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Tesi di Dottorato

Biomarcatori ecografici di funzione e struttura vascolare nell'uomo

Imaging biomarkers of vascular function and structure in humans

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Chapter 1: Introduction

Cardiovascular (CV) disease is the first cause of mortality and morbidity in the world (Ezzati et al., 2002). Prevention of this condition, which is responsible of more than 2200 deaths per day only in United States, is a public health priority (Roger et al., 2012). Thus in the last decades great efforts have been made in the search of non-invasive biomarkers, able to identify the individual at risk for CV events in the asymptomatic, subclinical stage **(Figure 1)** (Duivenvoorden et al., 2009). Some biomarkers are currently recommended in order to improve stratification of CV risk, whereas other are considered useful only for research purposes (Mancia et al., 2007;Mancia et al.;Greenland et al., 2010). In particular, increased intima-media thickness of the common carotid artery (C-IMT), representing a marker of early atherosclerosis significantly correlated with coronary or cerebrovascular disease (Chambless et al., 1997;Witte et al., 2005), has been considered as an intermediate stage in the continuum of vascular disease and as a predictor of CV risk. Current guidelines also introduced other vascular parameters evaluating mechanical and functional arterial properties of peripheral and central arteries (Mancia et al., 2007;Mancia et al., 2009). Increased aortic stiffness has been shown to predict future CV events (Vlachopoulos et al., 2010) and it has been recognized as a marker of subclinical target organ damage in hypertensive patients (Mancia et al., 2007). Earlier vascular abnormalities, such as endothelial dysfunction in the peripheral arteries (Charakida et al., 2010), have been also mentioned for their possible use in future. However, several questions in this field are still open, limiting the wide use of these tools in the clinical practice. The open issues concern methodological as well as pathophysiological and prognostic aspects, and in this thesis we will discuss only a small part of these ones. First, C-IMT and arterial stiffness represent two sides of vascular aging, atherosclerosis and arteriosclerosis respectively, and are generally considered structural alterations. The identification of a "functional" component in these alterations would be of interest, since it will suggest a possibility of reversibility of vascular aging.

Second, vascular aging is a process involving the whole organism, while different techniques explore different districts. The quantification of the impact of different CV risk factors on different vascular districts might indicate the most adequate biomarker to use for future studies and suggest specific mechanisms of disease in different conditions. Third, vascular aging is often accompanied by cardiac and renal damage, but the relationship between organ damage in different districts is still largely unexplored. Fourth, while hypertension and diabetes have become the main cause of end-stage renal disease, and chronic kidney disease has been recognized as a main cause of CV events, to date early markers of renal vascular damage have not been developed.

For this PhD thesis I examined cross-sectionally a cohort of healthy subjects and patients with traditional CV risk factors in order to elucidate some of the abovementioned aspects. My original contribution to the knowledge in this field consists in:

- the demonstration of a "functional" component in aortic stiffness, that is present only in diabetic patients and relies on endothelium-mediated mechanisms;
- the demonstration of a differential impact of different CV risk factors on carotid and aortic stiffness;
- the demonstration, in hypertensive patients, of an additive role of carotid and aortic stiffness in determining cardiac organ damage;
- the identification of a new marker of renal vascular damage.

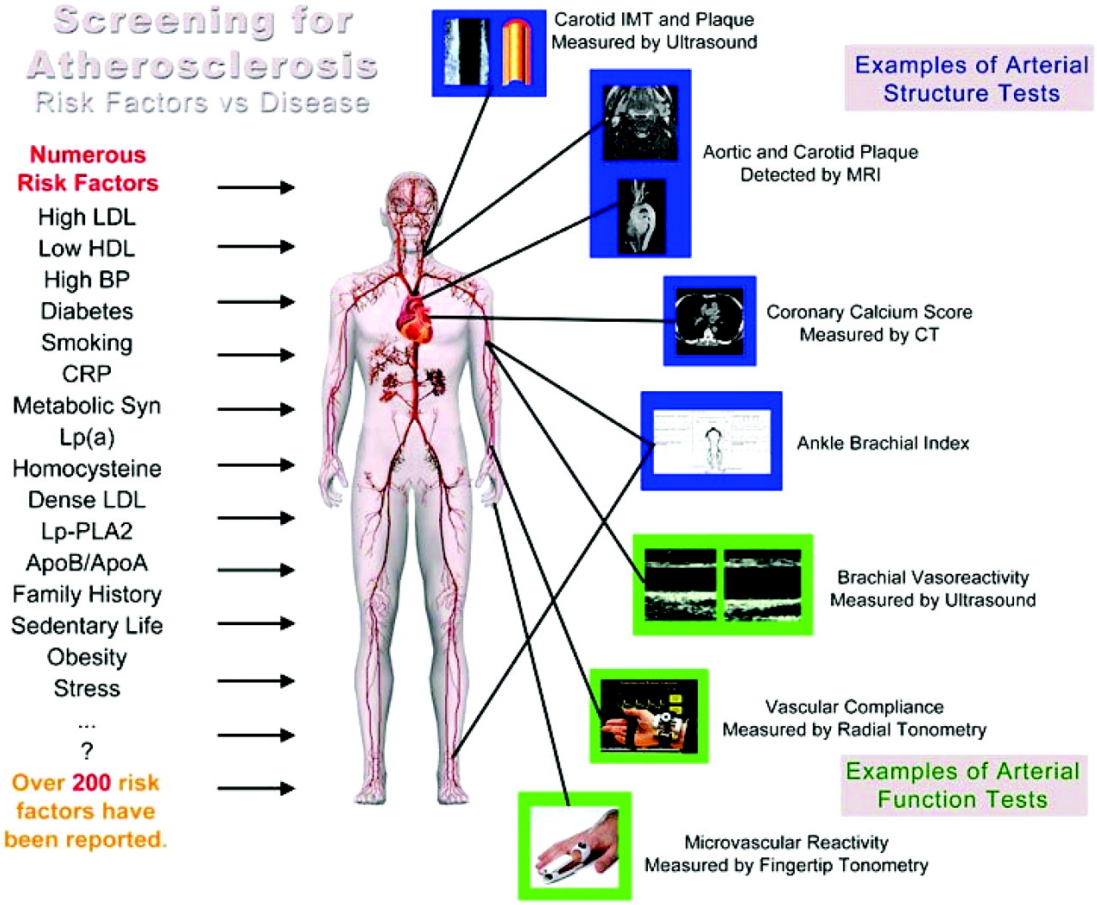
In the second part of the PhD thesis, I sought to demonstrate the usefulness of imaging biomarkers of vascular function and structure not only for risk stratification in patients with traditional CV risk factors, but also to explore CV consequences of non primarily CV diseases and conditions. We hypothesized that a comprehensive, multiparametric approach would be the best strategy to detect early vascular damage in one or more districts, possibly in a subclinical, reversible stage. This approach could allow identifying the "CV footprint" characterizing each condition, with a double aim: to elucidate the pathophysiology of CV complications in non-CV diseases and to propose the most useful test to be used in the clinical practice for screening and follow-up.

During my PhD thesis I applied this strategy to some primarily non-CV conditions that might qualify as emerging CV risk factors, such as exposure to environmental and iatrogenic radiation doses, obstructive sleep apnea syndrome (OSAS) in the absence of traditional CV risk factors, and chronic exposure to hypobaric hypoxia at high altitude.

My original contribution to the knowledge in this field consisted in:

- the demonstration of a selective reduction of circulating endothelial progenitor cells, in the presence of preserved vascular function and structure, in young adults exposed during childhood to environmental radiation doses after the Chernobyl disaster and to therapeutic radioiodine treatment after thyroid cancer;
- the demonstration that conduit artery endothelial dysfunction and impaired renal vasodilating capacity are part of the vascular phenotype of OSAS per se, since they are present even in the absence of traditional CV risk factors, while structural alterations such as arterial stiffness and increased C-IMT characterized only obese and/or hypertensive OSAS patients;
- the demonstration that Himalayan high altitude dwellers, chronically living above 2500 meters of altitude, present a mainly microcirculatory endothelial dysfunction and a maladaptive carotid remodeling even in the absence of traditional CV risk factors.

