased and D increased in healthy subjects but not in patients. Finally ElvI highly increased in controls but not in patients. Behaviours of the two populations during recovery were similar.

Hence, we can conclude that the developed approach provides a suitable reproducibility for clinical studies and was able to dynamically discriminate between different kind of subjects. In particular, in patients with known or suspected CAD, carotid distensibility, which at rest is lower than in healthy controls, remains unchanged during maximal exercise, despite a similar increase in mean blood pressure in the two populations. This difference is underlined also by the absence of strong cardiac response and carotid vasodilation in the pathologic population.

From our preliminary results, the importance of a dynamic assessment of carotid elasticity was confirmed, and there is evidence of a clinical need including a simple and robust device to more easily perform this kind of analysis than by ultrasound. In our lab a first prototype based on vibration approach was designed and might be the suitable solution for implementing low-cost and easy carotid elasticity dynamic evaluation.

In the future, the cardiologist ambulatory might provide, besides cardiac and pressure monitoring, additive relevant clinical information from an arterial elasticity 24-hours device.

To my family: the apples and the trees from which they are born!

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Introduction and Aim

Cardiovascular disease is one of the major causes of mortality in the developed countries even though several cardiovascular risk factors, such as aging, smoking, hypercholesterolemia, diabetes mellitus and hypertension are well known today. This kind of disease may result in substantial disability and then largely contributes to the cost of the health care system. Efficient primary prevention, which includes the assessment, management, and follow-up of patients who risk cardiovascular diseases, is the best approach to the problem. However, the assessment of classical risk factors cannot offer an accurate estimate of the

probability that a subject will suffer a cardiovascular event.

For this reason, the number of studies that propose methods for the evaluation of markers of subclinical cardiovascular disease have been increased significantly in the last years and these measurements proved to be independent predictors of cardiovascular events [1, 2, 3].

Among these great emphasis has been placed on the role of vascular parameters assessed by ultrasound data due to the non-invasiveness and low-cost of this technique. By processing ecographic data estimation of endothelial function by Flow-Mediated Dilation (FMD) [4], and analysis at the level of the carotid artery by Intima-Media Thickness (IMT) [5] and arterial stiffness [6], can be obtained. In particular, FMD ad IMT can be evaluated by ultrasonography, whereas indices of local arterial stiffness of superficial arteries can be estimated by measuring the diameter change during the heart cycle from ultrasound data in conjunction with the local pulse pressure.

The latter analysis, which is considered one of the most promising from a clinical point-of-view, is usually estimated from resting values; however, arterial stiffness is not a static characteristic but a function of pressure

and therefore, parameters of local elasticity quantified in-vivo over a range of pressures could enhance the characterization of the elastic properties of the vessel. Recently, some authors [7] have estimated the arterial elastance index during exercise in patients with hypertension suggesting a gender related difference in dynamic arterial stiffness. Others [8] have characterized carotid distensibility via the isometric exercise pressor and they hypothesized that differences in vascular function with gender and age may only be recognized if arterial elasticity is quantified over a range of pressures.
Besides the attention in dynamically evaluating the arterial elasticity, another recent hypothesis, based on the translation of flow-mediated dilation [9] assessment from the brachial artery to a less peripheral, larger and predisposed to atherosclerosis vessel, sounds interesting: some authors studied the carotid artery reactivity to hand grip (isometric exercise) by measuring the change in the vessel diameter following the stress with respect to the baseline, and concluded that this might be associated with coronary risk status [10]. Dynamic exercise, as well as hand grip increases sympathetic tone and is by far the most used stress test. The aim of the present project was to investigate the carotid artery's response to dynamic exercise during

exercise stress echocardiography, both in terms of diameter and local elasticity parameters.
During the first phase of the project the methodology was developed and then tested in a small group of healthy volunteers in terms of reproducibility. Subsequently, a clinical experimentation was performed; patients with known or suspected coronary artery disease (CAD), and healthy volunteers were analyzed in order to investigate if the assessment of dynamic parameters might allow a better characterization of the subjects and stronger discrimination between different populations, than a static evaluation.

Progress beyond state of the art

Indeed, although several studies reported an independent predictive value on cardiovascular events for arterial stiffness evaluated at rest, only a few studies, where parameters of vascular elasticity and reactivity were assessed dynamically, are available in literature. Hence, the originality of this project is due to the fact that it could provide analysis of the carotid site before, during and after exercise in order to obtain, at one time, a non-invasive and integrated picture of static and dynamic characteristics of this central district.

CHAPTER 1

Ultrasound Vascular Biomarkers

Part of this chapter is obtained from the paper "Functional and structural alterations of large arteries: methodological issues." ***Bianchini E****, Giannarelli C, Bruno RM, Armenia S, Landini L, Faita F, Gemignani V, Taddei S, Ghiadoni L.Curr Pharm Des. 2012 Nov 19.*
The paper is reported in Appendix A.

Cardiovascular disease is one of the major cause of mortality and morbidity worldwide. The prevention of this disease, which kills more people in the United States than do cancer, AIDS and car accidents together (data source: www.shapesociety.org) is a priority in public health. It is widely accepted by the scientific community that the description of the cardiovascular status of a person based only on classic risk factors such as smoking, obesity, hypercolesteromia and hypertension, is not enough in terms of clinical effectiveness nor in terms of cost to the NHS.
The ability to identify a "vulnerable" patient can be increased by the introduction of innovative biomarkers,

such as those based on laboratory analysis and omics techniques, or the ones objectively measurable on biomedical signals and images. This approach, beside diagnostics and pharmacology, is particularly important when applied to implement a model for effective prevention able to detect the presence of vascular damage at the sub-clinical, asymptomatic stage preceding the onset of decades of disease. In this way effective screening and prevention can be obtained, that reduce the transition of the disease to deadly (for the patient) and expensive (for the National Health Service - NHS) clinical stages.

Of the various cardiovascular biomarkers introduced and studied in the scientific literature in recent years great emphasis has been placed on parameters that can be assessed by ultrasound data, since this approach is widespread, non-invasive, feasible and non-ionizating. Thus, great emphasis has been placed on the role of markers such as brachial Flow-Mediated Dilation (FMD), carotid Intima-Media Thickness (IMT) and arterial stiffness [1,2,3,4]. An accurate, robust and reliable methodology for the evaluation of these markers could improve their predictive value and allow them to be used for analysis in a large population.

1.1 Carotid intima-media thickness

1.1.1 Clinical aspects and prognostic values

Carotid intima-media thickness (C-IMT), as measured by high resolution B-mode ultrasound of extra-cranial carotid arteries, is the most widely accepted non-invasive marker of subclinical atherosclerosis. C-IMT is considered an intermediate phenotype of atherosclerosis suitable for use in large-scale population studies [11]. Increased C-IMT has been associated with augmented cardiovascular risk [12, 13] as well as with presence of advanced atherosclerosis at different vascular sites including peripheral, cerebral and coronary areas [14,15]. Most importantly, epidemiological studies, including the Atherosclerosis and Risk in Communities Study (ARIC), the Rotterdam Study and the Cardiovascular Health Study, have consistently reported the predictive value of C-IMT for myocardial infarction or stroke independent of traditional cardiovascular risk factors [16-21].

1.1.2 Methodological and technical issues

C-IMT is included in the American College of Cardiology and American Heart Association guidelines as a class IIA recommendation for intermediate risk patients [22]. However, several methodological aspects should be

taken into consideration for its correct evaluation as recently suggested by an expert's review [23] since C-IMT value can be influenced by location of the measure, type of ultrasound data and features of the reading system.

A careful examination of previous studies on C-IMT reveals methodological discrepancies that must be taken into account for a proper interpretation of results. Inaba et al. [24] observed that 77% of the studies included in their meta-analysis did not indicate whether plaques were actually included in C-IMT analysis. In addition, 63% of the studies used maximal C-IMT, more likely reflecting focal thickening or plaque, instead of mean C-IMT. Furthermore, study design of C-IMT trials was heterogeneous since the definition of the landmarks of carotid segments (Common Carotid Artery, CCA, Carotid Bulb, CB or Internal Carotid Artery, ICA) selected to measure C-IMT differ significantly [25]. The far wall of CCA is the easiest of the three anatomical segments to examine, being the most commonly used measurement in clinical studies. Unfortunately plaques are rare at this site and studies of the relationship of C-IMT at this site are conflicting [26]. The carotid artery is a complex vessel, with differing associations for each segment regarding risk factors and outcome. Common

carotid C-IMT is a better predictor of stroke than myocardial infarction [27, 28] and shows a better correlation with left ventricular mass than with coronary artery disease [26].

These data suggest that different pathological processes occur at distinct vascular sites of the carotid artery in different stages of disease. Indeed, the hemodynamics of the carotid artery in its different segments explains why atherosclerotic plaques are located in the carotid bulb and internal carotid artery than in the common carotid segment, which is affected in more advanced stages of the disease [29].

Several studies suggest the CCA far wall as the best location in terms of feasibility and reproducibility of the measure [30, 31] and this has been considered as the standard segment for the evaluation.

However, it might also be interesting to investigate whether information provided at CB or ICA, more challenging from a technical point of view, may show stronger correlation with classic risk factors [31]. Another point to take into consideration specifically referring to CCA is the variability in morphology and in vessel appearance under pathology. In particular, a horizontal image of the carotid artery cannot always be obtained depending on the anatomy of the subject, and

this may be a problem for some automated segmentation techniques [32].

A post-hoc analysis to determine the best algorithm for determining CIMT using data from the METEOR study showed that ultrasound protocols that include CIMT measurements at multiple angles of both the near and far walls provide the best balance between reproducibility, rate of CIMT progression, treatment effect and their associated precision in this low-risk population with subclinical atherosclerosis [33].

Regarding the ultrasound data that can be used to obtain C-IMT evaluation, two main types are commercially available: B-mode image processing-based device and Radio-frequency (RF)-based echo-tracking system [32]. In the past, estimation was obtained manually, but currently the assessment of C-IMT is generally obtained by automatic processing of these ultrasound signals [32]. RF data devices are considered very accurate since they are based on signals with higher spatial resolution than B-mode data [34, 35]. However, when comparing the performance in terms of reproducibility of this kind of technique with that of robust image-based systems, similar results are obtained [32, 36]. The repeatability of the two approaches was recently tested in the same population,

obtaining similar coefficients of variation (5% for RF-based device and 6% for image processing systems, respectively) [36]. A good agreement between the two techniques in terms of Bland-Altman statistics was also reported. However, it must be pointed out that the quality of the final result of B-mode based systems is related to several issues which have to be carefully considered, such as quality of the scans and the system's setting. In particular, Potter et al. [37] studied the effects of changing dynamic range (DR), gain set and probe distance in C-IMT assessment by an image analysis software applied to an agar phantom. An increase in DR or gain causes a reduction in the measured wall thickness, whereas the distance of the probe did not influence the final result. Hence, DR and gain sets, but also other parameters such as depth gain compensation (DGC) or filtering should be standardized as suggested by international guidelines [38] or at least documented in follow-up analysis. Furthermore, Rossi et al. [39] analyzed the influence on carotid diameter evaluation of non-linear processing generally used in standard ultrasound equipment for better image visualization. In particular, these authors show that logarithmic compression and saturation can cause alteration when using approaches based on the gray

level gradient, and the consequent small deviation might affect also the assessment of C-IMT. Another point to consider when working with standard US equipment is whether values obtained with newer apparatus are comparable to those obtained with older ones. A recent paper [40] reported the effects of transducer frequency on the final result by semi-automated analysis in a small group of patients. In particular C-IMT measurements obtained with standard (8 MHz) and high (14 MHz) frequencies were comparable.

Other features of the reading systems should be also considered for the assessment of C-IMT. Low-cost and user-friendly devices can make the diffusion of this vascular biomarker easier and faster. Hence, reliable and robust software based on B-mode image processing, which can be adopted with any standard ultrasound equipment, could provide an effective solution. Nevertheless they should be used according to international guideline suggestions, with particular attention to machine settings. Finally, it is worth noting that despite guideline suggestions introducing standardization in the measure, different approaches are available for C-IMT estimation in terms of analyzed data, (i.e., B-mode images or RF signal processing) or for anatomical sites. Thus, future analysis providing the

agreement between different kinds of measurements and reference values for risk classification are needed in order to improve the clinical implications of C-IMT assessment.

1.2 Carotid distensibility and stiffness

1.2.1 Clinical aspects and prognostic value

Arterial distensibility is a measure of the artery's ability to expand and contract with cardiac pulsation and relaxation. Hypertension and other risk factors such as diabetes, dyslipidemia and smoking can alter the structural and functional properties of the arterial wall, leading to a decrease in arterial distensibility. This seems to be a common pathologic mechanism for many factors that lead to the occurrence and progression of the vascular changes associated with cardiovascular disease [35]. The aorta is a major vessel of interest when determining regional arterial stiffness, for at least two reasons: the thoracic and abdominal aorta makes the largest contribution to the arterial buffering function, [35] and aortic stiffness is an independent predictor of outcome in a variety of populations [8]. The measurement of aortic stiffness as carotid-to-femoral pulse wave velocity (PWV) by arterial tonometry is generally accepted as the most simple, non-invasive, robust, and reproducible method to determine arterial

stiffness. However, it should be recognized that carotid-femoral PWV is not a direct measurement, since it is based on the acceptance of a propagative model of the arterial system. Thus, other arterial sites have potentially more interest: the measurement of local carotid stiffness may also provide important prognostic information, since the carotid artery, which is a superficial vessell that can be easily analyzed, is a frequent site of plaque formation.

1.2.2 Methodological and technical issues

Arterial stiffness can be estimated at the systemic, regional and local levels [35]. The local measure is generally obtained at the common carotid site, a large superficial artery that is easily accessible; this evaluation is considered particularly accurate, since unlike the systemic and regional evaluation, in this case arterial stiffness is determined locally and is estimated directly by pressure changes, which in turn determine the changes of volume of the vessel. The local assessment can be obtained by measuring the diameter of the vessel and its variations during the cardiac cycle (stroke change in diameter or distension) by ultrasound signal in conjunction with local pulse pressure estimation by tonometry [35].

With ever-increasing attention focused on the clinical implications of arterial stiffening analysis, it is extremely important to take into consideration methodological aspects, regarding arterial diameter assessment by ultrasound influencing clinical study outcomes. Several factors should be considered when performing this kind of measurement, especially accuracy, precision and feasibility.

Two main approaches are available for arterial diameter assessment by ultrasound data: B-mode image processing based device [36, 41] and radio-frequency (RF)-based echo-tracking system [34]. Devices processing RF data are considered very accurate since they are based on signals with higher spatial resolution than B-mode data [35]. Furthermore, when adopting B-mode based systems some issues should be considered since the accuracy of this kind of device depends on many aspects such as the quality of the scans, and can be influenced by the system's setting. In particular, Potter et al [37] showed that dynamic range (DR), gain set and probe distance alter lumen diameter values obtained by an image analysis software applied to an agar phantom; an increase in DR, gain or distance causes a reduction in the measured diameter value. Hence, DR and gain sets, but also other parameters

such as depth gain compensation or filtering should be documented and replicated when performing follow-up analysis on the same subject, and consensus guidelines adopted. As regards the distance's influence on the final measurement, the authors suggest keeping in mind this aspect when interpreting the reported effects of weight changes on arterial diameter. Furthermore, Rossi et al. [39] showed that non-linear processing used to improve the B-mode image visualization on standard ultrasound equipment could affect the diameter measure obtained by edge-detection algorithms. In particular, the authors show that logarithmic compression and saturation can cause alteration when using approaches based on the grey-level gradient. Another aspect should be taken into consideration when using methods based on edge-detection for diameter assessment on ultrasound images: the location of a grey-level discontinuity corresponding to an artery interface depends on the mathematical operator adopted and on its particular configuration. Consequently, it is possible that an edge detector converges to a point slightly different from the real localization of the interface [36]. This issue is relevant when tracking the two walls of a longitudinal section of the vessel in order to compute the diameter; in fact, in this case the grey-level discontinuities of the

near and far border respectively are in opposite directions, and different convergence points result in different measures. On the other hand, when evaluating distance where the grey-level discontinuities corresponding to the two edges are in the same direction, like the C-IMT evaluation, the possible different point of convergence does not influence the resulting measurement. A similar consideration can be drawn regarding distension, since it is computed as the subtraction of two diameter values and hence is not influenced by the edge location [36].

RF-based devices are generally also considered more precise than video-image systems, which are limited by the spatial resolution of pixel analysis. For this reason, precision for video-image analyzers is usually estimated to be about 150 μm (i.e., the size of the pixel) [35] and this would be insufficient for determining arterial stroke change in diameter. However, it is important to point out that methods are available based on algorithms with sub-pixel precision, able to evaluate change in a diameter less than 15 μm [36, 41] and therefore suitable for local arterial stiffness assessment. In addition, studies investigating precision in terms of repeatability of instantaneous arterial diameter evaluation by ultrasound data processing are available in literature

[42]: coefficients of variation (CV) of the parameters involved in arterial elasticity evaluation which are considered appropriate for studying their physiological and pathophysiological variations, are shown in [43-45]. As an example Selzer et al. [45] reported a CV of 1.28% for arterial diameters and from 11.05 to 14.54% for carotid stiffness indices. Kool et al [44] found a CV of 4.5% for carotid diameters, 7.9% for distension and 8.3 to 9.1% for arterial stiffness parameters.

Furthermore, in a recent work [36] reproducibility of RF- and image-based techniques were assessed in the same population showing comparable reproducibility and good agreement. Hence, it might be concluded that high spatial resolution of RF-based methods is not mandatory for standard clinical examination. This point might be even more interesting when considering how important it is to document the independent predictive value of carotid stiffness on cardiovascular events; so far only a few studies where parameters of carotid elasticity were used are found in the literature and the development of user-friendly and relatively inexpensive systems for assessing carotid diameter and distension would be important. In addition, besides the RF-systems, B-mode based devices that can also provide the automatic measure of carotid C-IMT are available

and are able to furnish both functional and structural parameters of the analyzed vessel, as suggested by the international expert consensus [35].

RF-based echo-tracking devices are considered the reference technique providing optimal conditions in the simultaneous measurement of local arterial stiffness and C-IMT for their high precision; however, since this kind of data output is not easily available in standard ultrasound equipment, reproducible and robust B-mode based technique (that can be applied to any ultrasound equipment) in conjunction with international guidelines, can be considered an effective alternative.

1.3 Non-invasive assessment of endothelial function: brachial artery flow mediated dilation

1.3.1 Clinical aspects and prognostic values

Endothelium plays a primary role in the control of vascular function [46] by the production of nitric oxide (NO), which derives from the transformation of L-arginine into citrulline by the constitutive endothelial enzyme NO synthase (eNOS), under the stimulus of agonists (acetylcholine, bradykinin, and others) acting on specific endothelial receptors and of mechanical forces, namely shear stress [47]. In pathological conditions, the same stimuli determine the production of endothelium-derived contracting factors (EDCFs, e.g., thromboxane A2 and prostaglandin H2), which counteract the relaxing activity of NO, and reactive oxygen species (ROS) which impair endothelial function by causing NO breakdown. In such conditions, reduced NO availability and EDCF not only exert an opposite effect on vascular tone, but also facilitate the pathogenesis of thrombosis and atherosclerotic plaque by promoting platelet aggregation, vascular smooth

muscle cell proliferation and migration, and monocyte adhesion [48].

This pivotal role of the endothelium in the atherosclerotic process led to the development of different methods to assess endothelial function, which could provide novel insights into patho-physiology and 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 [49, 50].

Endothelial function in clinical research is mainly tested by vascular reactivity studies [49]. The most widely used technique is the so-called “flow-mediated dilation” (FMD) of the brachial artery. This is a non-invasive, Ultrasound-based method, introduced in 1992 [51]. FMD occurs as a result of local endothelial release of NO and it is measured as brachial artery diameter changes in response to increased shear stress, induced by reactive hyperemia and measured [52, 53]. To this aim the sphygmomanometer cuff placed on the forearm distal to the brachial artery is inflated to 200 mmHg and subsequently released 5 min later. Endothelium-independent dilator response can be tested by low-dose sublingual nitroglycerin [54]. FMD has been studied widely in clinical research as it enables serial evaluation

of young subjects, including children [51]. 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 [50].
Impaired FMD has been shown in hypertensive patients and in the presence of the other cardiovascular risk factors [54-58].

1.3.2 Methodological and technical issues

Assessment of brachial FMD in clinical investigation has increased because it is non-invasive and apparently easy to perform. However, several challenges must be overcome that are major limitations to a widespread application of this method in clinical studies. 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 [59]. Furthermore, other caveats should be considered in designing a study where FMD is investigated for the biological and technical variability of its measurement, including appropriate study design and sample size and efforts to achieve a uniform technique and minimize operator-dependency, including the adoption of probe-holding devices and automated systems to measure brachial artery diameter changes [60-63].

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 partly determined by the magnitude of post-ischemic forearm vasodilatation, which is a measure of microcirculatory function [50]. Interestingly, the use of upper cuff occlusion was associated with one of the few negative reports on the prognostic role of FMD [64], although a recent meta-analysis showed that studies applying the upper cuff occlusion technique showed similar prognostic predictive values compared with those using the lower cuff technique [65].

Training and certification of sonographers in FMD procedure has been well-described in guidelines [61] and proven by results in recent multicenter trials by the small number of rejected examinations, due to poor quality and/or instability of the images [66, 67].