Figure 1. Some biomarkers of vascular function and structure in humans proposed for CV risk stratification. From (Koenig, 2007)



Chapter 2. Imaging biomarkers in cardiovascular disease: review of current literature

2.1 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 (Stein et al., 2008). C-IMT has been considered for a long time an intermediate phenotype of atherosclerosis suitable for use in large-scale population studies (Salonen and Salonen, 1993). Increased C-IMT has been associated with augmented CV risk (Oren et al., 2003;Paul et al., 2005) as well as with presence of advanced atherosclerosis at different vascular sites, including peripheral, cerebral and coronary districts (Geroulakos et al., 1994;Amato et al., 2007). Most importantly, large 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 CV risk factors (Bots et al., 1997;Chambless et al., 1997;Hodis et al., 1998;Chambless et al., 2000;Rosvall et al., 2005;Lorenz et al., 2006). A recent meta-analysis of 8 population-based studies involving a total of 37197 subjects followed for a mean of 5.5 years confirmed the strong independent predictive value of cross-sectional C-IMT for future CV events (Lorenz et al., 2007). The predictive value of C-IMT for CV events has been confirmed also in asymptomatic type 2 diabetic patients, in whom, in combination with Framingham Risk Score (FRS), showed a greater predictive value than FRS alone (Yoshida et al., 2012).

Negative results on the independent predictive value of C-IMT for CV events have been also reported. The Thromso study, a prospective population-based study, in which total plaque area and C-IMT were measured in over 6000 healthy participants followed up for 6 years(Johnsen et al., 2007), and the Three-City Study, a large prospective study in which 5895 adults aged 65-85 years

with no history of coronary heart disease were followed up for 6 years (Plichart et al., 2011), showed that carotid plaques were a stronger predictor of myocardial infarction than was C-IMT. A recent meta-analysis of 11 population-based studies (54336 subjects) provided further evidence of a stronger predictive value for future CV events of carotid plaque than C-IMT (Inaba et al., 2012).

It has been proposed that reduced progression of C-IMT corresponds to a reduction in CV events (Espeland et al., 2005). This hypothesis has been documented in clinical trials designed to study the efficacy of statins where C-IMT was used as surrogate end-point (Mercuri et al., 1996;Smilde et al., 2001;Taylor et al., 2002), even if conflicting results exist (Crouse et al., 2007). The European Lacidipine Study on Atherosclerosis (ELSA), a randomized trial in which 2334 hypertensive patients under effective antihypertensive treatment were followed-up for 3.75 years, showed that, despite baseline C-IMT strongly predicted CV events rate, differences in C-IMT compared with baseline did not (Zanchetti et al., 2009). Furthermore, a meta-analysis including 41 trials with 18307 participants showed that, despite significant reduction in CV 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 CV drug therapies, could not reflect reduction in CV events (Costanzo et al., 2010).

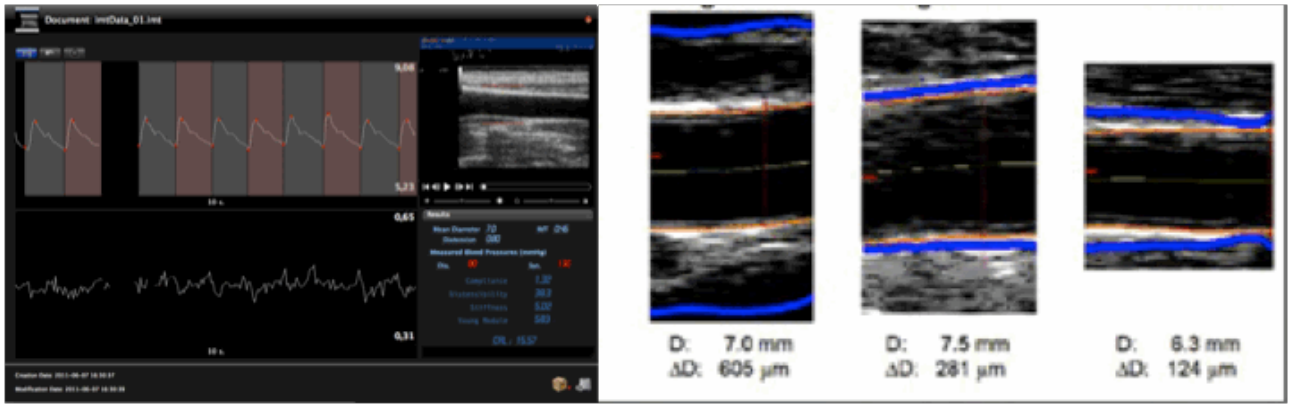
Taken together, the results of these studies question the accuracy of C-IMT as a marker of atherosclerosis. There are several explanations for these findings. First C-IMT, even if detected by high-resolution ultrasound, is not been able to distinguish between intima and media. The carotid artery is an 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 a progressive intimal thickening or medial hypertrophy occurs, determined by age, gender and hypertension, that do not necessarily reflect the atherosclerotic process (Finn et al., 2010). The observation that the classical risks factors for atherosclerotic disease poorly correlate with C-IMT further supports this hypothesis (Zanchetti et al., 2001;de Groot et al., 2004;Junyent et al., 2008). Istopathological studies indicate that C-IMT mainly represent hypertensive medial hypertrophy or thickening of smooth muscle

media, whereas atherosclerosis is largely an intimal process (Spence, 2006). In line with the hypothesis that C-IMT is biologically distinct from plaque, age-related thickening of intima-media layers of common carotid artery has been observed in the absence of overt atherosclerosis (Finn et al., 2010). Thus, it is not surprising that atherosclerosis plaque shows a better prognostic value than C-IMT for CV events. Furthermore, there have been great heterogeneity in methodological approaches to C-IMT measurements, which could have hampered its predictive value (O'Leary and Bots, 2010). C-IMT value can be influenced by: location of the measure, type of ultrasound data and features of the reading system. Inaba et al (Inaba et al., 2012) observed that 77% of the studies included in their meta-analysis did not explicit 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 may differ significantly (Lorenz et al., 2007). The far wall of CCA is the easiest of the three anatomical segments to be examined, being the most used measurement in clinical studies, but unfortunately plaques are rare at this site and studies on the relationship of C-IMT at this site are conflicting (O'Leary and Bots, 2010). No firm conclusion can be taken about which carotid district should be studied until large epidemiological studies will include measurement of C-IMT at different sites following standardized protocols. Ultrasound protocols that include common carotid CIMT measurements at multiple angles and multiple sites have been recently demonstrated to give the best results in terms of reproducibility, progression rate and treatment effect detection (Dogan et al., 2011), but it is still unknown whether this kind of approach can improve C-IMT predictive value. Noteworthy, the majority of the large prospective trials on predictive value of C-IMT used manual measurement by caliper, whereas automated systems are preferable. Regarding the latter, two main types are commercially available: B-mode image processing based devices and Radio-frequency (RF)-based echo-tracking systems **(Figure 2)** (Molinari et al., 2010). RF-based devices are

considered very accurate since RF signal has higher spatial resolution than B-mode (Brands et al., 1999;Laurent et al., 2006). However, when comparing the performance in terms of reproducibility of RF-based techniques with that of robust image-based systems, similar results are obtained (Bianchini et al., 2010;Molinari et al., 2010). However, it has to be pointed out that quality of the scans and system setting are crucial for the quality of the final result of B-mode based systems. In particular, dynamic range and gain settings, but also other parameters such as depth gain compensation or filtering, should be standardized as suggested by international guidelines (Touboul et al., 2007;Potter et al., 2008). Furthermore, Rossi et al. found that non-linear processing, such as compression and saturation, generally used in standard ultrasound equipment for a better image visualization, significantly influences carotid measurements (Rossi et al., 2009).

Future analysis providing the agreement between different kind of measurements and reference values for risk classification are needed in order to implement C-IMT assessment in clinical practice. In this sense, an important step forward has been recently made by Reference Values for Arterial Measurements Collaboration, which established reference values for C-IMT obtained by echotracking, based on subject-level data from different study centres worldwide (Engelen et al., 2012). This cross-sectional analysis, performed on 24871 subjects (4234 without any CV risk factor) enables now a comparison of C-IMT values for groups with different CV risk profiles, helping interpretation of such measures obtained both in research and clinical settings.

Figure 2. B-mode (on the left) and RF-mode (on the right) based echotracking systems for C-IMT measurement.



2.2 Carotid-femoral pulse wave velocity

Arterial stiffness represents the ability of the arterial system to cope with the systolic ejection volume through the distension of compliant large arteries during systole, and to relay cardiac contraction during diastolic runoff, through return to their initial dimensions, thus transforming the pulsatile ejection from the heart into continuous blood flow to the organs. Aorta is the vessel that gives the greatest contribution to the capacitive function of the arterial tree, and is a major determinant of the amplitude of the incident/forward pressure wave.

During ventricular contraction, part of the stroke volume is momentarily stored in the aorta and central arteries, stretching the arterial walls because of raising local blood pressure (BP). The arterial pressure wave generated in the aorta (forward or incident wave) is propagated to arteries throughout the body and is partly reflected back towards the aorta (reflected pressure wave). Arterial stiffness determines the propagation velocity of the pressure wave **(Figure 3)**. Pulse wave velocity (PWV) works bidirectional, from the proximal aorta towards peripheral vessels and back. If aortic stiffness is low, the reservoir function during systole, as well as the recoiling during diastole, are maintained; furthermore the reflected pressure wave is delayed enough to impact on central arteries during end-systole and diastole, thus increasing the aortic pressure in early-diastole but not during systole. This situation is physiologically advantageous, since the higher diastolic pressure boosts coronary perfusion, without increasing the left ventricular pressure load. Increased arterial stiffening causes loss of the reservoir function and disrupts the above-described desirable timing of reflected wave. Thus, an increased PWV acts amplifying aortic and ventricular pressures during systole, and reducing aortic pressure during diastole. As a consequence, raising myocardial pressure load and oxygen consumption are increased, favoring the development of left ventricular hypertrophy and impaired ventricular relaxation (Ghiadoni et al., 2009;London and Pannier, 2010). On the other hand, diastolic BP and subendocardial blood flow are decreased in the presence of

stiffer large arteries: this phenomenon is responsible for decreased coronary perfusion pressure and myocardial ischemia. Increased aortic stiffness also decreases the stiffness gradient because peripheral arteries stiffness does not increase with aging, the consequence being microvascular pressure overload with concomitant alterations (capillary rarefaction, inward remodeling of small arteries) (Avolio et al., 1983;O'Rourke and Safar, 2005;Mitchell, 2008).

Aging is physiologically accompanied by arterial dilatation, increase in wall thickness and reduction of the elasticity and compliance, all features characterizing atherosclerosis (O'Rourke, 2007). The main structural change occurring with aging is the degeneration of the tunica media, which causes a gradual stiffening of large elastic arteries (Kaplan and Opie, 2006). This corresponds on the 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 -3 collagen (Lakatta and Levy, 2003). Arterial stiffness seems to be a common pathophysiological mechanism for many conditions associated with CV disease, including aging, menopausal status, lack of physical activity, genetic background or polymorphisms, and CV risk factors (smoking, hypertension, hypercholesterolemia, diabetes) or diseases (coronary heart disease, congestive heart failure, stroke) (Laurent et al., 2006). Arterial stiffness also has been demonstrated in primarily non-CV diseases, such as end-stage renal disease or moderate chronic kidney disease, rheumatoid arthritis, systemic vasculitis, and systemic lupus erythematosus (Laurent et al., 2006).

Arterial stiffness can serve in clinical practice as “intermediate” or “surrogate” end points for CV events. A major reason for its routine measurement comes from the recent demonstration that these parameters have an independent predictive value for CV morbidity and mortality. The largest amount of evidence has been given for carotid-femoral PWV **(Figure 3)**, which showed independent predictive value for all-cause and CV mortality, fatal and nonfatal coronary events, and fatal strokes in patients with uncomplicated essential hypertension, type 2 diabetes, and end-stage renal disease, in the elderly and in the general population (Laurent et al., 2006;Vlachopoulos et al.,

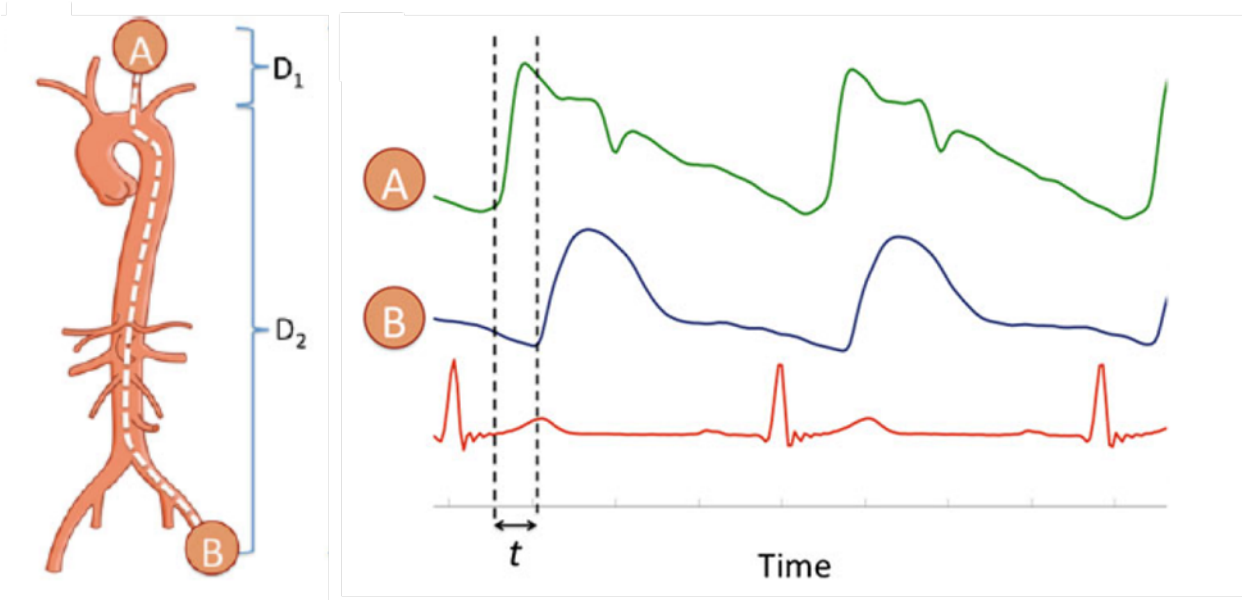
2010). The independent predictive value of aortic stiffness has been demonstrated after adjustment to classic CV risk factors, including brachial PP, suggesting that aortic stiffness adds value to a combination of CV risk factors (Boutouyrie et al., 2002). This finding may be related to the fact that aortic stiffness integrates the damage to the aortic wall of CV risk factors over a long period, whereas BP, blood fasting glucose, and lipids can fluctuate over time and the values recorded at the time of risk assessment may not reflect the true damage to the arterial wall. Another explanation may be that the identification of aortic stiffness reveals the patients in which risk factors are translated into real risk (Ghiadoni et al., 2009).

An indirect argument to support the predictive value of arterial stiffness for CV mortality is the relationship with well-accepted intermediate end points such as left ventricular hypertrophy (Watabe et al., 2006), microalbuminuria and estimated decrease in glomerular filtration rate (GFR) (Hermans et al., 2007).