The use of a clamp to hold and adjust probe position, as well as a computerized system to automatically measure brachial artery diameter are currently required to obtain the best reproducibility of this non-invasive technique [9, 49, 68] as recently also shown in multicenter settings [66, 67].

As of today, only a few experienced research centers apply a rigorous methodology to achieve a high standard of accuracy and reduce FMD variability [69]. The lack of uniform methodology, including all the above-mentioned procedures, is a major limitation, although not the only one, for the application of FMD assessment in large multicenter studies. We recently evaluated the time-dependent variability of FMD measurements obtained in more than 130 healthy volunteers by trained operators according to a uniform technique [67]. This included centralized analysis by an automated edge detection system, composed of a special-purpose hardware/software device for measuring changes in brachial artery diameter [70, 71]. The study showed for the first time that adherence to a rigorous protocol, with certified operator training as well as defined experimental settings (adjustable stereotactic probe-holding device, automated computer-assisted brachial artery measurements), is feasible in different research centers, ensures high quality examinations and, most of all, provides an optimal time-dependent reproducibility of FMD. In particular, a similar coefficient of variation (close to 10%) for intra-session (1 h apart) and inter-session (1 month apart) FMD assessment was shown and the overall FMD variability was comparable

with that observed by the authors who originally described the non-invasive method for FMD using a similar methodology [69]. Thus, this approach should be implemented in all studies investigating FMD as a surrogate marker of cardiovascular disease.

As already stated, automated, computer-based analysis of brachial artery diameter changes [70, 71] is fundamental for the assessment and reproducibility of FMD testing. At the present time, automatic systems for FMD assessment are based on both post-processing and real-time analysis, thus working offline and online, respectively. In particular, real-time systems offer several advantages enhancing reliability and precision of FMD measurement [72]. Mainly, a real-time feedback signal generated during the scan acquisition and strictly related to the algorithm performance could continuously inform the operator about the quality of the ultrasound images. This aspect is of particular importance in FMD studies because in these examinations, the quality of the image is a critical component that can compromise the success of the measurement. Indeed, a proper image must be maintained for several minutes to best quantify the transitory response induced by the endothelium. For this reason, adjustments of the position of the probe may be required during the

examination, especially to compensate for small movements of the patient. The sonographer is largely helped in this task by immediate feedback from the measurement system. As a final result, the number of examinations rejected due to low-quality post-processing analysis could be reduced [66, 67, 72].

Another advantage of online analysis is the reduction in time spent analyzing the images after acquisition and the absence of those drawbacks associated with video storing. Recording the video means a reduction of image quality, while an acquisition on a personal computer requires a large amount of memory. Moreover, the real-time characteristic improves the operator's learning curve, significantly reduced by this approach [72] another major challenge for FMD assessment [61].

Finally, another important characteristic of the FMD technique is the timing of the procedure, with respect to the cardiac cycle. In fact, vasodilatations induced by reactive hyperemia are not much larger than the diameter variations between systole and diastole [70, 71]. Guidelines suggest using electrocardiogram (ECG) gating during image acquisition [61], where the onset of the R-wave is used to identify the end diastole, and this is currently the method most commonly used both for

manual and automatic analyses. However, this requirement influences the complexity of the ultrasound equipment adopted for the examination. Nowadays, high frequency linear array transducers are also available in less expensive hand-carried ultrasound devices, which are being used more and more in research and clinical practice. Although such devices produce high quality B-mode images, they may lack ECG trigger capabilities, which are at times provided as an option with a significant increase in the overall cost of the system. On the other hand, modern automatic measurement methods used in FMD examinations have become faster and more precise, thus allowing a continuous measurement of the diameter curve with a sample rate of 25 to 30 samples/s. By using these systems, information on the timing with respect to the cardiac cycle can be obtained by directly analyzing the diameter curve, without the need for an ECG trigger. Also, working at 25/30 frames per second ensures greater reliability against noise, analyzing a greater number of frames for diameter measurements, so FMD technique is more suitable for centralized readings.

Some issues remain unresolved in FMD measurement. In particular, agreement was not reached on the normalization of the percentage variation brachial

diameter by the amount of the reactive stimulus (e.g. shear rate) that induced vasodilation. As a consequence, several papers present FMD values as not normalized, especially in the past. Recently, this problem has been recognized and a debate on how to normalize FMD values was started. At the present, the maximum shear rate, the full shear rate area under the curve and the shear rate area under the curve up to the peak of the FMD have been proposed as potential normalization factors with the last one as the most promising in terms of efficacy [60-63].

Lastly, some interesting new topics are still waiting answers in the FMD area. Among these, the need for reference values that could be used in clinical studies is the most interesting and imperative point. By this means, clinicians would be able to stratify populations and share results more easily.

CHAPTER 2

Materials and Methods

The elasticity of a vessell can be estimated by ultrasound data processing for arterial diameter assessment in conjunction with a local pressure measure. These direct measurements allow calculating arterial distensibility as the ratio between the stroke change in the lumen area (i.e. the variation of the area during the cardiac cycle) and the local pulse pressure, normalized by the diastolic lumen area.

During the first phase of this project we compared two different edge detectors applied to ultrasound images for the evaluation of the arterial diameter, in order to adopt the more suitable. Then we implemented the method for carotid diameter estimation and we used it in conjunction with tonometry for pressure evaluation, in order to measure carotid distensibility in a group of healthy controls during graded bicycle semi-supine exercise test. During this second part of the project we tested the robustness of the algorithm in terms of reproducibility: the control group was analyzed in two

different sessions and coefficients of variation suitable for clinical applications were obtained. Finally, we applied the approach to a group of subjects with known or suspected coronary artery disease (CAD) in order to investigate differences in dynamic behaviour between healthy controls and patients.

2.1 Development of a contour tracking algorithm for arterial diameter estimation

Arterial elasticity is an innovative non-invasive cardiovascular biomarker. In fact, vessel elasticity decreases in presence of cardiovascular disease and its accurate assessment permits early and efficient prevention. This parameter can be evaluated in superficial arteries by measuring instantaneous vessel diameter in conjunction with local pressure. The temporal changes of the diameter are highlighted on ultrasound images by movements of the vascular walls, which are usually not greater than few pixels. Therefore, to obtain a useful plot of arterial diameter variation, an algorithm which is able to locate edges on the image plane with a subpixel resolution is required. Moreover, in clinical analysis (that might require real-time

performances) the computational cost of the algorithm is also critical. With this work two subpixel edge operators were implemented in Matlab and compared in order to choose the most suitable for vascular analysis: the normalized gradient of Gaussian (NGoG) and the mass center of the gray level variability. First, from a significant set of B-mode images of a human artery we derived a simple model of the gray level discontinuity provided by the vessel borders. The model, which is given by the sum of a smoothstep discontinuity and a Gaussian function, was subsequently used to analyse the performances of the two operators. In this way, the best configurations of the two edge operators were defined by varying the work conditions. Subsequently, we generated a set of ultrasound synthetic images using the simulation software Field II [73] to verify the resolution of the two edge detectors in ultrasound applications. A sequence of 100 realistic images of an artery, with a resolution of 13 pixels/mm and with a diameter ranging from 3.9mm to 4mm, was obtained with steps of 0.01mm. In this way, a real ultrasound vascular exam was simulated, with temporal changes of the diameter generating movements of the arterial walls which were less than 2 pixels. Finally, the two

algorithms were used for analysis of arterial diameter in in-vivo studies.

2.1.1 The mass center of the gray level variability

The first absolute central moment is a statistical filter which measures the variability of the gray levels of the image with respect to the local mean. Let f(x,y) be the gray level map of an image, and let g(x,y,σi) be normalized Gaussian weight functions. The following relationship is used to compute the first absolute central moment at a point p of coordinates x,y.

$$e(\mathbf{p}) = \iint_{\Omega} | f(\mathbf{p}+\boldsymbol{\tau}) - f(\mathbf{p}) \otimes g(\mathbf{p},\sigma_1) | \, g(\boldsymbol{\tau},\sigma_3) d\tau_x d\tau_y \quad (1)$$

Moreover, the mass center b of the gray level variability associated to the first absolute central moment can be computed at p:

$$b(\mathbf{p}) = \begin{cases} \dfrac{1}{e(\mathbf{p})} \iint_{\Omega} | f(\mathbf{p}+\boldsymbol{\tau}) - f(\mathbf{p}) \otimes g(\mathbf{p},\sigma_1) | \, \boldsymbol{\tau} g(\boldsymbol{\tau},\sigma_3) d\tau_x d\tau_y & e(\mathbf{p}) \neq 0 \\ 0 & e(\mathbf{p}) = 0 \end{cases} \quad (2)$$

In [74] it is shown that if configurations of eq. (2) with σ1=σ3/π are used then vector b always locates a point p' which is closer to the nearest discontinuity than p, independently of the distance between p and the discontinuity. Therefore, given the points pi of an approximate starting contour, a discontinuity can be located by iteratively computing vector b at pi where the starting points for any new iteration are the points p'i which are located with the previous iteration.

2.1.2 The Normalized GoG

Another mathematical operator which can be used to process gray level discontinuities is known as the normalized gradient of Gaussian (NGoG). On real discontinuities when given an approximate starting contour, the points of the final contour can be located by computing iteratively NGoG from the points of the starting contour [74, 75]. Let f(x,y) be the image gray level map and g(x,y,σ) be a Gaussian function with σ2>a. A vector u, which locates a set of points p'i that are closer to the discontinuity than pi is obtained when computing NGoG at the points pi of a starting contour.

In this case also, given an approximate starting contour, the relative discontinuity can be located by iteratively computing vector u.

(3)

$$\mathbf{u}(x,y) = a\frac{\left[(f*g_x)(f*g_{xx})+(f*g_y)(f*g_{xy})\right]+\left[(f*g_x)(f*g_{xy})+(f*g_y)(f*g_{yy})\right]}{(f*g_x)^2+(f*g_y)^2}$$

2.2 Assessment of carotid elasticity: reproducibility evaluation

Part of this paragraph is obtained from the paper "Assessment of carotid elasticity during exercise: a reproducibility study". ***Bianchini E****, Bruno RM, Corciu AI, Faita F, Gemignani V, Ghiadoni L, Picano E, Sicari R. Ultrasound Med Biol. 2012 Feb;38(2):223-30.*
The paper is reported in Appendix A.

After choosing the most suitable edge-detector operator, we implemented in Matlab a contour tracking algorithm for the carotid analysis in exercise. This technique, in conjunction with a local pulse pressure evaluation, can provide estimation of arterial elasticity. In order to evaluate the robustness of the developed approach, a pilot study in a group of healthy volunteers was performed. Variability of the technique was estimated by coefficient of variation as reported in the subsequent paragraphs.

Study population

A group of 18 healthy untrained volunteers were recruited for the study (9 males, age 34 ± 3 years, BMI =

22 ± 6 kg/m^2, 3 smokers). Subjects with overt cardiovascular disease, diabetes, hypertension, major non-cardiovascular diseases or who engaged in competitive sports were excluded. None of the subjects were taking any medication at the time of the study or during the previous week. The study protocol was approved by the local ethics committee and informed consent was obtained from all subjects.

Experimental procedure

The subjects were analyzed in two different sessions 3 days apart, in order to evaluate the intersession repeatability of carotid elasticity parameters in exercise. The examinations were all performed in the afternoon after a light lunch in a temperature-controlled room according to current guidelines [6]. The subjects avoided taking caffeine-containing beverages and smoking in the 3 h preceding the experimental sessions. In each session a maximal exercise test was performed on a graded bicycle semi-supine ergometer (Fig. 1) [76]. Workload was increased by 25W every 2 min.

Theoretical maximal heart rate (HRmax) was computed for both male and female subjects, as:

HRmax = 220 - age [in years]

In order to estimate arterial elasticity, acquisitions of carotid ultrasound images, brachial blood pressure and

radial pressure waveform were obtained. All measurements were performed during the exercise test while the subject was riding on the cycle ergometer, with head and neck and right wrist lying on a dedicated support. The following different temporal steps were considered: at 60%, 70%, 80% and 85% (peak) of maximal heart rate and during the recovery. at 1, 2, 4, and 6 minutes after peak exercise.

The acquisitions were made by the same skilled operator in two sessions 3 days apart.

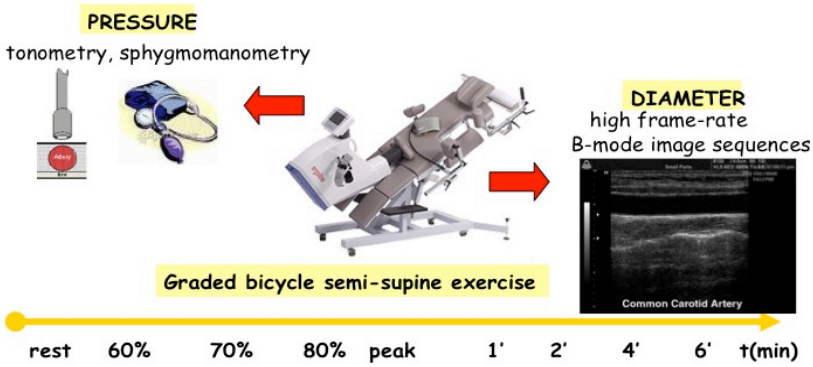

Figure 1. Experimental setup

Techniques

Diameter assessment

After the first part of the work described in the previous paragraphs, we developed the algorithm for the automatic evaluation of the instantaneous carotid diameter based on the most suitable mathematical operator and implemented it in Matlab (The MathWorks, Natick, MA, USA). The method is based on a contour tracking technique that allows the automatic evaluation of diameter stroke changes (i.e. difference between maximum and minimum values) during the heart cycle. The method assesses the instantaneous diameter of the artery by processing B-mode ultrasound sequences of the longitudinal section of the vessel. An example of B-mode image of the carotid artery and its interfaces is reported in Figure 2.

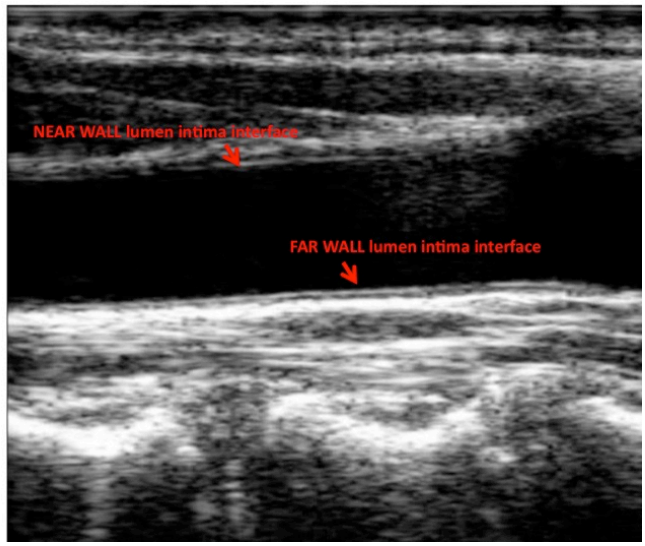

Figure 2. Ultrasound carotid longitudinal image

For each image, lumen-intima interfaces are automatically detected using the algorithm based on the edge operator "First Order Absolute Moment" and diameter is estimated as the distance between far and near lumen-intima interfaces.

The procedure must be initialized by providing two approximated starting borders, an operation that also defines the region of interest where the diameter will be computed. After this simple initialization procedure, the elaboration proceeds automatically: in each image of the video sequence the two borders of the vessel are located, then the diameter is calculated as the distance between such borders.

We have to point out that the two borders of the vessel are not of the same quality. The near wall (NW), that is the border which is closer to the probe, is generally less defined than the far wall (FW). The closeness of a multiple tissue layer, mainly the wall of the vessel, in the space between the transducer and the border is the reason for this. Such a structure, when crossed by the ultrasound beam, provokes a reverberation of the ultrasound energy which degrades the quality of the final image. The border which is further from the probe, by contrast, is better defined since this is the first interface which the ultrasound beam crosses.

Due to this characteristic of the images, the FW can be located more easily and more accurately than the NW. For this reason, in each image it was first located the FW, then it was used as a reference point for the computation of the NW.

The other feature we exploited is that the area internal to the vessel is less noisy than the external area. This difference is due to the presence of the blood, which generates weak echoes. The presence of a region with less noise is advantageously exploited by the edge detection algorithm. This is obtained simply by forcing the algorithm to locate the two borders by starting from the internal part of the vessel.

These two peculiarities were used to affect the way the approximated contours are obtained. As for the FW, it was assumed that for, each image, the contour computed on the previous image FW(n-1) is a good estimation of the current contour FW(n). This statement is generally true because there is a very little movement of the walls between two adjacent images. However, as it was previously mentioned, it was preferable to start from an approximated contour aFW(n) in the inner region of the vessel, where the noise is smaller. For this reason aFW(n) was computed as the segment parallel

to FW(n-1) at a distance ε1 towards the inner part of the vessel.

Concerning the NW, the same strategy was used to obtain an approximated contour aNW(n). However, since in most of the images the quality of the NW is worse than the quality of the FW, it was computed aNW(n) by starting from the FW(n) already computed on the current image instead that from the contour NW(n-1) computed on the previous image. Let d be an estimation of the diameter of the vessel computed as the mean value of the diameters calculated on the previous Nd images, then aNW(n) is obtained as the parallel to FW(n) at a distance (d-ε2). ε2 is a constant greater than zero, which was added to start from the inner region of the vessel, as well as it was done for FW.

Once the two borders of the vessel have been obtained, the diameter is computed. In theory, the two borders should be parallel but, in practice, this hypothesis is not exactly true and an approximation must be introduced in the computation of the diameter. Since it was assumed the FW is better estimated than the NW, the FW is taken as the reference line and the diameter is computed as the distance between the central point of the segment NW and the line FW.

Finally, the mean value of diameter is computed

on 10 beats of examination, in order to reduce the effect of cycle-to-cycle variability on the final result.

As regard data processing for the assessment of variations in diameter during the cardiac cycle (stroke change in diameter or distension [6]), several other computational stages are included:

- After a band-pass filtering, the maximum (systolic) and minimum (diastolic) diameter values are identified for each cardiac cycle.
- The stroke change in diameter is calculated for each cardiac cycle as the difference between the systolic and diastolic diameter values.
- The mean distension value is computed as the average of the results obtained during the last 10 beats.

The algorithm in this study was customized for high frame rate (i.e., > 25 frame/s) application. High frame rate, which ensures high temporal resolution, is needed in order to track the rapid wall movements of the vessel due to the high cardiac frequency in exercise. A graphical-user-interface (GUI) was developed in Matlab (Figure 3) in order to analyse the sequences: the user has just to trace an initial region-of-interest, then the algorithm is able to automatically track the vessel borders.

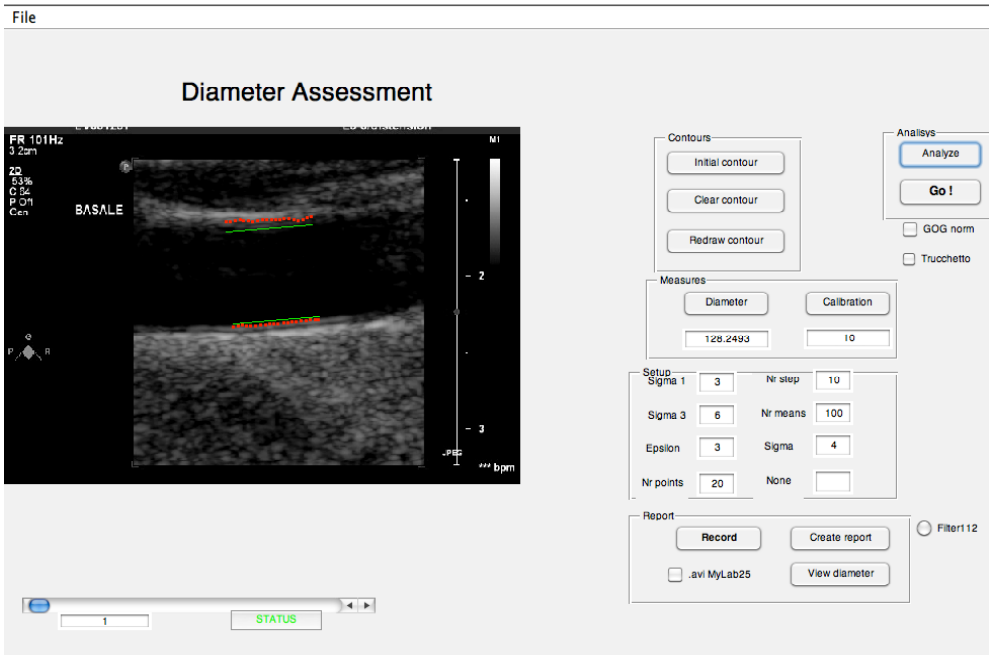

Figure 3. GUI for ultrasound image sequences analysis

Carotid image sequences in DICOM format (frame rate ~ 100 f/sec), were acquired and then analyzed off-line. Data were excluded when image quality was considered insufficient (i.e., the algorithm was not able to correctly track vessel borders).

Blood pressure measurement

Radial tonometry was performed in order to evaluate central pressure by radial to aortic transfer function (commercial device: Sphygmocor, Atcor Medical, Figure 4 and Figure 5). Central aortic pressures provide important information about cardiovascular status, but

direct measurements are invasive. Peripheral pressures can be measured noninvasively, and although they differ from central pressures, they can be mathematically transformed to approximate the latter. Hence transfer functions between aortic and radial pressures was calculated by parametric model and introduced in the scientific community more the 15 years ago [77].