Although measures of stiffness are useful in predicting the occurrence of CV events, the value of reduction in arterial stiffness as a measurement of the reduction by treatment of the risk of such events has not yet been unequivocally proven. A meta-analysis of individual data in 294 patients demonstrated that long-term and short-term antihypertensive treatment is able to reduce PWV and that this reduction is not entirely explained by BP reduction (Ong et al., 2011). Indeed, PWV is highly BP-dependent on short term, but not on long term (Ait-Oufella et al., 2010;Ong et al., 2011). This seems to suggest that antihypertensive treatment can improve aortic stiffness beyond BP reduction in essential hypertensive patients. The only clinical evidence that reducing arterial stiffness is associated to a decreased risk of CV events was obtained in patients with end-stage renal disease (Guerin et al., 2001). In a mean follow-up of 50 months, the absence of PWV decrease in response to BP decrease was one of the predictors of all-cause and CV mortality, together with increased left ventricular mass, age, and preexisting CV disease. After adjustment for all confounding factors, the risk ratio for the absence of PWV decrease was 2.59 for all-cause mortality

and 2.35 for CV mortality(Guerin et al., 2001). However, the effect of aortic stiffness attenuation on CV morbidity and mortality remains to be established in other populations, particularly those at lower but still high CV risk, such as those with hypertension, dyslipidemia, diabetes, and moderate chronic kidney disease.

Figure 3. Carotid-femoral PWV assessment.



2.3 Carotid distensibility and stiffness

The measurement of aortic stiffness as PWV by arterial tonometry is generally accepted as the most simple, non-invasive, robust, and reproducible method to determine arterial stiffness (Laurent et al., 2006). 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. Furthermore, other arterial sites have potential interest: the measurement of local carotid stiffness **(Figure 4)** may also provide important prognostic information, since the carotid artery is a frequent site of wall-thickening and plaque formation, as above discussed (Laurent et al., 2006). Arterial stiffness can be estimated at the systemic, regional and local level. Arterial stiffness evaluation at the local level, generally obtained at the common carotid site (a large, easily accessible superficial artery) presents as advantages a great accuracy, since, unlike the systemic and regional evaluation, it is estimated directly by BP changes, which in turn determine the changes of volume of the vessel **(Figure 4)** (Laurent et al., 2006).

Aging, along with BP, is the main determinant of stiffness both of carotid and aortic stiffness (Paini et al., 2006). However, histological differences exist between large artery districts, responsible for different behaviors in the presence of CV 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, seem to be resistant to age-induced stiffening when compared to the carotid artery (Laurent et al., 1994). 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 CV risk factors increases (Paini et al., 2006). Therefore, although carotid-femoral PWV and carotid stiffness provide similar information on the aging impact 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 seems to stiffen more than the carotid artery due to age and other CV risk factors (Paini et al., 2006).

Several studies 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 (Safar and Frohlich, 1995). 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 (Boutouyrie et al., 2000). Young's incremental elastic modulus of the common carotid artery, which estimates arterial stiffness controlling for C-IMT, has been shown to be greater in young never-treated hypertensive patients than in 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 (Bussy et al., 2000). Thus, the mechanisms involved in the arterial stiffening in younger hypertensive patients likely differ from those advanced to explain the stiffening of large arteries with aging (O'Rourke, 2007).

The Atherosclerosis Risk in Communities (ARIC) Study, a population study recruiting a biracial sample of 4701 men and women 45 to 64 years of age, investigated the relationship between carotid stiffness and different CV risk factors. Carotid stiffness was associated with hypertension, diabetes, trait anger, physical activity, and ethnicity (Salomaa et al., 1995;Liao et al., 1999;Schmitz et al., 2001;Din-Dzietham et al., 2004;Williams et al., 2006). In particular, metabolic factors, such as elevated glucose, insulin, and triglycerides, had a synergistic effect on Young's elastic modulus (Salomaa et al., 1995). 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 BP values, sex and age (Urbina et al., 2004). 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 metabolic syndrome and decreasing levels of testosterone (Scuteri et al., 2004). In a

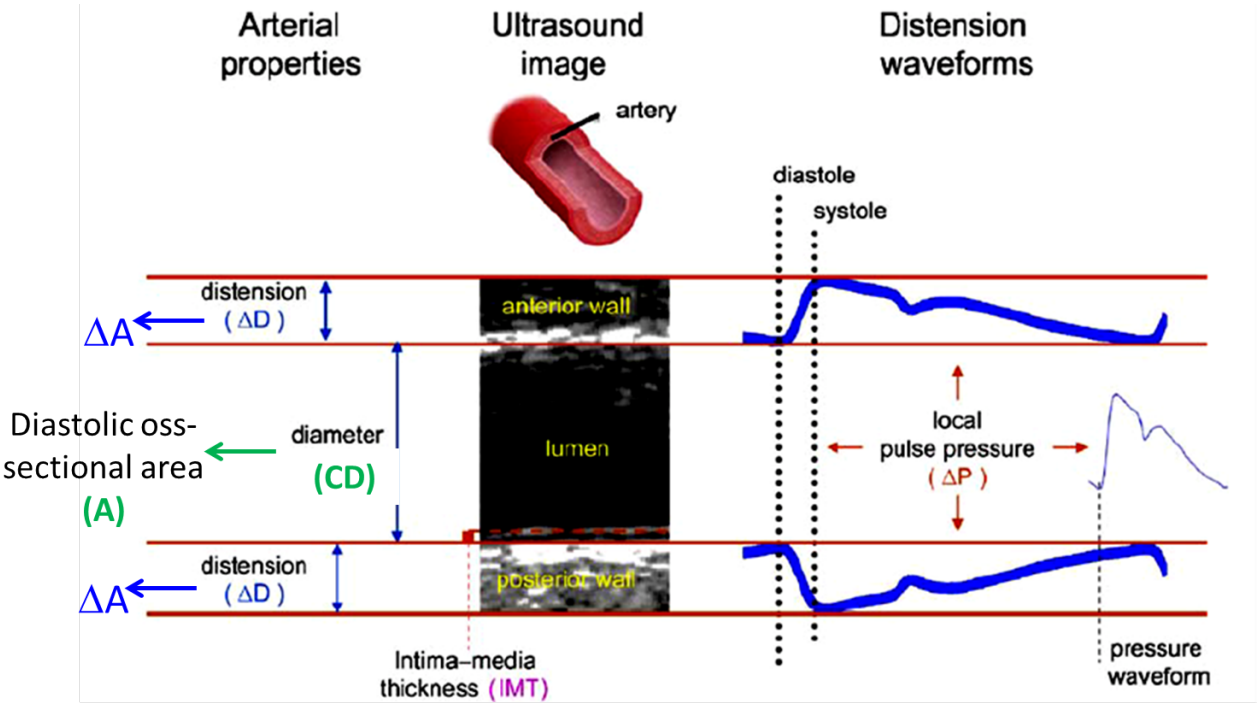
aged population with high prevalence of CV risk factors and disease, enrolled in the Hoorn study, low-grade inflammation appeared to have an important role in determining increased carotid stiffness, mainly through arterial enlargement (van Bussel et al., 2012). In the same population, metabolic syndrome has been associated with stiffness of muscular arteries (brachial and femoral), but not of musculo-elastic arteries (carotid and aorta) (Henry et al., 2009), again confirming that impact of different risk factors varies depending on the district considered. In the Second Manifestations of ARTerial disease (SMART) Study, decreased carotid distensibility was a marker of increased CV risk but in patients who already had vascular disease (Simons et al., 1999). Carotid stiffness was also able to predict incident hypertension in the ARIC cohort (Liao et al., 1999).

Arterial stiffness has been shown to be an independent predictor of CV morbidity and mortality (Vlachopoulos et al., 2010) and it is thought to play a crucial role in the development of CV disease. The predictive value of arterial stiffness has been shown mainly for aortic PWV (Vlachopoulos et al., 2010), while only few studies, with inconsistent results, were specifically aimed at investigating the predictive value of carotid stiffness for CV events. Blacher and colleagues first analyzed a cohort of 79 patients with end-stage renal disease undergoing hemodialysis, followed-up for 25 months, during which there were 10 fatal CV and 8 non-CV events. The study showed that an increased carotid stiffness is a powerful independent predictor of all-cause and CV mortality (Blacher et al., 1998). Carotid artery distensibility proved to be an independent predictor of CV disease after renal transplantation (Barenbrock et al., 2002). In the abovementioned SMART study, increased carotid stiffness was associated with an increased risk of CV events and mortality in not-corrected analysis, whereas the relationship disappeared after controlling for age (Dijk et al., 2005). However these study results are limited by the use of brachial instead of the local BP (BP) for the calculation of the carotid stiffness parameters, possibly leading to underestimation of the relationship between arterial stiffness and CV events. In contrast, in the Three-City study, enrolling 3337 elderly subjects (mean age 73 years) followed for a median of 44 months, patients who at baseline had a higher distension, showed an increased risk of coronary events compared to the ones

with lower distension (Leone et al., 2008). Finally, an independent association between increased carotid stiffness and a first-ever acute ischemic stroke has been reported (van Popele et al., 2001;Tsivgoulis et al., 2006), although other studies did not find the same relationship (Mattace-Raso et al., 2006). A tighter cause-effect relationship with cerebral rather than to coronary CV events is not surprising, given that the brain is the downstream district of the carotid artery. Accordingly, as a recent analysis conducted in 10407 subjects of the ARIC study followed-up for 13.8 years, showed that, after adjusting for CV risk factors, ultrasound measures of carotid arterial stiffness are associated with incident ischemic stroke but not incident coronary events, despite that the 2 outcomes sharing similar risk factors (Yang et al., 2012).

With an ever-increasing attention focused on the clinical implications of arterial stiffness analysis, it is extremely important to take into consideration methodological aspects. Indeed, inconsistent findings in trials exploring the clinical significance of carotid stiffness can be attributed, at least in part, to the large variance in technical measurements of carotid stiffness and local BP. The assessment of local stiffness 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 (PP) estimation by tonometry (Laurent et al., 2006). As already discussed also for C-IMT, two main approaches are available for arterial diameter assessment by ultrasound data, with similar accuracy (Bianchini et al., 2012): B-mode image processing based devices (Faita et al., 2008;Bianchini et al., 2010) and Radio-frequency (RF)-based echo-tracking systems **(Figure 2)** (Brands et al., 1999). In particular, when adopting B-mode based systems, attention should be posed to the quality of the scans and the system settings (Potter et al., 2008;Rossi et al., 2009;Bianchini et al., 2010). In addition, both RF- and B-mode based devices can also provide the automatic measure of carotid C-IMT, and thus are able to provide both functional and structural parameters of the analyzed vessel, as suggested by the international expert consensus (Laurent et al., 2006).

Figure 4. Local arterial distensibility assessment, based on echotracking techniques



2.4 Flow-mediated dilation

Endothelium plays a primary role in the control of vascular function by the production of nitric oxide (NO), which derives from the transformation of L-arginine into citrulline by the constitutive endothelial enzyme NO synthase. NO is produced under the stimulus of agonists (acetylcholine, bradykinin, and others), acting on specific endothelial receptors, and of mechanical forces, namely shear stress **(Figure 5)** (Furchgott and Zawadzki, 1980;Luscher and Barton, 1997). In pathological conditions, the same stimuli determine to the production of endothelium-derived contracting factors (e.g. thromboxane A2 and prostaglandin H2), which counteract the relaxing activity of NO, and reactive oxygen species, which impair endothelial function by causing NO breakdown (Luscher and Barton, 1997). In such conditions, reduced NO availability and endothelium-derived contracting factors 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 **(Figure 5)** (Ross, 1999).

This pivotal role of the endothelium in the atherosclerotic process led to the development of a different of methods to assess endothelial function, providing 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 (Deanfield et al., 2005;Deanfield et al., 2007).

Endothelial function in clinical research is mainly tested by vascular reactivity studies (Deanfield et al., 2005). The most widely used technique is the so-called “flow-mediated dilation” (FMD) **(Figure 6).** This is a non-invasive, ultrasound-based method, introduced in 1992 (Celermajer et al., 1992). 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 (Joannides et al., 1995;Ghiadoni et al., 2007). To this aim, a sphygmomanometer cuff placed on the forearm distal to the brachial artery is inflated to 300 mmHg and subsequently released 5 minutes

later. Endothelium-independent dilator response can be tested by low dose sublingual nitroglycerin (Ghiadoni et al., 2001). FMD has been studied widely in clinical research as it enables serial evaluation of young subjects, including children (Celermajer et al., 1992) 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 (Deanfield et al., 2007).

Impaired FMD has been show in hypertensive patients and in the presence of the other CV risk factors (Ghiadoni et al., 2001;Ghiadoni et al., 2003;Brunner et al., 2005;Ghiadoni et al., 2008a;Bruno et al., 2012b). A report from the Framingham study showed a progressive inverse relation between by FMD, and the increasing FRS (Benjamin, 2004). A meta-analysis, performed in over 200 available studies, observed that the relationship between FMD and risk factors was more evident in patient with lower CV risk (Witte et al., 2005).

Several studies have shown that endothelial dysfunction is an early indicator of atherosclerotic damage and is associated with target organ damage, including increased C-IMT (Juonala et al., 2004;Rundek et al., 2006;Halcox et al., 2009), and left ventricular hypertrophy (Ercan et al., 2003) Importantly, impaired FMD has been associated with major CV events (Modena et al., 2002;Muiesan et al., 2008;Yeboah et al., 2008;Yeboah et al., 2009). 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 CV risk, showed that endothelial dysfunction, evaluated also as FMD, significantly predicted CV events, independently of traditional CV risk factors (Lerman and Zeiher, 2005). These studies suggested 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 4 population-based cohort studies and 10 cohort studies, involving 5547 participants, showed a pooled relative risks of CV 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 (Inaba et al., 2010). However, the authors highlighted that the presence of heterogeneity in 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 CV risk factors (Inaba et al., 2010).

Lack of restoration of endothelial function despite conventional treatment might identify a subset of "non-responders", who might be suitable for more intensive or new therapeutic approaches. In a study conducted in 251 Japanese men with newly diagnosed stable coronary artery disease and concurrent endothelial dysfunction, FMD was repeated after 6 month of optimized individualized therapy. Those patients with persistently impaired FMD had significant higher event rates in the follow up period (26% over 31 months) (Kitta et al., 2009). Thus, correction of endothelial dysfunction might lead to an improvement of CV prognosis. Similarly, in a group of postmenopausal hypertensive women with impaired endothelial function, FMD was re-evaluated after 6 months of antihypertensive treatment (Modena et al., 2002). At 5-year follow-up, the incidence of CV events was significantly lower in the subgroup of women whose FMD was improved as compared to the subgroup without improvement, despite similar reduction in BP (Modena et al., 2002).

Furthermore, several studies have demonstrated that FMD can be improved with specific modifications of CV risk factors and with the use of drugs known to reduce CV risk (Ghiadoni et al., 2003;Plantinga et al., 2007;Charakida et al., 2009). Since the change in FMD occurring as a result of treatment can be obtained in a much shorter time (few months) than required for other vascular endpoints such as C-IMT (Halcox et al., 2009) or arterial stiffness (Laurent et al., 2006;Ghiadoni et al., 2009), FMD testing has a potential role for being included in clinical trials as a surrogate end-point (Charakida et al., 2010). Despite these considerable evidences, further large scale clinical trials are needed to demonstrate conclusively whether reversal of impaired FMD independently offers a better prognosis to patients with CV risk factor and disease.