For each step of exercise and recovery we applied this technique. In particular, the radial waveform was acquired and then calibrated by using the arm blood pressure automatically recorded at the right brachial artery (by a Dinamap XL device). In fact, diastolic and mean blood pressures can be assumed to be constant along the arterial tree and this allows to calibrate the radial waveform from the brachial values.

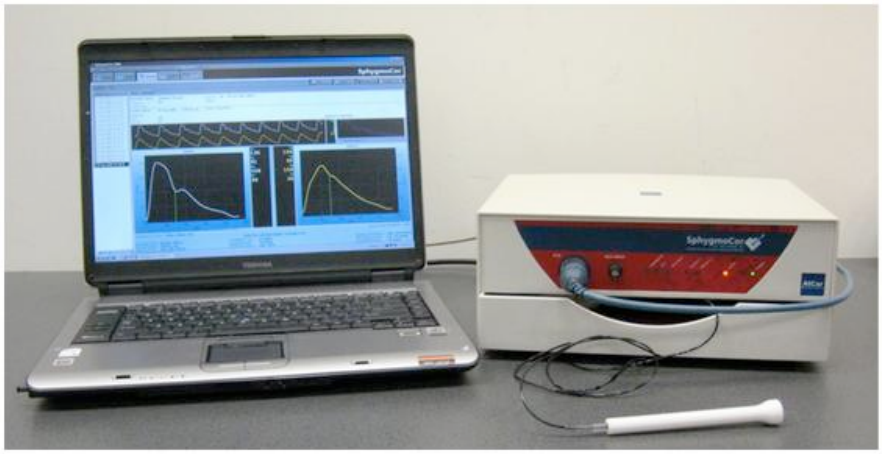

Figure 4. Tonometry for pressure evaluation

Figure 5. Acquistion of radial pressure waveform by tonometry

Quality index (QI) for central pressure waveform was automatically provided by Sphygmocor software: measurements were considered acceptable with QI > 75 at rest and QI > 50 during exercise.

Since carotid and aortic pressure estimations by tonometry (at least at rest) showed good agreement, with differences around 1-2 mmHg [78], final data obtained by the Sphygmocor system were used, together with diameter values, to assess carotid elasticity parameters.

Evaluation of carotid distensibility

Ultrasound B-mode image sequences (image resolution = 100 pixels/cm, DICOM format, 100 frame/s, 10 beats) of the right common carotid arteries were acquired and analyzed by the customized algorithm for evaluation of arterial diameter (D) and distension (ΔD). The common carotid arteries were scanned in longitudinal section using an iE33 Philips machine and a 10-MHz linear-array probe. Arterial diameter borders were tracked in the near and far wall lumen-intima interfaces 1 cm proximal to the carotid bulb in a region 1 cm wide and free of plaques. The bulb was considered an anatomic fiducial point and a picture of the adopted ultrasound image was printed in order to ensure similar location of the measurements between the first session and the second one performed 3 days later. During vascular scanning, time-gain-compensation and depth settings were fixed. In addition, systolic and diastolic brachial pressures were measured and central pulse pressure obtained by radial tonometry as described above. Local elasticity was then obtained for each step of the examination and expressed as:

cross sectional distensibility coefficient,

$$DC = \Delta A / (PPa * Ad)$$

where ΔA represents the stroke change in lumen area, PPa the central pulse pressure and Ad the diastolic

lumen area, respectively. ΔA and Ad were evaluated from the diameter values, assuming the cross-section of the artery to be circular.

Reproducibility data

The volunteers were analyzed in two sessions 3 days apart, in order to evaluate the intersession repeatability of the arterial elasticity measurements (CC and DC).

For each step of the examination, in each volunteer, variability was expressed as the coefficient of variation (CV), which is defined as the ratio of the standard deviation to the mean of the two measurements; the mean of CVs from all the subjects was then computed. CVs of pressure and diameter's evaluation were also obtained.

Furthermore, the reproducibility of each parameter for the whole examination was estimated by using the intraclass correlation coefficient. The two-way random effects model was adopted [79].

All calculations were made using SPSS software (SPSS version 13.0 for Windows, 2004).

2.3 Assessment of carotid elasticity: patients and controls comparison

After testing the robustness of the developed approach, carotid elasticity dynamic behaviour was assessed both in controls and patients. Besides this vascular analysis,

also a cardiac evaluation was performed during the exercise in order to investigate differences in arterial-ventricular coupling too.

Study population

In addition to the 18 healthy volunteers (9 men, 34±3 years), 36 consecutive patients with known or suspected coronary artery disease, CAD, (20 men, 61±8years) were analysed.

	Patients (n=36)	Controls (n=18)
age	61 ± 8*	34 ± 3
male (n)	20	9
BMI	26 ± 4*	22 ± 6
Systolic blood pressure	137.0 ± 12.5*	127.1 ± 8.1
Diastolic blood pressure	75.5 ± 4.3	82.5 ± 4.9
Heart rate	69.5 ± 19.9	68.3 ± 14.5
Diabetes(n)	8	0
Hypertension (n)	36	0
Hypercholesterolemia (n)	19	0
Smoking (n)	10	3
Known CAD	20	0
Ischemia	5	0

Table1: characteristics of the analyzed population

Vascular evaluation

Carotid elasticity was obtained as described in the previous paragraph. The subjects underwent a maximal exercise test on a graded bicycle semi-supine ergometer. Workload was increased by 25W every 2 min.

Also in this protocol theoretical maximal heart rate (HRmax) was computed for both male and female subjects, as:

$$HRmax = 220 - age \quad [years]$$

As previously described in order to estimate arterial elasticity, acquisitions of carotid ultrasound images, brachial blood pressure and radial pressure waveform were obtained. All measurements were performed at baseline and during the exercise test at peak of the age-dependent maximal heart rate while the subject was riding on the cycle ergometer, with head and neck and right wrist lying on a dedicated support. Recovery analysis was also obtained at 1, 2, 4 ad 6 minutes after peak.

Cardiac evaluation

Besides the carotid distensibility, also an important cardiac parameter, the left-ventricular elastance (Elvl), was evaluated in the analysed subjects during the exercise (Figure 6).

Cardiac volumes were estimated by 2D transthoracic echocardiography, and blood pressure by applanation tonometry. From these direct measurements Elvl was then calculated as the ratio between ESP and ESV, normalized by the body surface area, where ESP represents the end-systolic pressure, ESV the end-systolic volume. Elvl reflects left ventricular contractility, but it is also influenced by biochemical and morphological features of the cardiac tissues. In particular, changes during stress conditions (i.e.

exercise) can give us information about cardiac performance, and its behaviour in conjunction with the carotid analysis might give a more integrated and accurate picture of the cardiovascular status of a subject.

Also the cardiac measurements were performed at rest and peak of the age-dependent maximal heart rate, and during recovery.

Controls and patients data were analyzed by two-ways ANOVA, considering time intervals and population as factors, $p < 0.05$ was considered significant.

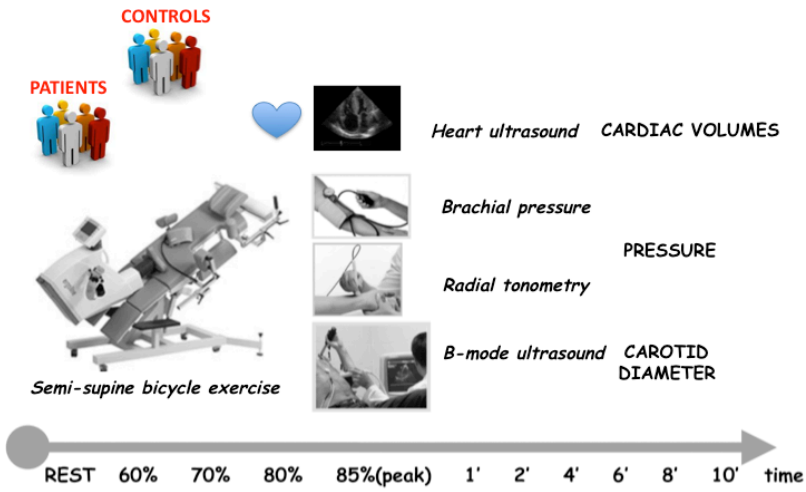

Figure 6. Experimental set-up for analysis in controls and patients

CHAPTER 3

Results

As described in the previous chapter, during the first phase of this project we compared two different edge detectors applied to ultrasound images for tracking vessell borders, in order to choose the best to implement a new approach for arterial diameter estimation in exercise. After developing the method for carotid diameter estimation, we adopted it in conjunction with pressure evaluation by tonometry, for dynamic carotid distensibility estimation in a group of healthy controls. During this second part of the project we tested the reproducibility of the algorithm in order to evaluate if it is suitable for clinical applications: the control group was analyzed twice and coefficients of variation were obtained. Finally, we applied the technique, in conjunction with a cardiac analysis, to a group of subjects with known or suspected coronary artery disease (CAD) to evaluate possible differences in dynamic behaviour between healthy controls and patients.

In this chapter we will summarize the obtained results.

3.1 Development of a contour tracking algorithm for arterial diameter estimation

3.1.1 Gray-level discontinuities modeling

We acquired and observed a set of real B-mode images of the carotid artery, in order to obtain a realistic model of the gray level discontinuity associated with the artery borders. In the figure below the gray level discontinuity associated to the artery border is shown.

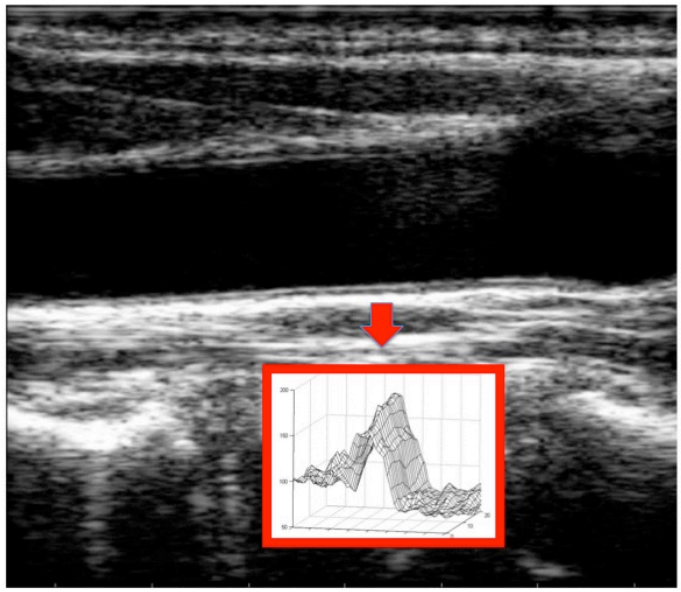

Figure 1. Carotid longitudinal B-mode image and profiles of the gray level discontinuities at the far artery border

After this analysis, we derived a synthetic discontinuity similar to this real profile from the sum of a smoothstep discontinuity and a Gaussian function. An image (1280x1280 pixels) with two gray levels 120/100 i.u. (intensity unit) was used as a base. The step was then smoothed with a 2D Gaussian kernel with σ2=30 pixels and a 1D Gaussian with σ=30 pixels was added. The maximum value of the obtained discontinuity model was equal to 180 i.u.. This mathematical model of the arterial gray level discontinuities is shown in the picture below.

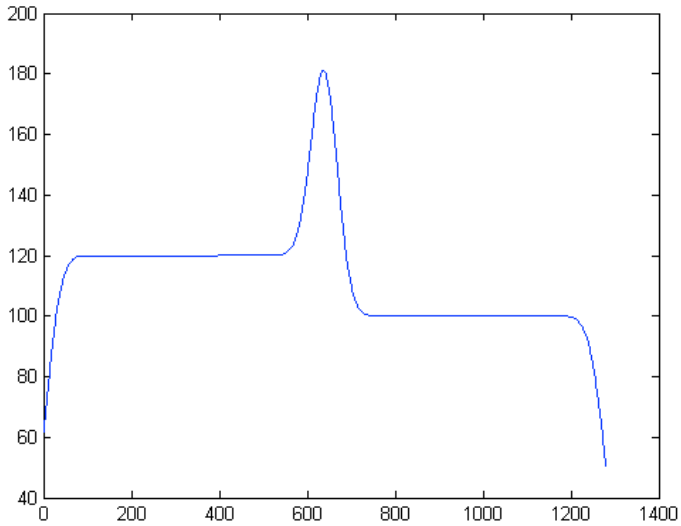

Figure 2. One-dimensional mathematical model of the gray level discontinuity associated to the artery borders

3.1.2 The mass center of the gray level variability and the normalized gradient in the presence of the synthetic vessell border discontinuity

The two operators were implemented in Matlab and then applied to the obtained model of discontinuity. The component bx of vector b was computed for different configurations of the operator which were obtained by varying the distance ε between the starting point p and the point which represents the discontinuity (the top of the Gaussian function). This first test underlined that, the point of convergence (the zero-crossing of the function bx(ε)), of the iterative procedure based on the computation of vector b, changes when varying the configuration of the mathematical operator.

Then, we applied also the algorithm based on the normalized gradient to the discontinuity model with different apertures of the Gaussian function and here again we observed that the point of convergence of the iterative procedure based on the computation of NGoG depends on the configuration of the operator.

Finally, we compared the mass center of the gray level variability and the normalized gradient in terms of behaviour when varying the distance of the starting point

from the discontinuity; the trends of the two edge detectors were found to be very similar. However, it is worth noting that the discontinuity is localized in two different points. The results of this part make clear that, when analyzing discontinuities such as those similar to a vessel border, the point of convergence depends both on the mathematical operator and on its configuration.

3.1.3 The mass center of the gray level variability and the normalized gradient applied to vascular ultrasound image sequences

Synthetic sequences

In this part of the project we applied the two edge detectors to more realistic synthetic images of the carotid artery obtained by using the software Field II. A 3D phantom was used to generate the images and the carotid artery was modelled as a cylinder positioned parallel to the probe. 100,000 scatterers were randomly distributed within the volume of the phantom and strong scatterers were placed to simulate boundaries, thus obtaining images which are very similar to the images obtained with standard echographic equipment. A 13 MHz linear array vascular probe with 192 elements spaced of 0.245mm was hypothesized. The obtained B-

mode images consisted of 256 gray levels with a resolution of 13 pixels/mm (Fig.3).

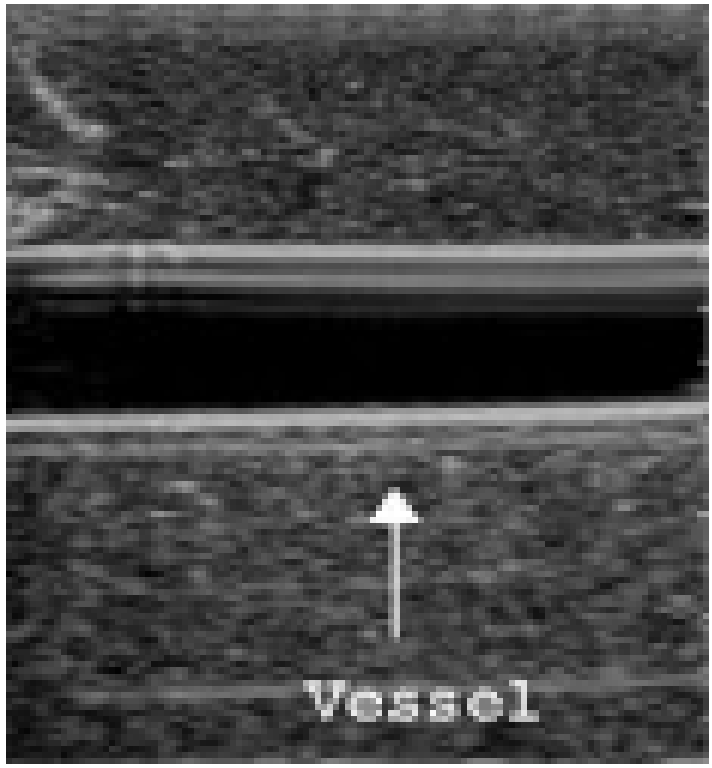

Figure 3. Image obtained with Field II.

From these data we synthesized set of 100 images that simulates five cardiac cycles where the diameter d of the artery varies between 3.9mm and 4.0mm (with a resolution of 13 pixels/mm, the diameter variation in the image sequence was 1.3 pixels, ranging from 50.7 to

52.0 pixels). Then we analysed this sequence with both the mathematical operators obtaining a fine measurement of the diameter. The two edge detectors were able to analyse this kind of images by detecting diameter variations in the order of 0.1 pixels (8μm in our test conditions).

The configuration σ1/σ3=2/π with σ3=4 pixels was chosen for the mass center of the gray level variability and a value a=0.72σ2 with σ=4 pixels was chosen for NGoG.

Let di be the diameter of the cylinder which is used to model the artery in the phantom and $\hat{d}_i$ be the diameter measured in the ith image, the error is $\xi_i = \hat{d}_i - d_i$.

The statistics of the error were calculated for the two edge detectors. The mean and the standard deviation of the error are -5.2 pixels (0.4 mm) and 0.04 pixels (0.003 mm), respectively, for NGoG and -2.3 pixels (-0.18mm) and 0.03 pixels (0.002 mm), respectively, for the mass center of the gray level variability. Results show that both the measurements have a significant bias, confirming that the location of these points depends on the algorithm and on the configuration of the mathematical operator. In addition, the mean values of the errors obtained with the mass center of the gray

level variability and with the NGoG are different and this is in line with the previous results.
Results confirm that the two algorithms converge to two different points giving rise to two different bias levels. Moreover, the low standard deviation of the error shows that both the two algorithms are accurate in measuring the absolute diameter changes.

Real sequences

Finally, the two algorithms were applied to B-mode images of longitudinal sections of carotid arteries in order to compute the diameter of the vessel. Fig.4 shows the plot of the diameter of an artery which was obtained in an in-vivo examination when using the mass center of the gray level variability. The conditions were similar to those set with Field II (13 MHz linear array probe and an image resolution of 15.7 pixel/mm). The curve of the diameter change during the cardiac cycle is correctly traced even when the difference between the maximum and minimum value is in the order of one pixel and this gave evidence of a sub-pixel precision. The two edge detectors were able to analyse this kind of images by detecting diameter variations in the order of 0.1 pixels (8μm in our test conditions). The implementation on real images highlighted that NGoG is computationally

twice more onerous than the mass center of the gray level variability and this was considered as a critical point. Moreover the mass center of the gray level variability has been proven to be particularly robust to speckle noise typical of ultrasound imaging. For these reasons, we decided to adopt it as edge detector to implement the algorithm to be used for carotid diameter evaluation in humans.

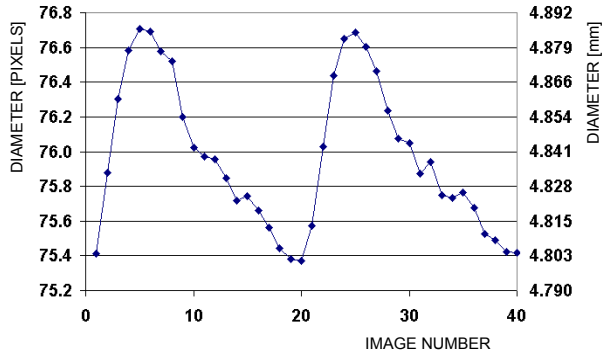

Figure 4. Real time in vivo analysis using the mass center of the gray level variability

3.2 Assessment of carotid elasticity: reproducibility evaluation

Part of this paragraph is obtained from the paper "Assessment of carotid elasticity during exercise: a reproducibility study". ***Bianchini E****, Bruno RM, Corciu*

AI, Faita F, Gemignani V, Ghiadoni L, Picano E, Sicari R. Ultrasound Med Biol. 2012 Feb;38(2):223-30.
The paper is reported in Appendix A.

The pilot study performed in the group of healthy volunteers allowed to estimate the precision of the approach in terms of reproducibility.
In particular, during the two sessions, 4.3% of the acquired B-mode images were considered of poor quality and hence rejected.
As regards tonometry acquisition, 2.7% of data presented an unacceptable quality index (QI) and were discarded. These resulted in a rejected percentage of the derived carotid elasticity measurements equal to 5.8%.
Reproducibility data
Mean coefficients of variation for each step of the exercise are summarized in Table 1.

	rest	60%	70%	80%	peak	1m	2m	4m	6m
SPb	3.1±3.4	3.2±2.5	3.1±1.8	3.2±3.3	4.1±3.4	3.4±3.5	4.1±3.2	4.6±2.8	3.2±2.7
DPb	4.2±5.3	4.9±6.1	5.1±6.3	5.4±6.3	6.2±6.5	5.2±4.9	6.1±4.6	5.6±6.1	5.8±5.2
PPb	7.3±6.1	9.6±5.9	9.5±5.9	9.9±3.2	10.1±6.8	9.3±8.4	11.2±6.7	11.9±7.9	11.3±9.1
SPa	4.2±4.4	4.3±3.1	4.4±2.5	5.2±4.3	3.5±3.6	4.9±4.3	4.2±3.9	4.9±4.4	3.3±2.9
DPa	4.4±4.7	6.2±5.9	7.2±5.1	7.9±4.4	8.2±7.8	8.1±7.2	5.4±4.1	7.8±8.7	6.2±4.9
PPa	8.3±6.1	11.3±8.2	11.2±6.9	10.3±7.4	10.5±7.2	12.3±104.	13.9±8.4	13.8±11.6	12.1±8.7
D	3.1±3.1	2.2±1.7	4.7±4.4	5.1±6.9	6.8±5.4	3.3±3.1	3.1±2.4	5.4±4.3	3.9±4.1
∆D	8.1±5.9	9.8±5.7	9.9±10.6	10.2±11.6	11.8±7.9	7.2±7.3	10.2±6.5	10.9±8.8	9.2±6.4
DC	8.3±7.8	15.9±10.6	13.5±10.4	16.4±17.2	24.2±14.9	12.5±11.1	14.9±12.2	14.2±15.1	18.1±14.8

Table 1. Mean ± standard deviation of coefficients of variation (percentage values) for each step of the exercise for repeated examinations that were performed in two different sessions, 3 days apart.