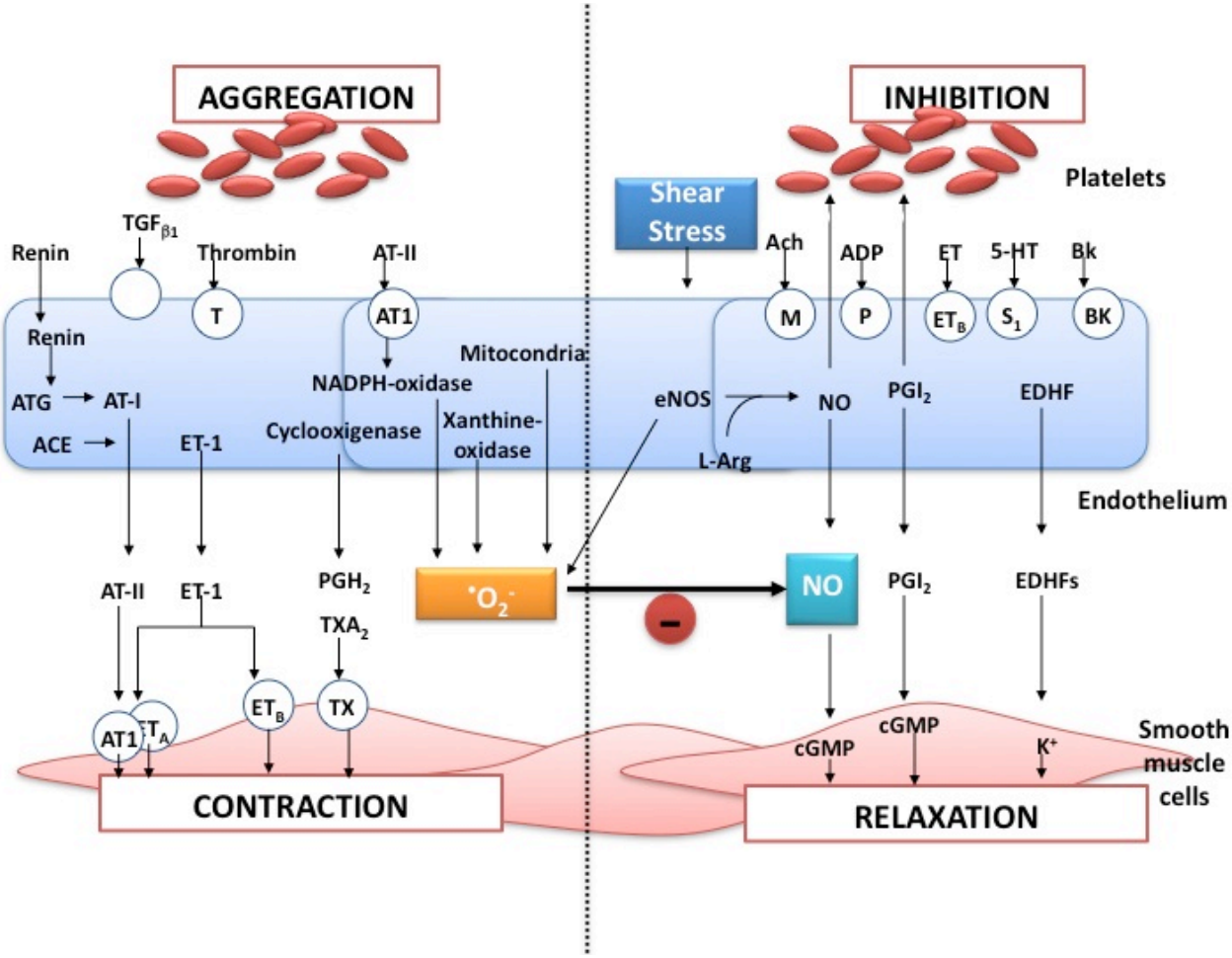
Assessment of brachial FMD in clinical investigation has increased because of it is noninvasive and apparently easy to perform. However, several challenges need to be overcome representing major limitations to a widespread application of this method in clinical studies (Corretti et al.,

2002;Deanfield et al., 2005;Ghiadoni et al., 2008a;Charakida et al., 2010;Thijssen et al., 2011a). These challenges include the need for highly trained operators, an adequate equipment including probe-holder with stereotactic clamp and automated image analysis **(Figure 6)**, and also the care required to minimize the effect of environmental or physiological influences (Ghiadoni et al., 2000). 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 to minimize operator-dependency (Corretti et al., 2002;Ghiadoni et al., 2008b;Charakida et al., 2010;Thijssen et al., 2011a).

It is important to note that variations in technique, such as the position of the occluding cuff and duration of inflation, may produce results that are less representative of local NO activity, since FMD is also determined by the magnitude of post-ischemic forearm vasodilatation, which is a measure of microcirculatory function (Deanfield et al., 2007). Interestingly, in a meta-analysis of 14 studies, FMD derived using upper cuff occlusion was at least as predictive as that derived using distal cuff placement, despite the latter being more NO dependent, suggesting that that any direct measure of vascular function may provide independent prognostic information in humans (Green et al., 2011). Training and certification of sonographers to FMD procedure has been well described in guidelines (Corretti et al., 2002), and proven by results in recent multicenter trials by the low number of rejected examinations, because of poor quality and/or instability of the images (Ghiadoni et al., 2012;Luscher et al., 2012). To date, only few experienced research centers apply a rigorous methodology to achieve a high standard of accuracy and reduce FMD variability (Donald et al., 2008). The lack of uniform methodology represents one of the major limitations for the application of FMD assessment in large multicenter studies. The time-dependent variability of FMD measurements obtained was recently evaluated in more than 130 healthy volunteers by trained operators according to a standardized technique (Ghiadoni et al., 2012). This included centralized analysis by an automated edge detection system, composed of a special-purpose hardware/software

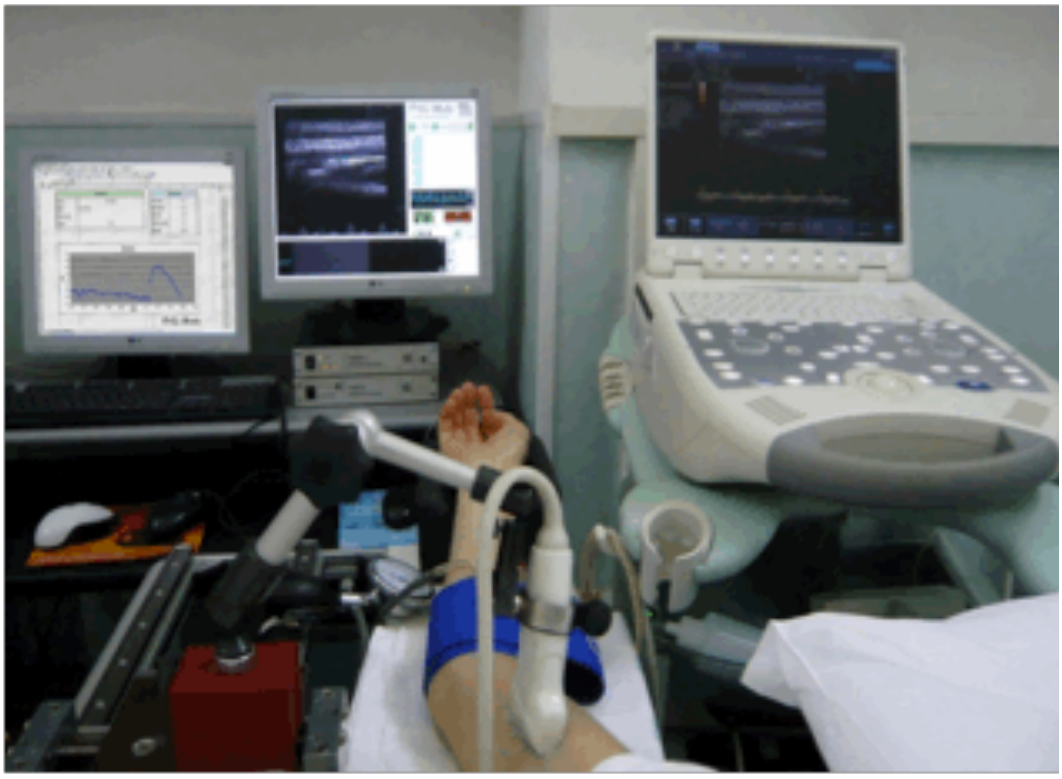
device for measuring changes of the brachial artery diameter (Gemignani et al., 2007;Gemignani et al., 2008). The study showed for the first time that the adherence to a rigorous protocol, with certified operator training as well as defined experimental settings, is feasible and provides an optimal time-dependent reproducibility of FMD (Ghiadoni et al., 2012).

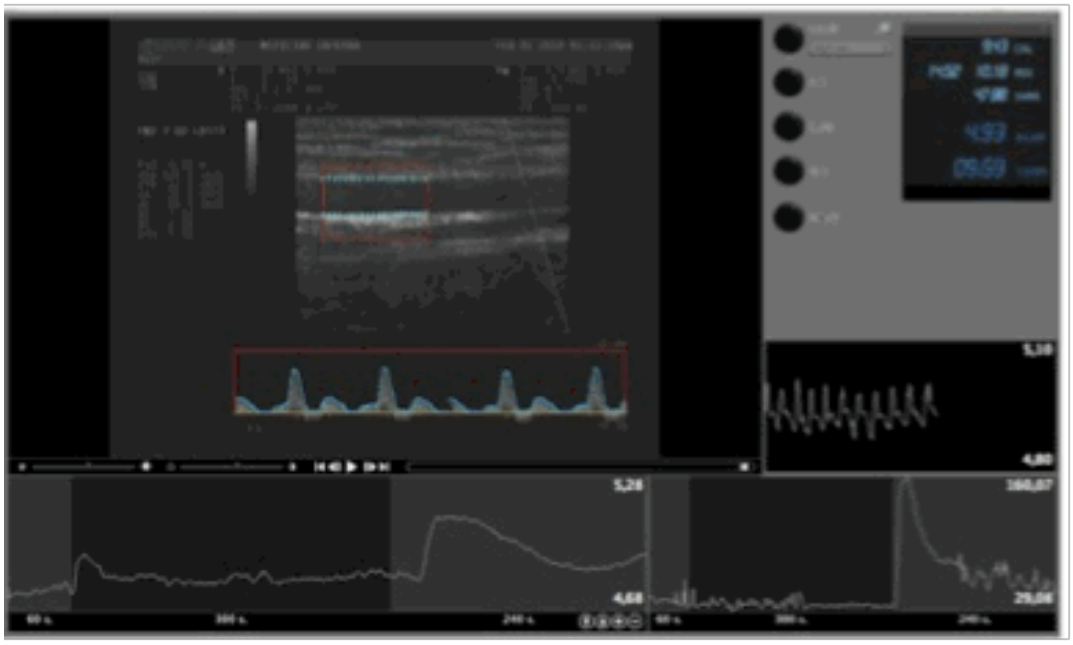
Figure 5. Role of NO in endothelial function



TGF: transforming growth factor. AT: angiotensin. ATG: angiotensinogen. ET: endothelin. ACE: Angiotensin converting enzyme. TX: tromboxane. PG: prostaglandin. NADPH: nicotinamide dinucleotide phosphate. eNOS: endothelial NO synthase. L-Arg: L-Arginine. Ach: acetylcholine. ADP: adenosin diphosphate. cyclic guanosine monophosphate: cGMP. EDHF: endothelium-derived hyperpolarizing factors. 5-HT: serotonin. BK: bradikynin.

Figure 6. Laboratory setup and automated image analysis system for FMD





Chapter 3. Rationale and objectives

There is increasing evidence that imaging biomarkers of vascular function and structure: 1) are associated with classical CV risk factors; 2) predict CV events independently of classical risk scores; 3) can be used as surrogate endpoints for pharmacological and non-pharmacological clinical interventions, thus possibly having a crucial role in CV drug development; 4) might identify a subset of patients in which conventional treatments are not sufficient. After decades of research, these non-invasive techniques are finally reaching solid standardization and good reproducibility. However, several questions in this field are still open, strongly limiting the wide use of these tools in the clinical practice. Methodological as well as pathophysiological and prognostic aspects still need to be clarified before a widespread use of imaging biomarkers for risk stratification in patients with traditional CV risk factors, as well as in primarily non-CV conditions. In this PhD thesis I focused mainly on the following issues:

Does vascular aging has a "functional component"?

Arterial stiffness has been commonly considered a consequence of structural alterations of the vessel wall. However, mechanistic studies have suggested that a "functional" component may contribute to the compliance of large arteries. In particular, endothelium-derived factors such as NO (Wilkinson et al., 2004) and endothelin-1 (McEniery et al., 2003) have been proposed as physiological modulators of arterial stiffness in healthy individuals. An inverse correlation between endothelial dysfunction and arterial stiffness has been reported in cross-sectional studies performed in mixed cohorts including healthy subjects as well as patients with isolated systolic hypertension (Wallace et al., 2007), type 2 diabetes (Ravikumar et al., 2002;Jadhav and Kadam, 2005;Gunarathne et al., 2009) and coronary artery disease (Nigam et al., 2003;Kobayashi et al., 2004;Kopec et al., 2009), although conflicting results were obtained in healthy subjects (McEniery et al., 2006;Koivistoinen et al., 2011). These aspects are of clinical relevance, since arterial stiffness is a powerful predictor of CV events in the diabetic population (Cruickshank et al., 2002).

Thus we hypothesized that the "functional" component of arterial stiffness might differ in relation to the presence of absence of CV risk factors. The aim of the study was to investigate whether conduit artery endothelial function could be a determinant of arterial stiffness in patients with arterial hypertension, and whether the concomitant presence of metabolic alterations such as type 2 diabetes mellitus might influence this relationship.

Is vascular aging similar in different districts?

Arterial tree ageing, expressed as stiffening of large arteries, is a key feature of arteriosclerosis and CV disease and is accelerated by the presence of CV risk factors. An increased carotid-femoral PWV was demonstrated in the presence of hypertension, smoking, diabetes, hypercholesterolemia, obesity and metabolic syndrome (Laurent et al., 2006). However it was recently suggested that only arterial hypertension and age are independently associated with increased aortic stiffness (Cecelja and Chowienczyk, 2009) and the role of CV risk factors on top of hypertension and aging is largely unexplored. Furthermore, vascular aging is a global process involving the whole organism, while different techniques explore different districts. The study of the impact of different CV risk factors on different vascular districts might indicate the most adequate biomarker to use for future studies and suggest specific mechanisms of disease in different disease conditions. The aim of the study was to evaluate whether classical CV risk factors on top of hypertension can worsen large artery stiffness in hypertensive patients. For this purpose we studied arterial stiffness in two different districts: aorta, which is the most important district in which stiffness is related to CV prognosis, by means of carotid-femoral PWV, and carotid artery, a musculo-elastic vessel which is the preferential site for development and assessment of arteriosclerosis but for which the prognostic significance of stiffness is less established, by means of an ultrasound-based analysis system.

Is arterial stiffness related to cardio-renal damage?

The presence of arterial hypertension and organ damage, at the cardiac, vascular and renal level, identifies a condition of high risk of future CV events (Jager et al., 1999;Verdecchia et al., 2001;Lorenz et al., 2007) and, in these patients, prompt initiation of pharmacological treatment is considered (Mancia et al., 2007). Carotid-femoral PWV was included among measures indicating target organ damage in hypertensive patients (Mancia et al., 2007) and has been shown to reclassify a significantly higher percentage of patients to the high risk category in comparison to routine workup but also added to microalbuminuria and to cardiac ultrasound in the general population (Muiesan et al., 2010), even if the prognostic significance of this reclassification is still unknown.

Early vascular aging is often accompanied by cardiac and renal damage, but the relationship between organ damage in different districts is still largely unexplored. Aortic stiffness, measured as PWV, has been associated in cross sectional studies with well-accepted intermediate endpoints such as left ventricular hypertrophy (Watabe et al., 2006), albuminuria, and estimated GFR (Hermans et al., 2007) in the general population. Arterial stiffness and albuminuria were associated even in nonhypertensive, nondiabetic individuals, which suggests the possibility of a similar pathophysiological mechanism involved in these two indices of subclinical target organ damage (Kim et al., 2011). To our knowledge, no data are available at the moment about the relationship between carotid stiffness and hypertensive organ damage. This would be of importance, because arterial hypertension and associated risk factors are able to damage in parallel different districts, but vascular damage might amplify organ damage in parenchymal districts per se (O'Rourke and Safar, 2005). Furthermore, stiffness in different arterial districts might cluster in a different manner with organ damage. Thus this study is aimed at evaluating whether carotid and aortic stiffness are associated in a similar way to cardiac and renal hypertensive organ damage.