SPb is the systolic brachial blood pressure, DPb diastolic brachial blood pressure, PPb brachial pulse pressure, SPa systolic central blood pressure obtained by tonometry, DPa diastolic central blood pressure obtained by tonometry, PPa central pulse pressure obtained by tonometry, D the carotid diameter, ∆D the carotid distension, DC the carotid cross-sectional distensibility coefficient.

In addition, agreement of elasticity evaluation for the whole examination between the two sessions resulted in an intraclass correlation coefficient of 0.694 (95% Confidence Interval: 0.574-0.780) for DC. Intraclass correlation coefficients of pressure and diameter measurements were: 0.934 (95% CI: 0.906-0.954) for SPa, 0.776 (95% CI: 0.660-0.848) for DPa, 0.962 (95% CI: 0.947-0.971) for SPb, 0.807 (95% CI: 0.712-0.867) DPb and 0.830 (95% CI: 0.759-0.879) for D. Finally, regarding differential measurements, intraclass correlation coefficient was: 0.876 (95% CI: 0.829-0.909) for PPa, 0.936 (95% CI: 0.911-0.953) for PPb and 0.897 (95% CI: 0.858-0.925) for ΔD.

For both physiological and reproducibility data, the analysis was repeated considering only non-smokers (n = 15), in order to evaluate whether he smokers' sub-group affected the final findings of our study, and we obtained super imposable results (data not shown).

3.3 Assessment of carotid elasticity: patients and controls comparison

After testing the robustness of the developed approach, carotid elasticity dynamic behaviour was evaluated both in controls and patients. In addition, a cardiac evaluation in terms of left-ventricular elastance was performed

during the exercise in order to provide information regarding arterial-ventricular coupling and hence a more integrated analysis of the subjects. Results regarding mean blood pressure, carotid diameter, carotid distensibility, left-ventricular elastance are summarized in the figures below as mean ± standard deviation. Finally, also the relationship linking carotid distensibility/left-ventricular elastance and cardiac frequency during the exercise is reported in order to give evidence of different behaviour between patients and controls.

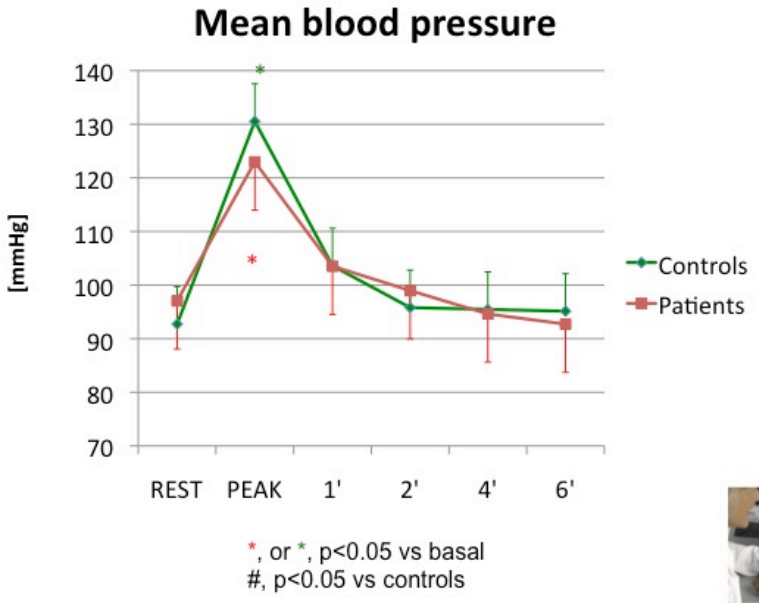

Figure 5. Mean ± standard deviation of mean blood pressure for each step of the exercise: controls in green, patients in red.

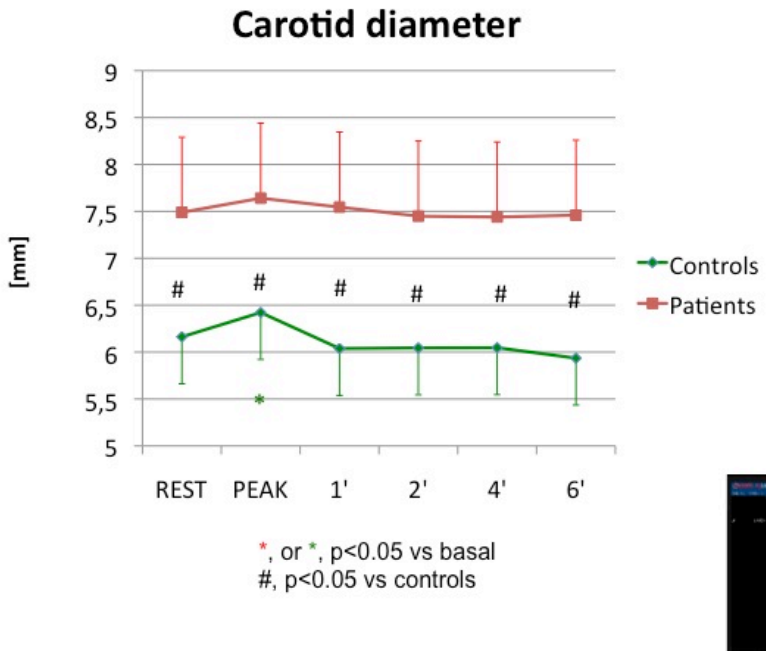

Figure 6. Mean ± standard deviation of carotid diameter for each step of the exercise: controls in green, patients in red.

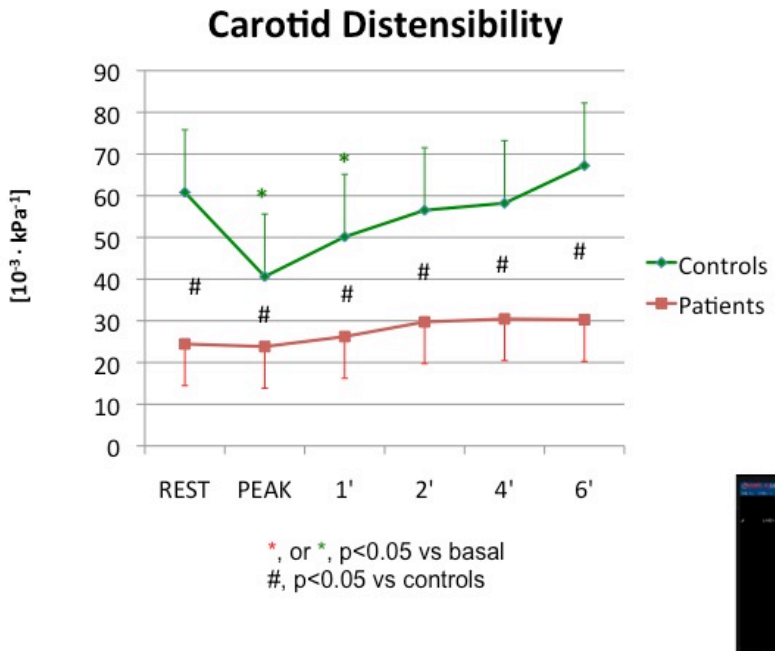

Figure 7. Mean ± standard deviation of carotid distensibility for each step of the exercise: controls in green, patients in red.

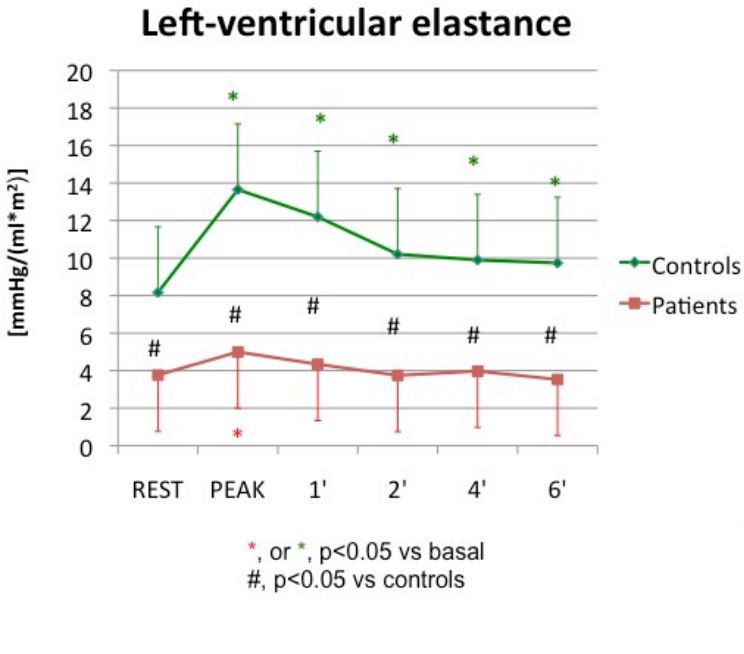

Figure 8. Mean ± standard deviation of left-ventricular elastance for each step of the exercise: controls in green, patients in red.

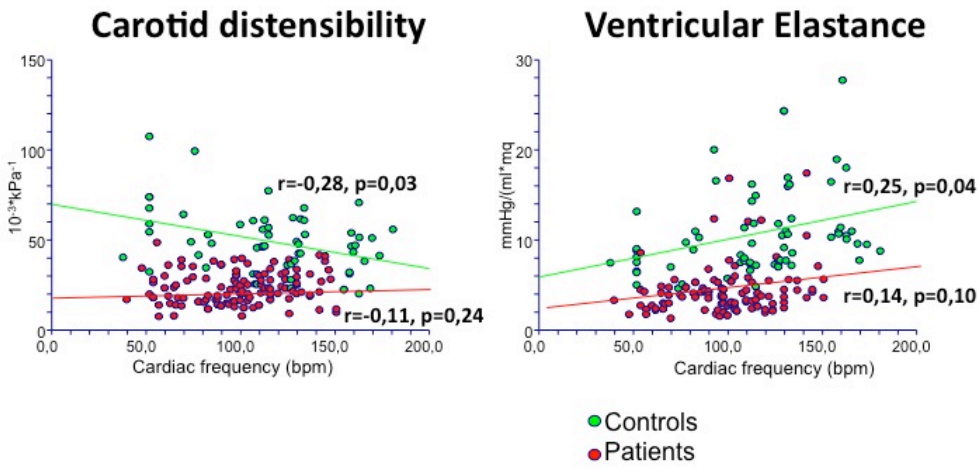

Figure 9. Relationship between carotid distensibility/left-ventricular elastance and cardiac frequency during the exercise: controls in green, patients in red.

CHAPTER 4

Discussion and Conclusions

Part of this chapter is obtained from the paper "Assessment of carotid elasticity during exercise: a reproducibility study". ***Bianchini E****, Bruno RM, Corciu AI, Faita F, Gemignani V, Ghiadoni L, Picano E, Sicari R. Ultrasound Med Biol. 2012 Feb;38(2):223-30.*
The paper is reported in Appendix A.

4.1 Discussion

Great emphasis has been placed on the role of vascular biomarker in evaluating the cardiovascular status of humans. In particular, arterial elasticity has been assessed in resting conditions for a more accurate stratification of cardiovascular risk that might allow a better clinical approach. However, the elastic behaviour of the arteries is not a static parameter and its analysis during exercise would be even more attractive, since it could provide information about dynamic conditions, mimicking a patient's real life.

The aim of this PhD project was the evaluation of vascular biomarkers in exercise and in particular of carotid elasticity by ultrasound images processing.

During the first part of the work we focused on the choice of the edge-detector operator to be adopted for the arterial diameter evaluation from B-mode imaging.

In particular, subpixel edge detection properties of NGoG and of the mass center of the gray level variability were analysed. These two operators were applied to a model of the gray level discontinuity similar to those generates by ultrasound imaging at vascular borders and it was highlighted that, for each operator, the point of convergence changes with the operator configuration. Moreover, it was clearly shown that when both algorithms are applied to the same discontinuity they converge to two different points. Therefore, from this first part of the project we concluded that the location of this kind of discontinuity depends on the edge detector and on its particular configuration.

Then both algorithms were applied to a set of synthetic images of the carotid artery obtained with Field II by simulating working conditions similar to the reality. This sequence reproduces five cardiac cycles where the difference between the maximum and minimum value of the diameter is in the order of one pixel. The results are

affected by a bias since the location of the discontinuity depends on the edge operator and on its configuration. However, small variations of the diameter were detected and a very low standard deviation of the error was obtained for both operators.

Thus the operators were applied to real ultrasound images of the carotid artery and they both traced the curve of the diameter with high precision on real ultrasound images of the vessel. However, the computational cost of the two edge detectors was compared and NGoG resulted to be about two times more onerous. Therefore, we decided to use the mass center of the gray level variability to measure the variation of carotid diameter during the heart cycle with subpixel precision in real analysis. Moreover, this mathematical operator has been proven to be particularly robust to speckle noise typical of ultrasound imaging [74].

After developing the algorithm to track the vessel borders during the exercise we tested its precision in terms of reproducibility. A group of healthy subjects were analyzed in two different sessions 3 days apart. At the moment, to our knowledge, repeatability studies for carotid elasticity during exercise are not available in the

scientific literature. On the other hand, coefficients of variation (CVs) at rest of the analyzed parameters, which are considered appropriate for studying their physiological and pathophysiological variations, are described in several studies using well-known and validated gold-standard techniques [80-83]. As an example Selzer reported CVs = 6.17 – 9.66% for blood pressure measurements, CV = 1.28% for arterial diameters and CVs from 11.05 to 14.54% for carotid stiffness indices; similarly Kool found CV = 4.5% for carotid diameters, CV = 7.9% for distension and CVs from 8.3 to 9.1% for arterial stiffness parameters. Our results regarding reproducibility of baseline data provided similar values and hence the reliability of the proposed approach is as good as the state-of-the-art technology.

Regarding results in exercise and recovery, CVs of carotid diameter (D and ΔD) and pressures (SPb, DPb, PPb, SPa, DPa and PPa) are slightly higher but comparable to the resting values reported in the abovementioned previous works [80-83]. Finally, coefficients of variability of carotid elasticity measurements in dynamic conditions are also slightly higher but comparable to values acquired at rest for all steps, except for exercise peak, where mean CV is 24 ±

15% for distensibility (DC). However, it is worth noting that repeatability of DC again improves (i.e., comparable to baseline) already starting from the first minute of recovery; at this time, the phenomenon of significant decrease in elasticity with respect to baseline detected at peak is still observable. We might speculate that if confirmed in future work, including both controls and patients, these results could imply the possibility of observing arterial elasticity variations by performing the measurement after peak exercise, when the subject is not riding the bike, artefacts are reduced, and as a consequence variability is smaller and data more reliable. Moreover, in this case the design of future clinical studies might be improved even more, since when analyzing a subject who is not moving, direct and more accurate carotid tonometry [6] (which we had to exclude and replace with the radial approach due to feasibility issues) can also be performed.

Regarding the reliability of the whole examination, the obtained intra-class correlation coefficients (ICC) result slightly worse than the resting values of validated techniques in previous works. As an example, Selzer [82] reported intraclass correlations around 0.98 for diameter measurements, around 0.89 for arterial distensibility and compliance, and between 0.77 and

0.83 for pressure estimation. Furthermore, in this case ICC values for arterial pressure resulting in a reliable dynamic evaluation were available in literature [84] and were similar to ours (ICC = 0.89).

Our results regarding parameters that were derived directly from the B-mode image analysis (i.e., D and ΔD) prove the robustness of the algorithm used in more critical conditions as well. Furthermore, these data show the feasibility of dynamic vascular diameter assessment from ultrasound imaging by using a robust contour tracking method together with an appropriate frame rate that ensures the temporal resolution needed to correctly observe rapid movements of the walls due to high cardiac frequency. Most modern ultrasound machines allow the acquisition of high frame rate image sequences and thus, in conjunction with a precise automatic method for arterial wall tracking, could be used in future clinical studies for evaluating instantaneous diameter at high heart rate.

As regards the pressure measurement needed in conjunction with diameter assessment for elasticity estimation, we opted for radial tonometry that allowed us to compute central pressure by radial to aortic transfer function. This choice, although less accurate than direct carotid tonometry for local pressure estimation, was

preferred since it is considered much more feasible in exercise. Since aortic and carotid pressure estimations by tonometry, at least at rest, are in good agreement [78], the data obtained were then used in conjunction with the diameter values to assess carotid elasticity parameters. However, although we tested the reproducibility of the radial tonometry-based technique, we did not show that it is the most appropriate method for carotid pressure estimation during exercise, from a physiological point of view. First, at this time we do not know whether differences between aortic and carotid pressure can be considered small in dynamic conditions as well. Second, there is no consensus [85, 86] on the capability of generalized transfer functions to accurately estimate central hemodynamic variables from radial pressure waveform in dynamic conditions. Some authors [85] compared the radial tonometry-derived measure with a catheter-based one in 30 patients during supine exercise, concluding that the two approaches show good agreement. However, others [86] observing eight healthy volunteers during the incremental hand-grip test concluded that changes in vascular tone due to dynamic conditions could compromise the assumptions for aortic evaluation derived by radial applanation tonometry. Indeed, further studies analyzing different

approaches for carotid or aortic pressure dynamic evaluation are warranted. It is worth noting that although some studies assessed vascular parameters dynamically [7,8] there are very few studies reporting the reliability of this evaluation in literature [87].

After testing the robustness of the developed approach we compared the physiological behaviour during exercise in controls and patients. In few previous works some results regarding healthy volunteers have been reported. Myers et al. [7] reported stiffness increase with isometric handgrip that varies with differences in gender and age and can be manifest even in young, healthy adults; other studies [88, 89] found elasticity to be reduced during exercise in healthy volunteers. Elasticity analysis during exercise is intriguing. In fact, accuracy of the estimation of cardiac afterload during exercise on the basis of brachial BP is debatable, since PP amplification from the center to the periphery of the arterial tree increases greatly during exercise [88]. Moreover, men with hypercholesterolemia have higher augmentation index and blunted pulse pressure amplification compared with age-matched healthy controls during light exercise, in spite of similar brachial SBP at baseline and during exercise [90]. Hence, an abnormal exercise central BP can underlie an abnormal

behaviour of arterial stiffness, which until now has been evaluated mainly by indirect measurement, such as the timing of the reflected wave. BP response to exercise is a negative prognostic factor for cardiovascular mortality, and a more in-depth study of the behaviour of arterial stiffness during exercise could provide not only better knowledge of physiological mechanisms, but possibly also a better prediction of cardiovascular risk.

In this study, carotid distensibility was assessed in healthy volunteers during exercise and then compared with a group of patients.

In controls, aside from the expected increase in mean central pressure, at peak as compared to baseline, a decrease in DC during exercise was documented; the phenomenon was observable at peak and remained evident until 1 min after peak exercise. These data indicate increased carotid stiffness during exercise, a phenomenon that might be partly due to the recruitment of a greater number of collagen fibers and consequently a different mechanical behaviour of the arterial wall at higher pressures [91]. These results are consistent with the non-linear relation between stress and strain that is thought to characterize the arterial borders: at higher stress (i.e., pulse pressure), the slope of the curve stress-strain (i.e., pressure-distension) increases and

thus also the corresponding stiffness. Besides arterial elasticity reduction, in controls also a vasodilation phenomenon was observed. In addition, the cardiac evaluation underlined an increase in left-ventricular elastance. As regards the comparison with patients with known or suspected coronary artery disease, those have an increased carotid diameter, reduced carotid distensibility, and reduced ventricular elastance at rest with respect to the group of young healthy volunteers.

As already mentioned in controls, exercise induced carotid dilatation, reduced carotid distensibility and increased left-ventricular elastance, but this does not occur in patients, despite a similar increase in mean blood pressure. Hence, in the presence of cardiovascular pathology an altered adaptation of carotid to exercise and an altered carotid-ventricular coupling was observed.

In healthy subjects the physiological reduction in arterial distensibility and the carotid vasodilation seems to be related to exercise intensity, and it is supported by an increase in cardiac work efficiency.

This relationship is altered in patients with cardiovascular disease. The non-physiological adaptation of carotid to exercise could be a result of

changes in carotid geometry and the inability to increase cardiac contractility.

4.2 Conclusions

In conclusion, this work shows that the reproducibility of carotid elasticity measurements obtained by the developed algorithm in healthy subjects during various exercise steps is comparable to resting variability reported in literature for well-known and validated gold-standard techniques. Moreover, we compared the results obtained in controls and patients with known or suspected coronary artery disease. Besides the vascular evaluation also cardiac analysis was provided by left-ventricular elastance assessment. The two populations showed different behaviour with an altered adaptation of carotid artery to exercise and an abnormal carotid-ventricular coupling in patients.

Thus, the proposed approach can be considered reliable and might be used in future population studies for investigating the dynamic behaviour of arterial elasticity and its role in arterial-ventricular coupling variation in stress conditions. Moreover, from these preliminary results, the importance of a dynamic assessment of carotid elasticity was confirmed, and there is evidence of a clinical need including a simple

and robust device to more easily perform this kind of analysis than by ultrasound.

CHAPTER 5

Future Perspective

Our work gave evidence of different carotid dynamic behaviour in controls and patients and of the huge clinical impact that this vascular analysis might have. The opportunities regarding our work can be divided in two main fields: i) the dynamic evaluation of other vascular biomarkers, ii) the development of a simple and low-cost device for dynamic carotid analysis.

Dynamic evaluation of other vascular biomarker

As mentioned in the first chapter, besides the evaluation of carotid elasticity, some other innovative biomarkers are available and their dynamic analysis might provide relevant and additional clinical information. The scientific attention includes the assessment of these parameters in different populations, such as healthy controls, subjects with risk factors, patients with cardiovascular diseases and extreme conditions (i.e. athletes, high altitude etc).

Among the known vascular biomarkers we put our attention on the analysis of carotid Intima-Media Thickness and in particular on its changes in athletes after competition. The evaluation of cardiovascular properties in extreme conditions of exercise could enhance the characterization of vessel elastic properties, elucidating dynamic behaviours and modifications due to training. In a group of thirteen ultra-marathon elite athletes (40.9±3.7 years, 8 males, BSA = 1.72±0.17 mq) we evaluated the acute effects of participation in a ultra-marathon on carotid Intima-Media Thickness, diameter and elasticity.

The subjects underwent ultrasound examinations at rest and at the end of a 100km race. Ten age- and sex-matched sedentary healthy controls were also studied in resting conditions. Ultrasound B-mode image sequences of right common carotid arteries were acquired before (rest) and immediately after (post) participation, and analyzed by our automatic system for the measurement of arterial diameter (D). The method was integrated with a new algorithm able to automatically assess also Intima-Media Thickness (IMT). In addition, carotid blood pressure (BP) values were estimated by applanation tonometry and cross-sectional distesibility coefficient (DC) was then obtained.

At rest athletes showed similar D and IMT, but lower mean BP and higher DC in comparison to sedentary controls.

With exercise athletes showed a significantly decreased BP, in the presence of unchanged total body water. D increased, DC tended to increase, and IMT decreased.

Elite ultramarathon competitors at rest did not show structural carotid remodeling but presented higher distensibility than sedentary controls. In these athletes, prolonged exercise induced blood pressure reduction and carotid dilation, in conjunction with a further increased of carotid elasticity and a reduction in IMT. From these preliminary data, we can speculate that athletes undergo a vascular adaptation consisting in IMT remodelling, higher carotid elasticity and enhanced vasodilator reserve. Further studies in this field are needed to completely understand the dynamic IMT behaviour and in general the vascular dynamic differences in controls, patients and athletes.

Development of a simple and low-cost device for dynamic carotid analysis

In order to make the dynamic analysis of carotid artery widespread, an easier and less expensive technique able to provide the information we obtained by

ultrasound image processing in conjunction with tonometry, should be available.

In our lab a first prototype based on vibration approach was designed and might be a suitable solution for implementing low-cost and simple carotid elasticity dynamic evaluation. The system diagram is summarised in the figure below.

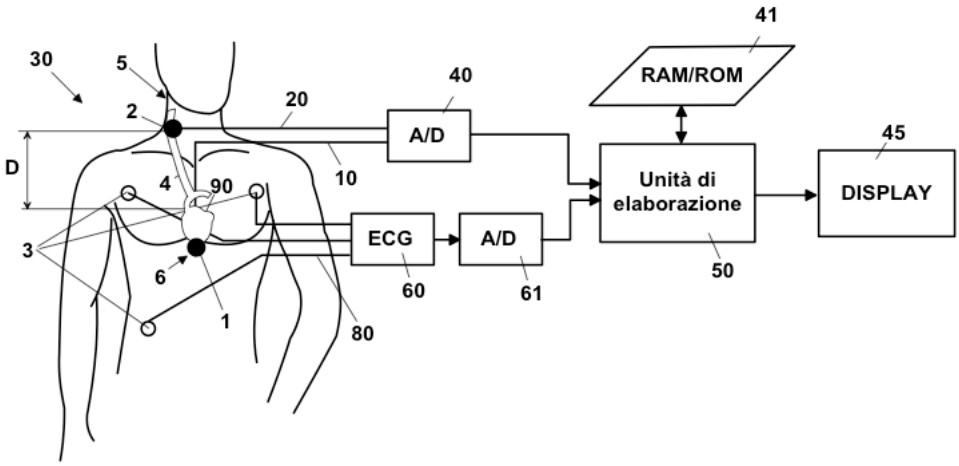

Figure 1. New simple and low-cost system for carotid elasticity evaluation

With this system the temporal distances (i.e. Pulse Transit Time, PTT) between two signals from two sites of the carotid artery can be obtained. The ratio between the geometrical distance of the two points and the PTT allows the estimation of the Pulse Wave Velocity (PWV);

PWV increases when arteries become stiffer and hence can give information regarding vessel elasticity [6].
The device includes two sensors based on MEMS accelerometers (Figure 2), an ECG (elettrocardiogram) signal acquisition, a wireless (Bluetooth®) portable unit and a laptop for signal processing.

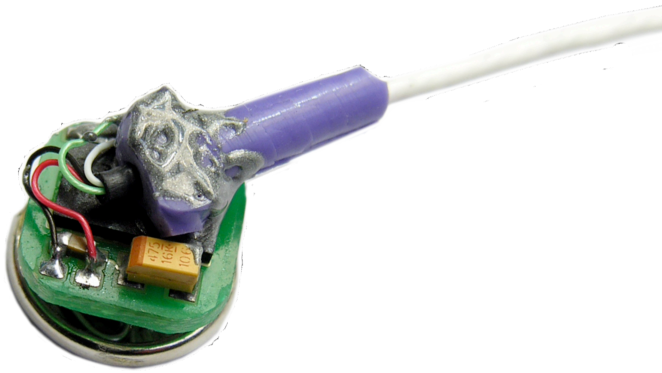

Figure 2. MEMS accelerometer

Data can be acquired at 500 Hz, ensuring the required temporale resolution also in dynamic conditions when human cardiac frequency becomes higher.

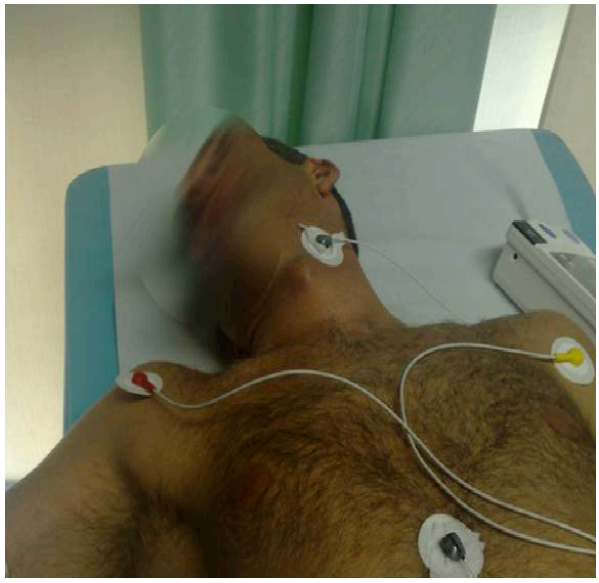

Figure 3. Application of the sensors: carotid assessment and contractility evaluation.

Moreover, the same idea might be applied also to obtain information regarding the cardiac contractility, whose behaviour is altered in exercise as shown by our data, and might allow a better stratification of cardiovascular status. Left-ventricular contractility might be measured by a third sensor applied to the chest [92] as shown in figure 3.

Studies regarding this new technology are in progress, in order to assess both the feasibility and the robustness of the system.

This simple device might allow the diffusion of a dynamic cardiovascular analysis able to improve the stratification of patients and their treatment.

Hence, in the future, the cardiologist ambulatory might provide, besides cardiac and pressure monitoring, additive relevant clinical information from a simple arterial elasticity 24-hours device.

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Appendix A

In this section the main papers reporting some of the results from this project are reported:

1. *"Functional and structural alterations of large arteries: methodological issues."* ***Bianchini E****, Giannarelli C, Bruno RM, Armenia S, Landini L, Faita F, Gemignani V, Taddei S, Ghiadoni L.Curr Pharm Des. 2012 Nov 19.*
2. *"Assessment of carotid elasticity during exercise: a reproducibility study".* ***Bianchini E****, Bruno RM, Corciu AI, Faita F, Gemignani V, Ghiadoni L, Picano E, Sicari R. Ultrasound Med Biol. 2012 Feb;38(2):223-30.*

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Functional and Structural Alterations of Large Arteries: Methodological Issues

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Abstract: Ultrasound assessment of vascular biomarkers has been implemented for screening, prevention and improvement of cardiovascular risk stratification beyond classical risk factors including smoking, diabetes, hypercholesterolemia and hypertension. Thus, the presence of vascular damage at the sub-clinical, asymptomatic stages can identify a "vulnerable" patient, and aid in implementing cardiovascular prevention strategies.

Increased intima-media thickness of the common carotid artery is a well-known marker of early atherosclerosis, which significantly correlates with the development of coronary or cerebro-vascular disease. More recently, guidelines for cardiovascular prevention in hypertension also introduced other vascular parameters evaluating both mechanical and functional arterial proprieties of peripheral arteries. Increased arterial stiffness, which can be detected by ultrasound at the common carotid, has been shown to predict future cardiovascular events and it is already considered a subclinical target organ of hypertensive patients.

Even earlier vascular abnormalities such as endothelial dysfunction in the peripheral arteries, detected as reduced flow-mediated dilation of the brachial artery by ultrasound, have also been mentioned for their possible clinical use in the future.

This manuscript reviews clinical evidence supporting the use of these different vascular markers for cardiovascular risk stratification, focusing on the need for an accurate, robust and reliable methodology for the assessment of vascular markers, which could improve their predictive value and increase their use in routine clinical practice.

Keywords: Ultrasound, carotid artery, intima-media thickness, stiffness, endothelium, automated edge detection

1. INTRODUCTION

Cardiovascular disease is one of the major causes of mortality and morbidity worldwide [1]. The prevention of this disease, which based on 2008 mortality rate data kills more than 2200 Americans each day (an average of 1 death every 39 seconds), is a priority in public health [2]. Cardiovascular risk estimated from classic risk factors such as smoking, obesity, hypercholesterolemia and hypertension may be not enough for effective prevention.

The possibility of identifying a "vulnerable" patient can be increased by the introduction of biomarkers such as those objectively measurable by ultrasound [3], thus implementing the model for effective prevention with the presence of vascular damage at the sub-clinical, asymptomatic stage preceding the onset of decades of disease (Fig. **1**).

Integrative vascular markers of risk that can be effective for the screening, prevention and improvement of cardiovascular risk stratification are well underlined by the European Hypertension Guidelines [4, 5]. In particular, increased intima-media thickness of the common carotid artery is a marker of early atherosclerosis, which significantly correlates with coronary or cerebrovascular disease [6, 7], and it has been considered an intermediate stage in the continuum of vascular disease and a determinant of total cardiovascular risk. These guidelines also introduced other vascular parameters evaluating mechanical and functional arterial proprieties of peripheral arteries [5]. Increased arterial stiffness has been shown to predict future cardiovascular events [8] and it is already considered a subclinical target organ of hypertensive patients [4].

Earlier vascular abnormalities, such as endothelial dysfunction in the peripheral arteries [9], have also been mentioned for their possible future use.

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This manuscript will review clinical evidence supporting the use of these different vascular markers for cardiovascular risk stratification. We will also focus on the need for an accurate, robust and reliable methodology, particularly for the evaluation of the newly proposed vascular markers, improving their predictive value for possible future use in clinical practice.

2. CAROTID INTIMA-MEDIA THICKNESS

2.1. Clinical Aspects and Prognostic Values

Carotid intima-media thickness (C-IMT), as measured by high resolution B-mode ultrasound of extra-cranial carotid arteries, is the most widely accepted non-invasive marker of subclinical atherosclerosis [10]. C-IMT is considered an intermediate phenotype of atherosclerosis suitable for use in large-scale population studies [11]. Increased C-IMT has been associated with augmented cardiovascular risk [12, 13] as well as with presence of advanced atherosclerosis at different vascular sites including peripheral, cerebral and coronary areas [14, 15]. Most importantly, epidemiological studies, including the Atherosclerosis and Risk in Communities Study (ARIC), the Rotterdam Study and the Cardiovascular Health Study, have consistently reported the predictive value of C-IMT for myocardial infarction or stroke independent of traditional cardiovascular risk factors [7, 16-21].

A recent meta-analysis of eight relevant general population-based studies involving a total of 37,197 subjects followed for a mean of 5.5 years confirmed the strong independent predictive value of cross-sectional C-IMT for future cardiovascular events [22]. The predictive value of C-IMT for cardiovascular events has also been confirmed in asymptomatic Type 2 diabetic patients. In particular, the combination of Framingham Risk Score (FRS) and C-IMT showed a greater predictive value than FRS alone in this population [23].

Given the predictive value of increased C-IMT for cardiovascular disease, its progression has been related to increase relative risk

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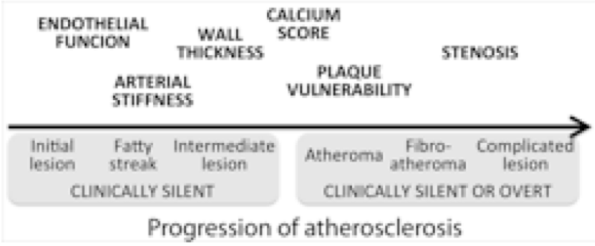

Fig. (1). Schematic representation of progression of atherosclerosis and its possible detection by vascular tests.

of cardiovascular disease. Consequently, it has been proposed that reduced progression of C-IMT is congruent with a reduction in cardiovascular events [24]. This hypothesis has been documented in clinical trials designed to study the efficacy of statins where C-IMT was used as surrogate end-point. The effect of statins on C-IMT progression appears to be correlated to potency and intensity of treatment in reducing LDL levels. Several clinical trials have shown a regression of C-IMT in subjects aggressively treated with atorvastatin 80 mg, whereas a reduced progression of C-IMT was observed in subjects less intensively treated with simvastatin or pravastatin 40 mg [25-27].

The results of the "Measuring Effects on Intima-Media Thickness: an Evaluation of Rosuvastatin" (METEOR) Study show a reduced progression of C-IMT in the rosuvastatin 40 mg arm [28]. In contrast with previous findings, no regression was observed despite a 48.8% reduction of LDL-C by rosuvastatin. A similar effect of rosuvastatin on progression of C-IMT was confirmed by the results of a smaller study [29]. A possible explanation for the discrepancy of results of studies could regard the different cardiovascular risk of the recruited populations. In the METEOR trial only asymptomatic subjects with no advanced atherosclerosis, no current requirement for statin use and low FRS (< 10%) were recruited, while most of the previous lipid-lowering studies showing C-IMT regression were performed in secondary prevention or in high-risk patients with elevated LDL-C, in whom a greater effect on C-IMT is conceivable.

The European Lacidipine Study on Atherosclerosis (ELSA), a randomized trial in which 2334 hypertensive patients received effective antihypertensive treatment for 3.75 years, showed that although baseline CIMT strongly predicted cardiovascular events during the follow-up period, differences in CIMT measured yearly compared with baseline did not [30]. Furthermore, a meta-analysis including 41 trials with 18,307 participants showed that despite a significant reduction in cardiovascular events and all-cause death induced by active treatments, there was no significant relationship between C-IMT regression and events, suggesting that regression or slowed progression of C-IMT, induced by cardiovascular drug therapies, may not reflect a reduction in cardiovascular events [31].

Conflicting results on the independent predictive value of C-IMT for cardiovascular events have also been recently reported. The Three-City Study is a large prospective study in which 5,895 adults aged 65-85 years with no history of coronary heart disease were scanned to measure C-IMT at a plaque-free site of the common carotid artery and followed-up for 6 years [32]. Mean C-IMT measured in areas without focal plaques in the common carotid was not an independent predictor for cardiovascular disease. In this study, carotid plaques were strictly quantified at different sites (near and far walls of common carotid artery, bifurcation, origin of the internal carotid arteries) and defined as localized echo structures encroaching into the vessel lumen for which the wall thickening was at least 50% greater than that of the surrounding vessel wall. The investigators of the Tromso study, a prospective population-based study, have previously reported similar observations [33]. In this study both total plaque area and C-IMT were measured in over 6000 healthy participants. After 6 years of follow-up, the results showed that carotid plaque area was a stronger predictor of myocardial infarction than was C-IMT.

A recent meta-analysis of 11 population-based studies (54.336 subjects) provided further evidence of a stronger predictive value for future cardiovascular events of carotid plaque than C-IMT [34].

Taken together, the results of these studies question the accuracy of C-IMT as a marker of atherosclerosis. C-IMT detected by high-resolution ultrasound represents the combined width of the carotid artery intima and media with the technical limitation of not being able to distinguish between intima and media. The carotid artery is an elastic artery and C-IMT in healthy subjects consists almost entirely of media. While the carotid artery is unaffected by age or gender until 18 years of age, thereafter there is a progressive intimal thickening or medial hypertrophy determined by age, gender and hypertension that do not necessarily reflect the atherosclerotic process [35]. The observation that the classic risks factors for atherosclerotic disease poorly correlate with C-IMT further supports this hypothesis [36-38]. Pathological studies indicate that C-IMT mainly represent hypertensive medial hypertrophy or thickening of smooth muscle media, whereas atherosclerosis is largely an intimal process [39]. In line with the hypothesis that C-IMT is biologically distinct from plaque, age-related thickening of intima-media layers of the common carotid artery has been observed in the absence of overt atherosclerosis [35]. Thus, it is not surprising that atherosclerotic plaque offers a better prognostic value than C-IMT for cardiovascular events.

No firm conclusion can be drawn either way on the prognostic value of C-IMT, until large epidemiological studies include measurement of C-IMT at different sites following standardized protocols that would provide useful information for clarifying the value of C-IMT as surrogate marker of atherosclerosis and/or more generically of cardiovascular disease. In particular, C-IMT evaluation at Carotid Bulb (CB) or Internal Carotid Artery (ICA), the two vascular sites most frequently affected by atherosclerosis, should be routinely included in future clinical trials. Since total plaque area might provide a better prognostic value for future cardiovascular events than C-IMT, this evaluation should be part of the same studies. Nevertheless, plaque burden (area or volume) evaluation should be performed following precise protocols established in a future consensus of experts on ultrasound carotid examination.

2.2. Methodological and Technical Issues

C-IMT is included in the American College of Cardiology and American Heart Association guidelines as a class IIA recommendation for intermediate risk patients [40]. However, several methodological aspects should be taken into consideration for its correct evaluation as recently suggested by an expert's review [41] since C-

IMT value can be influenced by location of the measure, type of ultrasound data and features of the reading system.

A careful examination of previous studies on C-IMT reveals methodological discrepancies that must be taken into account for a proper interpretation of results. Inaba *et al*. [34] observed that 77% of the studies included in their meta-analysis did not indicate whether plaques were actually included in C-IMT analysis. In addition, 63% of the studies used maximal C-IMT, more likely reflecting focal thickening or plaque, instead of mean C-IMT. Furthermore, study design of C-IMT trials was heterogeneous since the definition of the landmarks of carotid segments (Common Carotid Artery, CCA, Carotid Bulb, CB or Internal Carotid Artery, ICA) selected to measure C-IMT differ significantly [22]. The far wall of CCA is the easiest of the three anatomical segments to examine, being the most commonly used measurement in clinical studies. Unfortunately plaques are rare at this site and studies of the relationship of C-IMT at this site are conflicting [41]. The carotid artery is a complex vessel, with differing associations for each segment regarding risk factors and outcome [41]. Common carotid C-IMT is a better predictor of stroke than myocardial infarction [42, 43] and shows a better correlation with left ventricular mass than with coronary artery disease [41].

These data suggest that different pathological processes occur at distinct vascular sites of the carotid artery in different stages of disease. Indeed, the hemodynamics of the carotid artery in its different segments explains why atherosclerotic plaques are located in the carotid bulb and internal carotid artery than in the common carotid segment, which is affected in more advanced stages of the disease [35].

Several studies suggest the CCA far wall as the best location in terms of feasibility and reproducibility of the measure [44, 45] and this has been considered as the standard segment for the evaluation.

However, it might also be interesting to investigate whether information provided at CB or ICA, more challenging from a technical point of view, may show stronger correlation with classic risk factors [45]. Another point to take into consideration specifically referring to CCA is the variability in morphology and in vessel appearance under pathology. In particular, a horizontal image of the carotid artery cannot always be obtained depending on the anatomy of the subject, and this may be a problem for some automated segmentation techniques [46].

A post-hoc analysis to determine the best algorithm for determining CIMT using data from the METEOR study showed that ultrasound protocols that include CIMT measurements at multiple angles of both the near and far walls provide the best balance between reproducibility, rate of CIMT progression, treatment effect and their associated precision in this low-risk population with subclinical atherosclerosis [47].

Regarding the ultrasound data that can be used to obtain C-IMT evaluation, two main types are commercially available: B-mode image processing-based device and Radio-frequency (RF)-based echo-tracking system [46]. In the past, estimation was obtained manually, but currently the assessment of C-IMT is generally obtained by automatic processing of these ultrasound signals [46]. RF data devices are considered very accurate since they are based on signals with higher spatial resolution than B-mode data [48, 49]. However, when comparing the performance in terms of reproducibility of this kind of technique with that of robust image-based systems, similar results are obtained [46, 50]. The repeatability of the two approaches was recently tested in the same population, obtaining similar coefficients of variation (5% for RF-based device and 6% for image processing systems, respectively) [50]. A good agreement between the two techniques in terms of Bland-Altman statistics was also reported. However, it must be pointed out that the quality of the final result of B-mode based systems is related to several issues which have to be carefully considered, such as quality of the scans and the system's setting. In particular, Potter *et al*. [51] studied the effects of changing dynamic range (DR), gain set and probe distance in C-IMT assessment by an image analysis software applied to an agar phantom. An increase in DR or gain causes a reduction in the measured wall thickness, whereas the distance of the probe did not influence the final result. Hence, DR and gain sets, but also other parameters such as depth gain compensation (DGC) or filtering should be standardized as suggested by international guidelines [52] or at least documented in follow-up analysis. Furthermore, Rossi *et al*. [53] analyzed the influence on carotid diameter evaluation of non-linear processing generally used in standard ultrasound equipment for better image visualization. In particular, these authors show that logarithmic compression and saturation can cause alteration when using approaches based on the gray level gradient, and the consequent small deviation might affect also the assessment of C-IMT. Another point to consider when working with standard US equipment is whether values obtained with newer apparatus are comparable to those obtained with older ones. A recent paper [54] reported the effects of transducer frequency on the final result by semi-automated analysis in a small group of patients. In particular C-IMT measurements obtained with standard (8 MHz) and high (14 MHz) frequencies were comparable.

Other features of the reading systems should be also considered for the assessment of C-IMT. Low-cost and user-friendly devices can make the diffusion of this vascular biomarker easier and faster. Hence, reliable and robust software based on B-mode image processing, which can be adopted with any standard ultrasound equipment, could provide an effective solution. Nevertheless they should be used according to international guideline suggestions, with particular attention to machine settings. Finally, it is worth noting that despite guideline suggestions introducing standardization in the measure, different approaches are available for C-IMT estimation in terms of analyzed data, (i.e., B-mode images or RF signal processing) or for anatomical sites. Thus, future analysis providing the agreement between different kinds of measurements and reference values for risk classification are needed in order to improve the clinical implications of C-IMT assessment.

3. CAROTID DISTENSIBILITY AND STIFFNESS

3.1. Clinical Aspects and Prognostic Value

Arterial distensibility is a measure of the artery's ability to expand and contract with cardiac pulsation and relaxation. Hypertension and other risk factors such as diabetes, dyslipidemia and smoking can alter the structural and functional properties of the arterial wall, leading to a decrease in arterial distensibility. This seems to be a common pathologic mechanism for many factors that lead to the occurrence and progression of the vascular changes associated with cardiovascular disease [49]. The aorta is a major vessel of interest when determining regional arterial stiffness, for at least two reasons: the thoracic and abdominal aorta makes the largest contribution to the arterial buffering function, [49] and aortic stiffness is an independent predictor of outcome in a variety of populations [8]. The measurement of aortic stiffness as carotid-to-femoral pulse wave velocity (PWV) by arterial tonometry is generally accepted as the most simple, non-invasive, robust, and reproducible method to determine arterial stiffness [49]. However, it should be recognized that carotid-femoral PWV is not a direct measurement, since it is based on the acceptance of a propagative model of the arterial system. Thus, other arterial sites have potential interest: the measurement of local carotid stiffness may also provide important prognostic information, since the carotid artery is a frequent site of plaque formation [49].

Aging is physiologically accompanied by arterial dilatation, increase in wall thickness and reduction of the elasticity and compliance, all features characterizing atherosclerosis [55]. The main structural change occurring with aging is the degeneration of the tunica media, which causes a gradual stiffening of large elastic

arteries [56]. This correspond on one hand to a reduced synthesis and an increased degradation of elastin, and on the other hand to an increased synthesis and reduced degradation of type 1 and type 3 collagen [57]. Aging, along with blood pressure, is the main determinant of stiffness in both carotid and aortic stiffness [58]. However, histological differences exist between large artery areas, responsible for different behaviors in the presence of cardiovascular risk factors. In particular, even if the carotid artery and the aorta are both classified as elastic vessels, the ultra-structure of the carotid artery is intermediate between muscular and elastic arteries, being more similar to the abdominal than to the ascending aorta. The radial, brachial and femoral arteries, which have a muscular structure, are resistant to age-induced stiffening when compared to the carotid artery [59]. This implies that carotid and arterial stiffness are strictly correlated in the healthy population, while the correlation becomes weaker as soon as the number of cardiovascular risk factors increases [58]. Therefore, although carotid-femoral PWV and carotid stiffness provide similar information on the impact of aging on the stiffness of large arteries in healthy subjects, this is not the same for the hypertension and/or diabetes. In these cases, the aorta stiffens more than the carotid artery due to age and other cardiovascular risk factors [58].

Several studies have investigated the physiopathology of carotid stiffness in essential hypertension. The increased arterial stiffness observed in patients with essential hypertension was generally attributed to arterial wall hypertrophy [60]. However, further studies have shown that the increased carotid stiffness observed in hypertensive patients was due to an increase in distending pressure and not to hypertension-associated changes in structural properties, suggesting a functional adaptation of the wall material [61]. Young's incremental elastic modulus of the common carotid artery has been shown to exist in young never-treated hypertensive patients in comparison to age- and gender-matched normotensive subjects, at a given circumferential wall stress, whereas it did not differ between the two groups in middle-aged and older individuals [62]. Thus, the mechanisms involved in arterial stiffening in younger hypertensive patients probably differ from those advanced to explain the stiffening of large arteries with aging [55].

The Atherosclerosis Risk in Communities (ARIC) Study, a population study recruiting a biracial sample of 4701 men and women 45-64 years of age, investigated the relationship between carotid stiffness and different cardiovascular risk factors. Carotid stiffness was associated with hypertension, diabetes, trait anger, physical activity, and ethnicity [63-67]. In particular, metabolic factors, such as elevated glucose, insulin, and triglycerides had a synergistic effect on Young's elastic modulus, which estimates arterial stiffness controlling for intima-media thickness [63]. Metabolic factors such as body mass index and triglycerides were independent correlates of Young's elastic modulus also in the Bogalusa study, enrolling a younger multiracial population sample (516 asymptomatic subjects aged 25-38 years), beyond blood pressure values, sex and age [68].

In the Baltimore Longitudinal Study on Aging, an independent association between suppressed anger and carotid stiffness was reported, as well as an increase in stiffness with the clustering of components of metabolic syndrome and decreasing levels of testosterone [69]. In an aged population with high prevalence of CV risk factors and disease, such as that of the Hoorn study, low-grade inflammation appeared to have an important role in determining increased carotid stiffness, mainly through arterial enlargement [70]. In the same population, metabolic syndrome has been associated with stiffness of muscular arteries (brachial and femoral), but not of muscular-elastic arteries (carotid and aorta) [71], again confirming that impact of different risk factors varies depending on the area considered. In the Second Manifestations of ARTerial disease (SMART) Study, decreased carotid distensibility was a marker of increased cardiovascular risk but in patients who already had vascular disease [72]. Carotid stiffness was also able to predict incident hypertension in the ARIC cohort [64].

Arterial stiffness has been shown to be an independent predictor of cardiovascular morbidity and mortality [8] and is thought to play a crucial role in the development of cardiovascular disease. The predictive value of arterial stiffness has been shown mainly for aortic PWV [8], but also from local carotid artery stiffness [73]. Currently these techniques are widely used in interventional clinical studies to assess the effect of either non-pharmacological or pharmacological treatments on cardiovascular risk [74, 75].

Some studies were specifically directed towards the association between carotid stiffness parameters and the risk of cardiovascular events. Blacher *et al.* were the first to analyze a cohort of 79 patients with chronic renal failure undergoing hemodialysis, followed-up for 25 months, during which there were 10 fatal cardiovascular and 8 non-cardiovascular events. The study shows that in patients with chronic renal failure, increased carotid stiffness is a powerful independent predictor of all-cause and cardiovascular mortality [73]. Also after renal transplantation carotid artery distensibility proved to be an independent predictor of cardiovascular disease, confirming the importance of chronic kidney disease in influencing carotid wall mechanics [76]. In the SMART study, a group of patients with manifest cerebrovascular, aortic, coronary or peripheral disease was prospectively examined, to assess whether the carotid arterial stiffness was related to the occurrence of cardiovascular events and mortality [77], showing that increased carotid stiffness was associated with an increased risk of cardiovascular events and mortality in not-corrected analysis, whereas the relationship disappeared after controlling for age. However, an important limitation of this study is the fact that brachial instead of the local blood pressure was used for calculation of the carotid stiffness parameters, possibly leading to underestimation of the relationship between arterial stiffness and cardiovascular events.

In the Three-City study the mechanical properties of the carotid artery wall were studied in 3,337 elderly subjects (mean age 73 years), followed for a median of 44 months [78]. In this study, patients who had a higher distension at baseline showed an increased risk of coronary events compared to the patients with lower distension. This relationship was independent of age, sex, brachial and carotid pulse pressure (PP), heart rate, anti-hypertensive treatment, C-IMT, carotid plaques and other major cardiovascular risk factors. In the study, in agreement with the previous results [79], carotid PP was closely related to coronary events. However, the association observed between carotid artery distension and coronary events was independent of carotid PP, suggesting that arterial wall distension was not completely determined by wall stress. However, no association between coronary events and distensibility was found, while the association between carotid distension, carotid compliance, Young's elastic modulus and coronary events, was independent from the presence of atherosclerosis [78].

Finally, an independent association between increased carotid stiffness and a first-ever acute ischemic stroke has already been reported [80, 81], although other studies did not find the same relationship [82]. Although the large variance in technical measurements of carotid stiffness and blood pressure were most likely responsible for conflicting data, a tighter cause-effect relationship with cerebral rather than coronary cardiovascular events is not surprising, as confirmed by a recent analysis conducted in 10,407 subjects of the ARIC study followed-up for 13.8 years. The study showed that after adjusting for cardiovascular risk factors, ultrasound measures of carotid arterial stiffness are associated with incident ischemic stroke but not incident coronary events, although the two outcomes shared similar risk factors [83].

3.2. Methodological and Technical Issues

Arterial stiffness can be estimated at the systemic, regional and local levels [49]. The local measure is generally obtained at the

common carotid site, a large superficial artery that is easily accessible; this evaluation is considered particularly accurate, since unlike the systemic and regional evaluation, in this case arterial stiffness is determined locally and is estimated directly by pressure changes, which in turn determine the changes of volume of the vessel. The local assessment can be obtained by measuring the diameter of the vessel and its variations during the cardiac cycle (stroke change in diameter or distension) by ultrasound signal in conjunction with local pulse pressure estimation by tonometry [49].

With ever-increasing attention focused on the clinical implications of arterial stiffening analysis, it is extremely important to take into consideration methodological aspects, regarding arterial diameter assessment by ultrasound influencing clinical study outcomes. Several factors should be considered when performing this kind of measurement, especially accuracy, precision and feasibility.

Two main approaches are available for arterial diameter assessment by ultrasound data: B-mode image processing based device [50, 84] and radio-frequency (RF)-based echo-tracking system [48]. Devices processing RF data are considered very accurate since they are based on signals with higher spatial resolution than B-mode data [49]. Furthermore, when adopting B-mode based systems some issues should be considered since the accuracy of this kind of device depends on many aspects such as the quality of the scans, and can be influenced by the system's setting. In particular, Potter *et al*. [51] showed that dynamic range (DR), gain set and probe distance alter lumen diameter values obtained by an image analysis software applied to an agar phantom; an increase in DR, gain or distance causes a reduction in the measured diameter value. Hence, DR and gain sets, but also other parameters such as depth gain compensation or filtering should be documented and replicated when performing follow-up analysis on the same subject, and consensus guidelines adopted. As regards the distance's influence on the final measurement, the authors suggest keeping in mind this aspect when interpreting the reported effects of weight changes on arterial diameter. Furthermore, Rossi *et al*. [53] showed that non-linear processing used to improve the B-mode image visualization on standard ultrasound equipment could affect the diameter measure obtained by edge-detection algorithms. In particular, the authors show that logarithmic compression and saturation can cause alteration when using approaches based on the grey-level gradient. Another aspect should be taken into consideration when using methods based on edge-detection for diameter assessment on ultrasound images: the location of a grey-level discontinuity corresponding to an artery interface depends on the mathematical operator adopted and on its particular configuration. Consequently, it is possible that an edge detector converges to a point slightly different from the real localization of the interface [50]. This issue is relevant when tracking the two walls of a longitudinal section of the vessel in order to compute the diameter; in fact, in this case the grey-level discontinuities of the near and far border respectively are in opposite directions, and different convergence points result in different measures. On the other hand, when evaluating distance where the grey-level discontinuities corresponding to the two edges are in the same direction, like the C-IMT evaluation, the possible different point of convergence does not influence the resulting measurement. A similar consideration can be drawn regarding distension, since it is computed as the subtraction of two diameter values and hence is not influenced by the edge location [50].

RF-based devices are generally also considered more precise than video-image systems, which are limited by the spatial resolution of pixel analysis. For this reason, precision for video-image analyzers is usually estimated to be about 150 µm (i.e., the size of the pixel) [49] and this would be insufficient for determining arterial stroke change in diameter. However, it is important to point out that methods are available based on algorithms with sub-pixel precision, able to evaluate change in a diameter less than 15 µm [50, 84], and therefore suitable for local arterial stiffness assessment. In addition, studies investigating precision in terms of repeatability of instantaneous arterial diameter evaluation by ultrasound data processing are available in literature [85]: coefficients of variation (CV) of the parameters involved in arterial elasticity evaluation which are considered appropriate for studying their physiological and pathophysiological variations, are shown in [86-88]. As an example Selzer *et al*. [88] reported a CV of 1.28% for arterial diameters and from 11.05 to 14.54% for carotid stiffness indices. Kool *et al*. [87] found a CV of 4.5% for carotid diameters, 7.9% for distension and 8.3 to 9.1% for arterial stiffness parameters.

Furthermore, in a recent work [50] reproducibility of RF- and image-based techniques were assessed in the same population showing comparable reproducibility and good agreement. Hence, it might be concluded that high spatial resolution of RF-based methods is not mandatory for standard clinical examination. This point might be even more interesting when considering how important it is to document the independent predictive value of carotid stiffness on cardiovascular events; so far only a few studies where parameters of carotid elasticity were used are found in the literature and the development of user-friendly and relatively inexpensive systems for assessing carotid diameter and distension would be important. In addition, besides the RF-systems, B-mode based devices that can also provide the automatic measure of carotid C-IMT (Fig. **2**) are available and are able to furnish both functional and structural parameters of the analyzed vessel, as suggested by the international expert consensus [49].

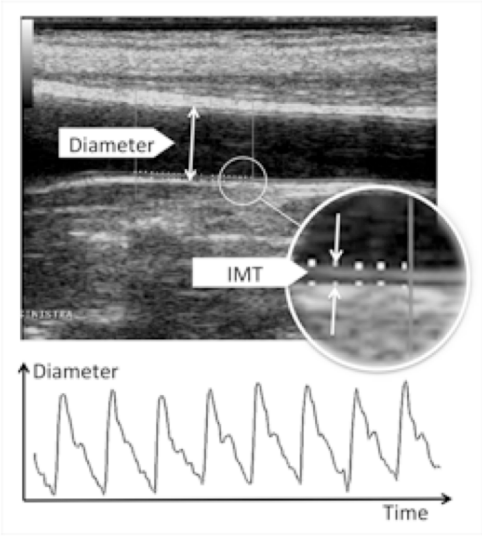

Fig. (2). Example of automatic edge detection of intima-media thickness (IMT) and diameter on B-mode scan of a common carotid artery (Top). Detected changes in carotid diameter (distension) over 8 cardiac cycles (bottom).

RF-based echo-tracking devices are considered the reference technique providing optimal conditions in the simultaneous measurement of local arterial stiffness and C-IMT for their high precision; however, since this kind of data output is not easily available in standard ultrasound equipment, reproducible and robust B-mode based technique (that can be applied to any ultrasound equipment) in conjunction with international guidelines, can be considered an effective alternative.

4. NON-INVASIVE ASSESSMENT OF ENDOTHELIAL FUNCTION: BRACHIAL ARTERY FLOW MEDIATED DILATION

4.1. Clinical Evidence and Prognostic Value

Endothelium plays a primary role in the control of vascular function [89] by the production of nitric oxide (NO), which derives from the transformation of L-arginine into citrulline by the constitutive endothelial enzyme NO synthase (eNOS), under the stimulus of agonists (acetylcholine, bradykinin, and others) acting on specific endothelial receptors and of mechanical forces, namely shear stress [90]. In pathological conditions, the same stimuli determine the production of endothelium-derived contracting factors (EDCFs, e.g., thromboxane A2 and prostaglandin H2), which counteract the relaxing activity of NO, and reactive oxygen species (ROS) which impair endothelial function by causing NO breakdown [90]. In such conditions, reduced NO availability and EDCF not only exert an opposite effect on vascular tone, but also facilitate the pathogenesis of thrombosis and atherosclerotic plaque by promoting platelet aggregation, vascular smooth muscle cell proliferation and migration, and monocyte adhesion [91].

This pivotal role of the endothelium in the atherosclerotic process (Fig. **1**) led to the development of different methods to assess endothelial function, which could provide novel insights into pathophysiology and 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 [92, 93].

Endothelial function in clinical research is mainly tested by vascular reactivity studies [92]. The most widely used technique is the so-called "flow-mediated dilation" (FMD) of the brachial artery. This is a non-invasive, ultrasound-based method, introduced in 1992 [94]. FMD occurs as a result of local endothelial release of NO and it is measured as brachial artery diameter changes in response to increased shear stress, induced by reactive hyperemia and measured [95, 96]. To this aim the sphygmomanometer cuff placed on the forearm distal to the brachial artery is inflated to 200 mmHg and subsequently released 5 min later (Fig. **3**). Endothelium-independent dilator response can be tested by low-dose sublingual nitroglycerin [97]. FMD has been studied widely in clinical research as it enables serial evaluation of young subjects, including children [94]. 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 [93].

Impaired FMD has been shown in hypertensive patients and in the presence of the other cardiovascular risk factors [97-101]. A report from the Framingham study showed a progressive inverse relation between FMD and the increased Framingham risk score [102]. A meta-analysis performed in over 200 available studies observed that the relationship between FMD and risk factors was more evident in patients with a lower cardiovascular risk [6].

Several studies have shown that endothelial dysfunction is an early indicator of atherosclerotic damage associated with target organ damage, including increased C-IMT [103-105] and left ventricular hypertrophy [106]. Importantly, impaired FMD has been associated with major cardiovascular events [107-110]. A meta-analysis, evaluating longitudinal studies on the prognostic impact of endothelial dysfunction and including around 2500 patients with atherosclerotic coronary disease or characterized by high cardiovascular risk, showed that endothelial dysfunction, also evaluated as FMD, significantly predicted cardiovascular events, independently of traditional cardiovascular risk factors [111]. These studies suggested the prognostic relevance of endothelial dysfunction in high-risk patients. This concept could be extended to lower risk populations according to the results of a more recent meta-analysis of four population-based cohort studies, and ten cohort studies, involving 5,547 participants, showed a pooled relative risk of cardiovascular events per 1% increase in brachial FMD, adjusted for confounding risk factors of 0.87 (95% CI, 0.83-0.91), consistent among all subgroups evaluated. However, the authors highlighted that the presence of heterogeneity in the study quality, the remaining confounding factors, and publication bias in the available literature prevent a definitive evaluation of the additional predictive value of brachial FMD beyond traditional cardiovascular risk factors [112].

Thus, correction of endothelial dysfunction might lead to improved cardiovascular prognosis. So far, only few studies have tested this hypothesis [107, 113]. In a group of 400 postmenopausal hypertensive women with impaired FMD, it was retested after 6 months of anti-hypertensive treatment [107]. At 5-year follow-up, the incidence of cardiovascular events was significantly lower in the subgroup of women whose FMD was improved as compared to the subgroup without improvement, despite a similar reduction in blood pressure. In a similarly designed study, 251 patients with

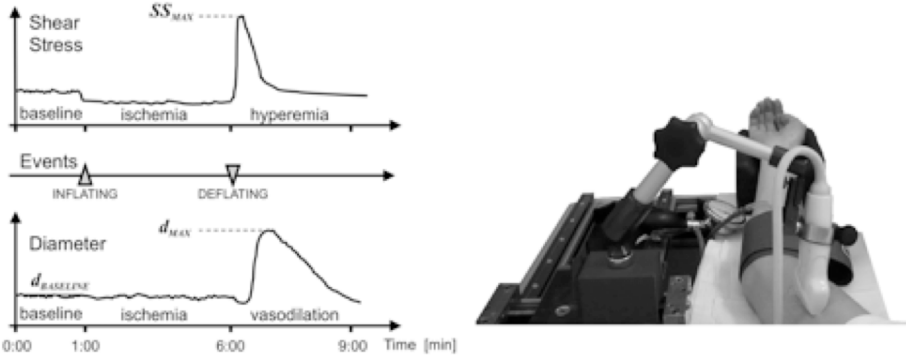

Fig. (3). Assessment of brachial artery flow mediated dilation (FMD). Right panel shows the stereotactic clamp to hold the probe and to adjust the images by means of micrometric screws. Graphs on the left panel show changes in diameter (d, bottom) and shear stress (SS, top) obtained by real-time analysis of B-mode and doppler signal during FMD assessment. Timing of cuff inflating and deflating for inducing and stopping forearm ischemia are also shown in the cartoon.

newly diagnosed coronary artery disease and an impaired FMD, measurement of FMD was repeated after 6 months for individualized and optimized therapy to reduce risk factors, and patients were followed-up for 36 months or until one cardiovascular event [113]. Multivariate Cox hazards analysis showed that persistent impairment of FMD was an independent predictor of events, while baseline FMD before the optimized therapy to reduce risk factors had no significant prognostic information in this high-risk population.

Several studies have shown that FMD can be improved with specific modifications of cardiovascular risk factors and with the use of drugs known to reduce cardiovascular risk [98, 114, 115]. Since the change in FMD occurring as a result of treatment can be obtained in a much shorter time (a few months) than required for other vascular endpoints such as carotid intima-media thickness [105] or arterial stiffness [49, 75], FMD testing has a potential role for inclusion in clinical trials as a surrogate end-point [116]. Despite this considerable evidence, further large-scale clinical trials are needed to demonstrate conclusively whether reversal of impaired FMD independently offers a better prognosis to patients with essential hypertension.

4.2. Methodological and Technical Issues

Assessment of brachial FMD in clinical investigation has increased because it is noninvasive and apparently easy to perform. However, several challenges must be overcome that are major limitations to a widespread application of this method in clinical studies [92, 116-119]. 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 [120]. Furthermore, other caveats should be considered in designing a study where FMD is investigated for the biological and technical variability of its measurement, including appropriate study design and sample size and efforts to achieve a uniform technique and minimize operator-dependency, including the adoption of probe-holding devices and automated systems to measure brachial artery diameter changes [116-119].

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 partly determined by the magnitude of post-ischemic forearm vasodilatation, which is a measure of microcirculatory function [93]. Interestingly, the use of upper cuff occlusion was associated with one of the few negative reports on the prognostic role of FMD [121], although a recent meta-analysis showed that studies applying the upper cuff occlusion technique showed similar prognostic predictive values compared with those using the lower cuff technique [122].

Training and certification of sonographers in FMD procedure has been well-described in guidelines [117], and proven by results in recent multicenter trials by the small number of rejected examinations, due to poor quality and/or instability of the images [123, 124].

The use of clamps to hold and adjust probe position, as well as a computerized system to automatically measure brachial artery diameter (Fig. **3**) are currently required to obtain the best reproducibility of this non-invasive technique [9, 92, 125], as recently also shown in multicenter settings [123, 124].

As of today, only a few experienced research centers apply a rigorous methodology to achieve a high standard of accuracy and reduce FMD variability [126]. The lack of uniform methodology, including all the above-mentioned procedures, is a major limitation, although not the only one, for the application of FMD assessment in large multicenter studies. We recently evaluated the time-dependent variability of FMD measurements obtained in more than 130 healthy volunteers by trained operators according to a uniform technique [124]. This included centralized analysis by an automated edge detection system, composed of a special-purpose hardware/software device for measuring changes in brachial artery diameter [127, 128]. The study showed for the first time that adherence to a rigorous protocol, with certified operator training as well as defined experimental settings (adjustable stereotactic probe-holding device, automated computer-assisted brachial artery measurements), is feasible in different research centers, ensures high quality examinations and, most of all, provides an optimal time-dependent reproducibility of FMD. In particular, a similar coefficient of variation (close to 10%) for intra-session (1 h apart) and inter-session (1 month apart) FMD assessment was shown and the overall FMD variability was comparable with that observed by the authors who originally described the non-invasive method for FMD using a similar methodology [126]. Thus, this approach should be implemented in all studies investigating FMD as a surrogate marker of cardiovascular disease.

As already stated, automated, computer-based analysis of brachial artery diameter changes [127, 128] is fundamental for the assessment and reproducibility of FMD testing. At the present time, automatic systems for FMD assessment are based on both post-processing and real-time analysis, thus working offline and online, respectively. In particular, real-time systems offer several advantages enhancing reliability and precision of FMD measurement [129]. Mainly, a real-time feedback signal generated during the scan acquisition and strictly related to the algorithm performance could continuously inform the operator about the quality of the ultrasound images. This aspect is of particular importance in FMD studies because in these examinations, the quality of the image is a critical component that can compromise the success of the measurement. Indeed, a proper image must be maintained for several minutes to best quantify the transitory response induced by the endothelium. For this reason, adjustments of the position of the probe may be required during the examination, especially to compensate for small movements of the patient. The sonographer is largely helped in this task by immediate feedback from the measurement system. As a final result, the number of examinations rejected due to low-quality post-processing analysis could be reduced [123, 124, 129].

Another advantage of online analysis is the reduction in time spent analyzing the images after acquisition and the absence of those drawbacks associated with video storing. Recording the video means a reduction of image quality, while an acquisition on a personal computer requires a large amount of memory. Moreover, the real-time characteristic improves the operator's learning curve, significantly reduced by this approach [129], another major challenge for FMD assessment [117].

Finally, another important characteristic of the FMD technique is the timing of the procedure, with respect to the cardiac cycle. In fact, vasodilatations induced by reactive hyperemia are not much larger than the diameter variations between systole and diastole [127, 128]. Guidelines suggest using electrocardiogram (ECG) gating during image acquisition [117], where the onset of the R-wave is used to identify the end diastole, and this is currently the method most commonly used both for manual and automatic analyses. However, this requirement influences the complexity of the ultrasound equipment adopted for the examination [130]. Nowadays, high frequency linear array transducers are also available in less expensive hand-carried ultrasound devices, which are being used more and more in research and clinical practice. Although such devices produce high quality B-mode images, they may lack ECG trigger capabilities, which are at times provided as an option with a significant increase in the overall cost of the system. On the other hand, modern automatic measurement methods used in FMD examinations have become faster and more precise, thus allowing a continuous measurement of the diameter curve with a sample rate of 25 to 30 samples/s. By using these systems, information on the timing with respect to the cardiac cycle can be obtained by directly

analyzing the diameter curve, without the need for an ECG trigger. Also, working at 25/30 frames per second ensures greater reliability against noise, analyzing a greater number of frames for diameter measurements, so FMD technique is more suitable for centralized readings.

Some issues remain unresolved in FMD measurement. In particular, agreement was not reached on the normalization of the percentage variation brachial diameter by the amount of the reactive stimulus (e.g. shear rate) that induced vasodilation (Fig. **3**). As a consequence, several papers present FMD values as not normalized, especially in the past. Recently, this problem has been recognized and a debate on how to normalize FMD values was started. At the present, the maximum shear rate, the full shear rate area under the curve and the shear rate area under the curve up to the peak of the FMD have been proposed as potential normalization factors with the last one as the most promising in terms of efficacy [116-119].

Lastly, some interesting new topics are still waiting answers in the FMD area. Among these, the need for reference values that could be used in clinical studies is the most interesting and imperative point. By this means, clinicians would be able to stratify populations and share results more easily.

5. CONCLUSIONS

Cardiovascular disease, although many of the major risk factors, such as age, smoking, hypercholesterolemia and diabetes are well-known today, remains a major cause of disability and mortality worldwide. Prevention remains the best approach to this health problem, but classic risk stratification is unable to provide an accurate estimate of probability that a subject will suffer from a cardiovascular event. Vascular biomarkers, which are parameters of subclinical cardiovascular disease, could increase the estimation of the individual cardiovascular risk and improve strategies for effective prevention.

Several vascular markers obtained by ultrasound have been shown to be independent predictors of cardiovascular events. Greater evidence is available for increased C-IMT, which is currently used as subclinical target organ damage. However, recent analyses of clinical studies criticized that the predictive role of C-IMT changes with therapies. This limitation could be overcome by automated measurements with greater precision and more reliable reference values for risk classification. An automated system would also allow simultaneously measuring stiffness of the carotid artery and providing adjunctive analysis of functional and mechanical properties of the carotid artery, which are related to cardiovascular prognosis [131].

Finally, since endothelium plays a central role in the maintenance of vascular homeostasis, the implementation of the methodology of non-invasive ultrasound-based tests such as brachial artery FMD will allow assessment of changes in endothelial function after therapy in relation to subclinical target organ damage and cardiovascular prognosis. However, this intriguing hypothesis requires more testing in specific ongoing and future clinical trials.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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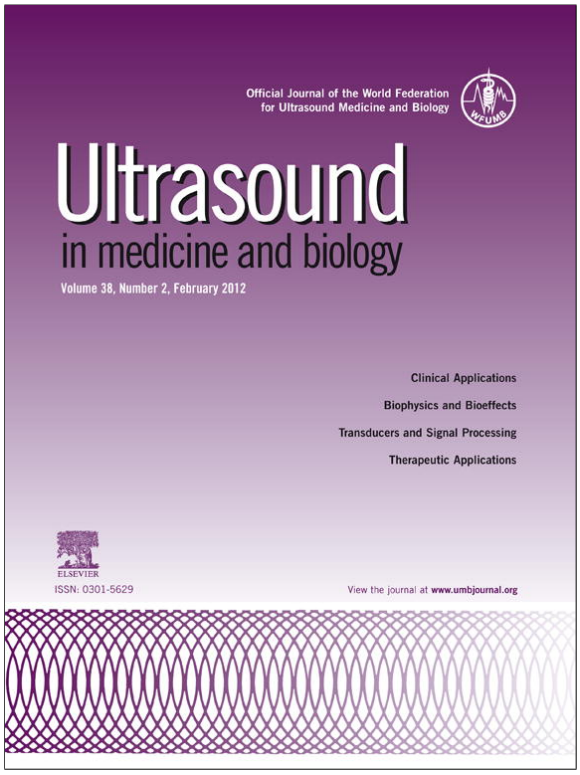

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● *Original Contribution*

ASSESSMENT OF CAROTID ELASTICITY DURING EXERCISE: A REPRODUCIBILITY STUDY

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Abstract—The study aimed to evaluate the reproducibility of carotid elasticity during exercise. Eighteen healthy volunteers (nine males, age 34 ± 3 years, BMI 22 ± 6 kg/m^2) underwent maximal exercise testing on a graded semi-supine cycle ergometer in two different sessions 3 days apart. Ultrasound B-mode image sequences of the right common carotid were acquired at different steps and analyzed by an automatic system; pressures were estimated by tonometry. Compliance (CC) and distensibility (DC) were significantly decreased at exercise peak and in the first recovery minute (CC from 1.6 ± 0.8 to 1 ± 0.6 mm^2/KPa, DC from 56.2 ± 25.3 to 34.5 ± 20 10^−3/KPa, $p < 0.05$). For the whole examination, intraclass coefficient was 0.780 for CC and 0.694 for DC. Mean coefficient of variation was maximum at peak exercise (CC = 19 ± 6%, DC = 24 ± 15%), but at first minute of recovery it was comparable to resting values (CC = 12 ± 9%, DC = 12 ± 11%). When designing future studies, acquisitions during first recovery minute might be preferred to peak measures. (E-mail: betta@ifc.cnr.it) © **2012 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Carotid stiffness, Carotid elasticity, Ultrasound, Exercise, Reproducibility.**

INTRODUCTION AND LITERATURE

An increasing number of studies propose methods for evaluating subclinical cardiovascular disease biomarkers, showing that some of these measurements are independent predictors of cardiovascular events (Mancia et al. 2007; Lorenz et al. 2007; Mattace-Raso et al. 2006). Among these, there has been great interest in the role of vascular parameters assessed at the level of the carotid artery, such as intima-media thickness (Touboul et al. 2007) and arterial stiffness (Laurent et al. 2007). In particular, important indices of local arterial stiffness of superficial arteries can be estimated by measuring the diameter and its change during the heart cycle (stroke change in diameter or distension) from ultrasound data in conjunction with the local pulse pressure.

This analysis is usually performed in resting conditions; however, arterial stiffness is not a static characteristic (Nichols and O'Rourke 1998). Therefore, quantification of parameters of local elasticity in dynamic conditions, when several physiologic changes occur (*i.e.*, in heart rate, blood pressure, sympathetic activity), could enhance characterization of the elastic properties of the vessel.

For this reason, carotid distensibility was recently assessed during isometric exercise (Myers et al. 2002), indicating a decrease in elasticity parallel to blood pressure increase, with different behavior according to gender and age. This phenomenon can manifest even in young, healthy adults, when static analysis might fail to find relevant differences. Other studies (Studinger et al. 2003) found carotid distensibility to be decreased in healthy volunteers during strenuous exercise, accompanied by an increase in mean baroreceptor activity level during exercise, thus, implying a role for mechanical factors in arterial baroreflex control.

Although analysis of local stiffness during exercise is intriguing, the dynamic conditions make this evaluation technically challenging. Also, reproducibility of this analysis during exercise remains to be evaluated. The aim of this work was to evaluate the reliability of the assessment of carotid artery elasticity parameters (*i.e.*, distensibility and compliance) during exercise in a group of healthy volunteers, in terms of reproducibility of the measurement.

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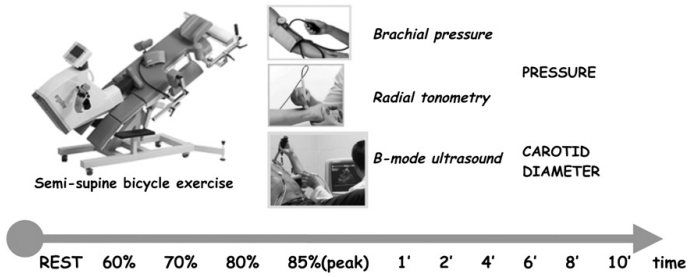

Fig. 1. Experimental set-up. Maximal exercise test was performed on a graded bicycle semi-supine ergometer. Measurements were performed during the examination, at 11 different temporal steps: 60%, 70%, 80% and 85% of maximal heart rate and during recovery at 1, 2, 4, 6, 8 and10 min after peak exercise.

MATERIALS AND METHODS

Study population

A group of 18 healthy untrained volunteers were recruited for the study (nine males, age 34 ± 3 years, BMI = 22 ± 6 kg/m^2, three smokers). Subjects with overt cardiovascular disease, diabetes, hypertension, major noncardiovascular diseases or who engaged in competitive sports were excluded. None of the subjects were taking any medication at the time of the study or during the previous week. The study protocol was approved by the local ethics committee and informed consent was obtained from all subjects.

Experimental procedure

The subjects were analyzed in two different sessions 3 days apart, to evaluate the intersession repeatability of carotid elasticity parameters in exercise. The examinations were all performed in the afternoon after a light lunch in a temperature-controlled room according to current guidelines (Laurent et al. 2006). The subjects avoided taking caffeine-containing beverages and smoking in the 3 h preceding the experimental sessions. In each session, a maximal exercise test was performed on a graded bicycle semi-supine ergometer (Fig. 1) (Armstrong et al. 1998). Workload was increased by 25 W every 2 min.

Theoretical maximal heart rate (HRmax) was computed for both male and female subjects, as:

$$\mathrm{HRmax} = 220 - \mathrm{age\ (in\ years)}$$

To estimate arterial elasticity, acquisitions of carotid ultrasound images, brachial blood pressure and radial pressure waveform were obtained. All measurements were performed during the exercise test while the subject was riding on the cycle ergometer, with head and neck and right wrist lying on a dedicated support. The following 11 different temporal steps were considered: at 60%, 70%, 80% and 85% of maximal heart rate and during the recovery at 1, 2, 4, 6, 8 and 10 min after peak exercise.

The acquisitions were made by the same skilled operator in two sessions 3 days apart: R.B. for applanation tonometry, V.G. for brachial blood pressure and A.C. for carotid ultrasound images.

Techniques

Diameter assessment. An algorithm (Carotid Studio, Institute of Clinical Physiology-CNR, Italy) for the automatic evaluation of the instantaneous carotid diameter in exercise was implemented in Matlab (The MathWorks, Natick, MA, USA). The method is based on a well-validated contour tracking technique (Bianchini et al. 2010; Faita et al. 2008) that allows automatic evaluation of diameter stroke changes during the heart cycle. The method assesses the diameter of the artery by processing B-mode ultrasound sequences of the longitudinal section of the vessel. For each image, lumen-intima interfaces are automatically detected using an algorithm based on the edge operator "first order absolute moment" and on a pattern recognition approach. Diameter is estimated as the distance between far and near lumen-intima interfaces and its mean value is computed on 15 beats of examination, to reduce the effect of cycle-to-cycle variability on the final result.

Moreover, data processing for the assessment of variations in diameter during the cardiac cycle (stroke change in diameter or distension, (Laurent et al. 2006) includes several other computational stages:

- The maximum (systolic) and minimum (diastolic) diameter values are identified for each cardiac cycle.
- The stroke change in diameter is calculated for each cardiac cycle as the difference between the systolic and diastolic diameter values.
- The mean distension value is computed as the average of the results obtained during the last 15 beats.

The algorithm in this study was customized for high frame rate (*i.e.*, >25 frame/s) application. High frame

rate, which ensures high temporal resolution, is needed to track the rapid wall movements of the vessel due to the high cardiac frequency in exercise.

Carotid image sequences in DICOM format were acquired and then analyzed off-line. Data were excluded when image quality was considered insufficient (*i.e.*, the algorithm was not able to correctly track vessel borders).

Blood pressure measurement. Radial tonometry was performed to evaluate central pressure by radial to aortic transfer function (Sphygmocor, Atcor Medical, West Ryde, New South Wales, Australia). For each step of exercise and recovery, the radial waveform was acquired and then calibrated by using the arm blood pressure automatically recorded at the right brachial artery (by a Dinamap XL device, Critikon Co., TX, USA).

Quality index (QI) for central pressure waveform was automatically provided by Sphygmocor software: measurements were considered acceptable with QI $>$ 75 at rest and QI $>$ 50 during exercise.

Since carotid and aortic pressure estimations by tonometry (at least at rest) showed good agreement, with differences around 1–2 mm Hg (Segers et al. 2005), final data obtained by the Sphygmocor system were used, together with diameter values, to assess carotid elasticity parameters.

Evaluation of carotid distensibility and compliance. Ultrasound B-mode image sequences (image resolution = 100 pixels/cm, DICOM format, 100 frame/s, 15 beats) of the right common carotid arteries were acquired and analyzed by the customized algorithm for evaluation of arterial diameter (D) and distension (ΔD). The common carotid arteries were scanned in longitudinal section using an iE33 Philips machine (Philips, Amsterdam, The Netherlands) and a 10-MHz linear-array probe. Arterial diameter borders were tracked in the near and far wall lumen-intima interfaces 1 cm proximal to the carotid bulb in a region 1 cm wide and free of plaques. The bulb was considered an anatomic fiducial point and a picture of the adopted ultrasound image was printed to ensure similar location of the measurements between the first session and the second one performed 3 days later. During vascular scanning, time-gain-compensation and depth settings were fixed. In addition, systolic and diastolic brachial pressures were measured and central pulse pressure obtained by radial tonometry as described above. Local elasticity parameters were then obtained for each step of the examination as: cross sectional compliance coefficient $\rightarrow$ CC = ΔA/PPa cross-sectional distensibility coefficient $\rightarrow$ DC = CC/Ad.

where ΔA represents the stroke change in lumen area, PPa the central pulse pressure and Ad the diastolic lumen area, respectively. ΔA and Ad were evaluated from the diameter values, assuming the cross-section of the artery to be circular.

Data analysis

Physiologic data. The mean values +/− standard deviation of the measured parameters were evaluated for each step of the examination. In addition, one-way analysis of variance (ANOVA) for repeated measures was used to evaluate differences between values at rest and during exercise, with Bonferroni or Kruskal-Wallis post hoc tests, for variables normally distributed or not, respectively; $p < 0.05$ was considered significant.

Reproducibility data. The volunteers were analyzed in two sessions 3 days apart, to evaluate the intersession repeatability of the arterial elasticity measurements (CC and DC).

For each step of the examination, in each volunteer, variability was expressed as the coefficient of variation (CV), which is defined as the ratio of the standard deviation to the mean of the two measurements; the mean of CVs from all the subjects was then computed. CVs of pressure and diameter's evaluation were also obtained.

Furthermore, the reproducibility of each parameter for the whole examination was estimated by using the intraclass correlation coefficient. The two-way random effects model was adopted (Fleiss 1986).

All calculations were made using SPSS software (version 20.0, 2011 by IBM Corporation, New York, NY, USA).

RESULTS

In the two sessions, 4.3% of the acquired B-mode images were considered of poor quality and hence rejected. An example of B-mode ultrasound images acquired for a subject at rest and peak and considered of acceptable quality is shown in Figure 2.

Regarding the tonometry acquisition, 2.7% of data presented an unacceptable quality index (QI) and were discarded. These resulted in a rejected percentage of the derived carotid elasticity measurements equal to 5.8%.

Physiologic data

For each step the mean values +/- standard deviation and the one-way ANOVA analysis of the measured parameters during the first day are summarized in Table 1a (exercise) and Table 1b (recovery).

Compared with the baseline, HR, SPb, DPb, PPb, SPa, DPa, PPa and MPa increased significantly ($p <$ 0.05) at each exercise intensity. ΔD was significantly greater at 70%, 80% and 85% HRmax steps, whereas D did not vary. Finally, CC and DC significantly decreased at 80% and 85% HRmax with respect to

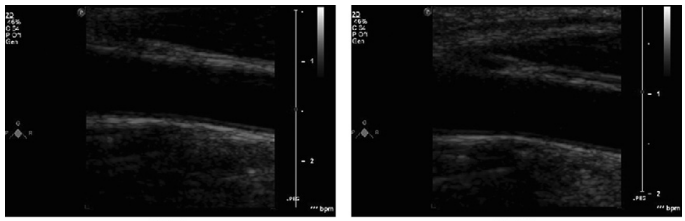

Fig. 2. Example of ultrasound B-mode images with accepted quality at rest (left) and peak exercise, respectively.

baseline. The significant variations persisted in the first recovery minute, then were superimposable to baseline values in the rest of the recovery period.

Reproducibility data

Mean coefficients of variation for each step of the exercise are summarized in Table 2.

In addition, agreement of elasticity evaluation for the whole examination between the two sessions resulted in an intraclass correlation coefficient of 0.780 (95% confidence interval [CI]: 0.686–0.844) for CC, and 0.694 (95% CI: 0.574–0.780) for DC. Intraclass correlation coefficients of pressure and diameter measurements were: 0.934 (95% CI: 0.906–0.954) for SPa, 0.776 (95% CI: 0.660–0.848) for DPa, 0.962 (95% CI: 0.947–0.971) for SPb, 0.807 (95% CI: 0.712–0.867) DPb and 0.830 (95% CI: 0.759–0.879) for D. Finally, regarding differential measurements, intraclass correlation coefficient was: 0.876 (95% CI: 0.829–0.909) for PPa, 0.936 (95% CI: 0.911–0.953) for PPb and 0.897 (95% CI: 0.858–0.925) for ΔD.

For both physiologic and reproducibility data, the analysis was repeated considering only non-smokers ($n = 15$), to evaluate whether the smokers' sub-group affected the final findings of our study, and we obtained superimposable results (data not shown).

DISCUSSION

Recently, there has been great interest in evaluating arterial elasticity in resting conditions for stratification of cardiovascular risk. Analysis of elastic properties during exercise would be even more attractive, since it could provide information about dynamic conditions, mimicking a patient's real life. Although some studies assessed vascular parameters dynamically (Myers et al. 2002; Studinger et al. 2003; Sharman et al. 2005; Bia et al. 2009), there are very few studies reporting the reliability of this evaluation in literature (Harris et al. 2007). Similar effects of dynamic conditions on arterial elasticity were observed in these studies: Myers et al. 2002 reported stiffness increase with isometric handgrip that varies with differences in gender and age and can be manifest even in young, healthy adults; other studies (Studinger et al. 2003; Sharman et al. 2005; Bia et al. 2009) found elasticity to be reduced during exercise in

Table 1a. Mean values ± standard deviation of the measured parameters at rest and in exercise

	Units of measure	Rest	60%	70%	80%	85%
HR	[beat/min]	64 ± 26 (0.6/0.7/0.8/P)	104 ± 30 (R/0.7/0.8/P)	122 ± 33 (R/0.6/0.8/P)	140 ± 37 (R/0.6/0.7/P)	147 ± 41 (R/0.6/0.7/0.8)
SPb	[mm Hg]	118 ± 33 (0.6/0.7/0.8/P)	146 ± 44 (R/0.8/P)	155 ±44 (R/P)	169 ± 50 (R/0.6)	176 ± 54 (R/0.6/0.7)
DPb	[mm Hg]	78 ± 21 (0.6/0.7/0.8/P)	85 ± 25 (R/P)	88 ± 24 (R/P)	90 ± 25 (R)	93 ± 27 (R/0.6/0.7)
PPb	[mm Hg]	40 ± 13 (0.6/0.7/0.8/P)	61±23 (R/0.8/P)	67 ± 25 (R)	79 ± 30 (R/0.6)	83 ± 30 (R/0.6)
SPa	[mm Hg]	103 ± 28 (0.6/0.7/0.8/P)	122 ± 36 (R/0.8/P)	128 ± 37 (R/0.8/P)	139 ± 44 (R/0.6/0.7)	140 ± 45 (R/0.6/0.7)
DPa	[mm Hg]	79 ± 22 (0.6/0.7/0.8/P)	88 ± 26 (R/0.8/P)	91 ± 26 (R/P)	95 ± 30 (R/0.6)	99 ± 31 (R/0.6/0.7)
MPa	[mm Hg]	86 ± 30 (0.6/0.7/0.8/P)	103 ± 31 (R/0.8/P)	108 ± 32 (R/P)	115 ± 37 (R/0.6)	117 ± 39 (R/0.6/0.7)
PPa	[mm Hg]	24 ± 7 (0.6/0.7/0.8/P)	34 ± 13 (R/0.8)	37 ± 14 (R)	40 ± 20 (R/0.6)	38 ± 20 (R)
D	[mm]	5.6 ± 1.5	5.6 ± 1.7	5.5 ± 1.6	5.6 ± 1.8	5.7 ± 1.7
ΔD	[mm]	0.5 ±0.2 (0.7/0.8/P)	0.6 ±0.3	0.7 ±0.3 (R)	0.8 ±0.4 (R)	0.7 ±0.3 (R)
CC	[mm^2/kPa]	1.6 ± 0.77 (0.8/P)	1.2 ± 0.5	1.2 ± 0.5	1.2 ± 0.6 (R)	0.9 ± 0.6 (R)
DC	[10^−3/kPa]	56.2 ± 25.3 (0.8/P)	43.5 ± 19.2	43.2 ± 21.8	41.6 ± 20.9 (R)	34.5 ± 20.0 (R)

HR = heart rate; SPb = systolic brachial pressure; DPb = diastolic brachial pressure; PPb = pulse brachial pressure; SPa = systolic aortic pressure; DPa = diastolic aortic pressure; PPa = pulse aortic pressure; D = carotid diameter; ΔD = carotid distension; CC = carotid cross-sectional compliance; DC = distensibility coefficient.

Data were analyzed by one-way ANOVA. Significant differences ($p < 0.05$) compared with baseline (R), 60% (0.6), 70% (0.7), 80% (0.8) and peak (P) are indicated. Sample $n = 18$.

Table 1b. Mean values ± standard deviation of the measured parameters at rest and during recovery

	Units of measure	Rest	1 m	2 m	4 m	6 m	8 m	10 m
HR	[beat/min]	64 ± 26 (1/2/4/6/8/10)	120 ± 35 (R/2/4/6/8/10)	103 ± 33 (R/1/10)	96 ± 31 (R/1)	94 ± 30 (R/1)	93 ± 29 (R/1)	91 ± 29 (R/1/2)
SPb	[mm Hg]	118 ± 33 (1/2)	142 ± 42 (R/2/4/6/8/10)	138 ± 40 (R/1/4/6/8/10)	150 ± 36 (1)	137 ± 34 (1/2)	128 ± 34 (1/2)	120 ± 33 (1/2)
DPb	[mm Hg]	78 ± 21	80 ± 21	69 ± 20	74 ± 21	73 ± 21	74 ± 21	76 ± 21
PPb	[mm Hg]	40 ± 13 (1/2)	60 ± 26 (R/2/4/6/8/10)	69 ± 25 (R/1/2/6/8/10)	76 ± 20 (1/10)	64 ± 17 (1/2)	53 ± 17 (1/2)	45 ± 14 (1/2/4)
SPa	[mm Hg]	103 ± 28 (1)	118 ± 33 (R/4/6/8/10)	110 ± 31 (8/10)	120 ± 30 (1)	110 ± 28 (1)	107 ± 28 (1/2)	103 ± 28 (1/2)
DPa	[mm Hg]	79 ± 22	84 ± 23	74 ± 21	80 ± 23	76 ± 21	75 ± 22	78 ± 22
MPa	[mm Hg]	86 ± 30 (1)	98 ± 28 (R)	90 ± 26	90 ± 26	89 ± 25	89 ± 25	90 ± 25
PPa	[mm Hg]	24 ± 7 (1)	33 ± 14 (R/6/8/10)	36 ± 14 (8/10)	40 ± 16	34 ± 9 (1)	32 ± 9 (1/2)	24 ± 7 (1/2/4)
D	[mm]	5.6 ± 1.5	5.5 ± 1.5	5.5 ± 1.5	5.5 ± 1.5	5.4 ± 1.5	5.4 ± 1.5	5.4 ± 1.5
ΔD	[mm]	0.5 ± 0.2 (1/2)	0.7 ± 0.3 (R/6/8/10)	0.7 ± 0.3 (R/8/10)	0.6 ± 0.3	0.5 ± 0.2 (1)	0.5 ± 0.2 (1/2)	0.5 ± 0.2 (1/2)
CC	[mm^2/kPa]	1.6 ± 0.7 (1/2)	1.2 ± 0.5 (R/6/8/10)	1.4 ± 0.6 (R)	1.4 ± 0.6	1.6 ± 0.7 (1)	1.6 ± 0.8 (1)	1.7 ± 0.7 (1)
DC	[10^{-3}/kPa]	56.2 ± 25.3 (1)	46.1 ± 16.5 (R/6/8/10)	52.2 ± 23.0	53.7 ± 21.4	61.4 ± 25.8 (1)	64.3 ± 29.3 (1)	65.6 ± 26.4 (1)

HR = heart rate; SPb = systolic brachial pressure; DPb = diastolic brachial pressure; PPb = pulse brachial pressure; SPa = systolic aortic pressure; DPa = diastolic aortic pressure; PPa = pulse aortic pressure; D = carotid diameter; ΔD = carotid distension; CC = carotid cross-sectional compliance; DC = distensibility coefficient.

Data were analyzed by one-way ANOVA. Significant differences ($p < 0.05$) compared with baseline (R), 1 min (1), 2 min (2), 4 min (4), 6 min (6), 8 min (8) and10 min (10) are indicated. Sample $n = 18$.

Table 2. Mean ± standard deviation of coefficients of variation (percentage values) for each step of the exercise for repeated examinations that were performed in two different sessions, 3 days apart

	Rest	60%	70%	80%	85%	1 m	2 m	4 m	6 m	8 m	10 m
SPb	3.1 ± 3.4	3.2 ± 2.5	3.1 ±1.8	3.2 ± 3.3	4.1 ±3.4	3.4 ± 3.5	4.1 ± 3.2	4.6 ± 2.8	3.2 ± 2.7	2.8 ± 3.6	2.1 ± 2.3
DPb	4.2 ± 5.3	4.9 ± 6.1	5.1 ± 6.3	5.4 ± 6.3	6.2 ± 6.5	5.2 ± 4.9	6.1 ± 4.6	5.6 ± 6.1	5.8 ± 5.2	4.1 ± 5.3	3.6 ± 3.4
PPb	7.3 ± 6.1	9.6 ± 5.9	9.5 ± 5.9	9.9 ± 3.2	10.1 ± 6.8	9.3 ± 8.4	11.2 ± 6.7	11.9 ± 7.9	11.3 ± 9.1	12.1 ± 8.7	9.9 ± 6.4
SPa	4.2 ± 4.4	4.3 ± 3.1	4.4 ± 2.5	5.2 ± 4.3	3.5 ± 3.6	4.9 ± 4.3	4.2 ± 3.9	4.9 ± 4.4	3.3 ± 2.9	3.5 ± 2.1	3.1 ± 2.9
DPa	4.4 ± 4.7	6.2 ± 5.9	7.2 ± 5.1	7.9 ± 4.4	8.2 ± 7.8	8.1 ± 7.2	5.4 ± 4.1	7.8 ± 8.7	6.2 ± 4.9	4.1 ± 3.6	3.1 ± 2.8
PPa	8.3 ± 6.1	11.3 ± 8.2	11.2 ± 6.9	10.3 ± 7.4	10.5 ± 7.2	12.3 ± 10.4	13.9 ± 8.4	13.8 ± 11.6	12.1 ± 8.7	13.9 ± 8.9	13.9 ± 9.1
D	3.1 ± 3.1	2.2 ± 1.7	4.7 ± 4.4	5.1 ± 6.9	6.8 ± 5.4	3.3 ± 3.1	3.1 ± 2.4	5.4 ± 4.3	3.9 ± 4.1	3.1 ± 3.5	4.9 ± 4.6
ΔD	8.1 ± 5.9	9.8 ± 5.7	9.9 ± 10.6	10.2 ± 11.6	11.8 ± 7.9	7.2 ± 7.3	10.2 ± 6.5	10.9 ± 8.8	9.2 ± 6.4	9.9 ± 5.2	8.1 ± 4.8
CC	8.2 ± 9.8	15.1 ± 12.4	14.7 ± 10.3	14.5 ± 18.1	19.2 ± 6.4	12.7 ± 9.1	15.2 ± 11.4	13.4 ± 11.9	15.1 ± 9.8	15.9 ± 10.6	14.8 ± 13.6
DC	8.3 ± 7.8	15.9 ± 10.6	13.5 ± 10.4	16.4 ± 17.2	24.2 ± 14.9	12.5 ± 11.1	14.9 ± 12.2	14.2 ± 15.1	18.1 ± 14.8	17.7 ± 9.7	16.2 ± 12.8

SPb = systolic brachial pressure; DPb = diastolic brachial pressure; PPb = pulse brachial pressure; SPa = systolic aortic pressure; DPa = diastolic aortic pressure; PPa = pulse aortic pressure; D = carotid diameter; ΔD = carotid distension; CC = carotid cross-sectional compliance; DC = distensibility coefficient.

healthy volunteers. Elasticity analysis during exercise is intriguing. In fact, accuracy of the estimation of cardiac afterload during exercise on the basis of brachial BP is debatable, since PP amplification from the center to the periphery of the arterial tree increases greatly during exercise (Sharman et al. 2005). Moreover, men with hypercholesterolemia have higher augmentation index and blunted pulse pressure amplification compared with age-matched healthy controls during light exercise, in spite of similar brachial SBP at baseline and during exercise (Sharman et al. 2007). Hence, an abnormal exercise central BP can underlie an abnormal behavior of arterial stiffness, which until now has been evaluated mainly by indirect measurement, such as the timing of the reflected wave (Sharman et al. 2005). BP response to exercise is a negative prognostic factor for cardiovascular mortality (Mundal et al. 1996) and a more in-depth study of the behavior of arterial stiffness during exercise could provide not only better knowledge of physiologic mechanisms but possibly also a better prediction of cardiovascular risk. In this study, carotid distensibility and compliance were assessed in a group of healthy volunteers during exercise. In this article, aside from the expected increase in mean central pressure and pulse pressure, at peak compared with baseline, a decrease in CC and DC during exercise was documented; the phenomenon was observable starting from 80% of maximal HR and remained evident until 1 min after peak exercise. These data indicate increased carotid stiffness during exercise, a phenomenon that might be partly due to the recruitment of a greater number of collagen fibers and, consequently, a different mechanical behavior of the arterial wall at higher pressures (Nichols and O'Rourke, 1998). These results are consistent with the non-linear relation between stress and strain that is thought to characterize the arterial borders: at higher stress (*i.e.*, pulse pressure), the slope of the curve stress-strain (*i.e.*, pressure-distension) increases and, thus, also the corresponding stiffness. Furthermore, the observed reduction in carotid elasticity due to exercise is in accordance with results from the abovementioned previous works.

The subjects were analyzed in two different sessions 3 days apart, to evaluate the intersession repeatability. At the moment, to our knowledge, repeatability studies for carotid elasticity during exercise are not available in the scientific literature. On the other hand, coefficients of variation (CVs) at rest of the analyzed parameters, which are considered appropriate for studying their physiologic and pathophysiologic variations, are described in several studies using well-known and validated gold-standard techniques (Kool et al. 1994; Benetos et al. 1993; Selzer et al. 2001; Bianchini et al. 2008, 2010). As an example Selzer reported CVs = 6.17%–9.66% for blood pressure measurements, CV = 1.28% for arterial diameters and CVs from 11.05% to 14.54% for carotid stiffness indices; similarly Kool found CV = 4.5% for carotid diameters, CV = 7.9% for distension and CVs from 8.3% to 9.1% for arterial stiffness parameters. Our results regarding reproducibility of baseline data provided similar values and hence the reliability of the proposed approach is as good as the state-of-the-art technology.

Regarding results in exercise and recovery, CVs of carotid diameter (D and ΔD) and pressures (SPb, DPb, PPb, SPa, DPa and PPa) are slightly higher but comparable to the resting values reported in the abovementioned previous works (Kool et al. 1994, Benetos et al. 1993; Selzer et al. 2001, Bianchini et al. 2008, 2010). Finally, coefficients of variability of carotid elasticity measurements in dynamic conditions are also slightly higher but comparable to values acquired at rest for all steps, except for exercise peak, where mean CVs are higher and equal to 19% $\pm$ 6% and 24% $\pm$ 15% for compliance (CC) and distensibility (DC), respectively. However, it is worth noting that repeatability of CC and DC again improves (*i.e.*, comparable to baseline) already starting from the first minute of recovery; at this time, as shown in Table 1b by the one-way ANOVA analysis of the measured parameters, the phenomenon of significant decrease in elasticity with respect to baseline detected at peak is still observable. We might speculate that if confirmed in future work, including both controls and patients, these results could imply the possibility of observing arterial elasticity variations by performing the measurement after peak exercise, when the subject is not riding the bike, artifacts are reduced, and as a consequence, variability is smaller and data more reliable. Moreover, in this case the design of future clinical studies might be improved even more, since when analyzing a subject who is not moving, direct and more accurate carotid tonometry (Laurent et al. 2006) (which we had to exclude and replace with the radial approach due to feasibility issues) can also be performed.

Regarding the reliability of the whole examination, the obtained intraclass correlation coefficients (ICC) results are slightly worse than the resting values of validated techniques in previous works. As an example, Selzer (Selzer et al. 2001) reported intraclass correlations around 0.98 for diameter measurements, around 0.89 for arterial distensibility and compliance and between 0.77 and 0.83 for pressure estimation. Furthermore, in this case ICC values for arterial pressure resulting in a reliable dynamic evaluation were available in literature (Holland et al. 2008) and were similar to ours (ICC = 0.89).

Our results regarding parameters that were derived directly from the B-mode image analysis (*i.e.*, D and ΔD) prove the robustness of the algorithm used in more

critical conditions as well. Furthermore, these data show the feasibility of dynamic vascular diameter assessment from ultrasound imaging by using a robust contour tracking method together with an appropriate frame rate that ensures the temporal resolution needed to correctly observe rapid movements of the walls due to high cardiac frequency. Most modern ultrasound machines allow the acquisition of high frame rate image sequences and, thus, in conjunction with a precise automatic method for arterial wall tracking, could be used in future clinical studies for evaluating instantaneous diameter at high heart rate.

Regarding the pressure measurement needed in conjunction with diameter assessment for elasticity estimation, we opted for radial tonometry that allowed us to compute central pressure by radial to aortic transfer function. This choice, although less accurate than direct carotid tonometry for local pressure estimation, was preferred since it is considered much more feasible in exercise. Since aortic and carotid pressure estimations by tonometry, at least at rest, are in good agreement (Segers et al. 2005; Vermeersch et al. 2008), the data obtained were then used in conjunction with the diameter values to assess carotid elasticity parameters. However, although we tested the reproducibility of the radial tonometry-based technique, we did not show that it is the most appropriate method for carotid pressure estimation during exercise, from a physiologic point of view. First, at this time we do not know whether differences between aortic and carotid pressure can be considered small in dynamic conditions as well. Second, there is no consensus (Sharman et al. 2006; Dawson et al. 2009) on the capability of generalized transfer functions to accurately estimate central hemodynamic variables from radial pressure waveform in dynamic conditions. Some authors (Sharman et al. 2006) compared the radial tonometry-derived measure with a catheter-based one in 30 patients during supine exercise, concluding that the two approaches show good agreement. However, others (Dawson et al. 2009) observing eight healthy volunteers during the incremental hand-grip test concluded that changes in vascular tone due to dynamic conditions could compromise the assumptions for aortic evaluation derived by radial applanation tonometry. Indeed, further studies analyzing different approaches for carotid or aortic pressure dynamic evaluation are warranted.

Limitations of the study

Some limitations of our study should be acknowledged. First, the sample size is relatively small and a subsequent verification on a larger sample should be conducted. Moreover, since only healthy volunteers were analyzed, the reproducibility of the present approach should be investigated in patients with various cardiovascular risk factors to provide findings for additional scenarios. We must also point out that during the tests we were not able to process all the acquired data: 4.3% of the total of B-mode image sequences and 2.7% of the total pressure waveforms were discarded because of bad quality issues due to movement artifacts. Finally, as discussed above for carotid pressure estimation, we adopted the radial tonometry-based technique, without showing it to be the most appropriate methodology to use during exercise.

CONCLUSIONS

In conclusion, this study shows that the reproducibility of carotid elasticity measurements in healthy subjects during various exercise steps is comparable to resting variability reported in literature for well-known and validated gold-standard techniques, with the exception of peak data where it is higher. On the other hand, according to our results, the observed decrease in arterial elasticity is still evident during the first minute of recovery and, hence, might be observed after the test peak, when the subject is not moving on the cycle ergometer and measurement is more precise. Thus, the proposed approach can be considered reliable and might be used in future population studies for investigating the dynamic behavior of arterial elasticity and its role in arterial-ventricular coupling variation in stress conditions.

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