How to evaluate renal vascular damage?

Early diagnosis of target organ damage has a relevant role for patients at high CV risk, since both the presence and the regression of initial alterations, including left ventricle hypertrophy, C-IMT and microalbuminuria, are independent determinants of CV prognosis (Mancia et al., 2007). On regard of large arteries, endothelial dysfunction and arterial stiffness have been proved to be indexes of early vascular pathological changes, which might lead to end-organ dysfunction (Laurent et al., 2006;Virdis et al., 2008;Ghiadoni et al., 2009). Microalbuminuria is recognized as a clinical marker of first stages of nephropathy in type 1 and 2 diabetes (de Zeeuw, 2007;Bakker et al., 2009), whereas in essential hypertension it is mainly considered an indicator of CV risk (Mancia et al., 2007). However, microalbuminuria identifies an already established glomerular damage, which is likely to be preceded by earlier structural and functional alterations undetectable so far, unless by kidney biopsy (Futrakul et al., 2009;Ruilope and Segura, 2009;Weir, 2009). Moreover, a variable percentage of type 2 diabetic patients shows an impairment of GFR even in the presence of normal albumin excretion (MacIsaac et al., 2004). Thus, new reliable markers of early renal dysfunction, specifically linked to vascular renal impairment, are needed, in order to better identify the responsible mechanisms and the ideal timing for the treatment, aiming to delay the onset and/or slow the progression of renal damage.

Renal resistive index (RI) measured by duplex ultrasound is a useful parameter for quantifying the alterations in intraparenchimal renal circulation that may occur during the course of chronic renal disease. RI is tightly related to renal arteriolosclerosis, as demonstrated by bioptic studies (Ikee et al., 2005), and represents an integrated index of arterial compliance, pulsatility and downstream microvascular impedance (Derchi et al., 2005;Krumme, 2006). High RI has a negative prognostic value in diabetic patients in terms of progression of renal disease (Hamano et al., 2008) and in hypertensive patients with renal artery stenosis for the success of percutaneous revascularization (Radermacher et al., 2001). However, some limitations must be considered concerning the use of RI in the early phases of nephropathy. First, reference values of RI in the general population have not

been unanimously defined and validated; second, RI is critically influenced by the aging process (Platt et al., 1991a); third, in the presence of normal renal function, the narrow range of RI values might not be able to highlight initial renal microvascular alterations. Therefore, the use of a pharmacological stimulus inducing vasodilation applied to RI measurements (Dynamic Resistive INdex, DRIN) might increase the discriminating power of duplex ultrasound, configuring a method potentially able to unmask renal abnormalities even in the early phases. A reduced renal vasodilation to nitroglycerine has been demonstrated in patients with overt diabetic nephropathy (Frauchiger et al., 2000); whether an impaired renal vasodilation is present even before the onset of microalbuminuria in patients with type 2 diabetes or with other CV risk factors, and its potential relation to markers of systemic vascular dysfunction, is still unknown.

The aim of the study was to evaluate RI in basal conditions and under pharmacological vasodilator stimulus in newly diagnosed, treatment-naive type 2 diabetic patients with normal urinary albumin excretion and glomerular filtration rate, comparing them to a group of essential hypertensive patients, in order to investigate whether a reduced DRIN could be a specific feature of diabetes. As a secondary aim, this study investigated the possible association of DRIN with established indexes of systemic vascular damage, such as endothelial function and arterial stiffness, and increased oxidative stress.

Multiparametric assessment of vascular function and structure in primarily non-cardiovascular conditions

Imaging biomarkers of vascular function and structure can be useful not only for risk stratification in patients with traditional CV risk factors, but also to explore CV consequences of primarily non-CV diseases and conditions (Laurent et al., 2006). We hypothesized that a comprehensive, multiparametric approach would be the best strategy to detect early vascular damage in one or more districts involved in each condition, possibly in a subclinical, reversible stage. This approach could

allow identifying the "CV footprint" characterizing each condition, with a double aim: to elucidate the pathophysiology of CV complications in primarily non-CV conditions and, in long term, to propose the most useful test to be used in the clinical practice for screening and follow-up.

During my PhD thesis I applied this strategy to some primarily non-CV conditions that might qualify as emerging CV risk factors, such as exposure to environmental and iatrogenic radiation doses, obstructive sleep apnea syndrome (OSAS) in the absence of traditional CV risk factors, and chronic exposure to hypobaric hypoxia at high altitude.

Chapter 4. Methods

In order to fulfill the objectives of this PhD thesis, several cohorts of volunteers with or without CV risk factors and other non-CV conditions were examined in a cross-sectional design. A set of non-invasive biomarkers of vascular function and structure were performed.

4.1 Carotid intima-media thickness and distensibility

Common carotid artery scans were obtained by high-resolution ultrasound with a 10 MHz linear array transducer (MyLab25; ESAOTE). Two 10'-clips were acquired from each common carotid artery (1 cm proximal to the carotid bulb in a region 1 cm wide and free of plaques) and then analyzed offline by means of Carotid Studio (Quipu srl, Pisa, Italy), an algorithm for the automatic evaluation of the instantaneous carotid diameter **(Figure 2)**. The method is based on a well-validated contour tracking technique, allowing automatic evaluation of diameter stroke changes during the heart cycle, and was validated for accuracy against the gold standard measurement by radiofrequency (Bianchini et al., 2010). The following parameters were calculated:

- Carotid distension (ΔD), that is the stroke change in diameter, calculated as the difference between the systolic and diastolic diameter values;
- Cross-sectional distensibility coefficient (DC), calculated as DC = ΔA/(A*carotid PP), where A is the diastolic lumen area, and ΔA is the stroke change in lumen area;
- Carotid stiffness (CS), calculated using the Moens-Korteweg equation, $(\rho*DC)^{-1/2}$, where ρ is the blood density.

Carotid lumen area was derived from the diameter values, assuming the cross-section of the artery to be circular. Carotid systolic BP and PP were obtained by applanation tonometry from carotid pressure waveforms, using central diastolic and mean BP for calibration (SphygmoCor, AtCor Medical). C-IMT was automatically measured on the same image sequences as the mean of the

relative values of 10 s. Parameters are indicated as the mean of the right and left common carotid values.

4.2 Carotid-femoral pulse wave velocity

Arterial tonometry was performed according to international recommendations (Laurent et al., 2006). Carotid-femoral and carotid-radial PWV were assessed by SphygmoCor device (AtCor Medical, Sydney, Australia), recording waveforms at the two recording sites, sequentially. PWV was calculated as the ratio between the subtracted distance between the two recording sites and wave transit time **(Figure 3 and 7)**. Applanation tonometry was also performed on the radial artery in order to obtain central BP values, by using a validated transfer function (SphygmoCor, AtCor Medical), and augmentation index normalized at 75 bpm (AIx). Two consecutive measurements were recorded and averaged. Carotid systolic BP and PP were then obtained from carotid pressure waveforms, using central diastolic and mean BP for calibration.

4.3 Flow-mediated dilation

Brachial artery scans were obtained by high-resolution ultrasound with a 10 MHz linear array transducer (MyLab25; ESAOTE, Florence, Italy). Endothelium-dependent response was assessed as dilation of the brachial artery in response to increased blood flow (flow-mediated dilation, FMD) (Bruno et al., 2011). Briefly, a pediatric cuff was positioned around the right forearm below the elbow and the right brachial artery was located and scanned longitudinally between 5 cm and 10 cm above the elbow using a 10 MHz linear array transducer (MyLab 25, ESAOTE Florence, Italy). The transducer has been held at the same point throughout the scan by a stereotactic clamp to ensure consistency of the image **(Figure 6)**. After 1-min baseline recording, the cuff was inflated for five min at 300 mmHg and then deflated to induce reactive hyperemia (Figure 1). Endothelium-independent vasodilation was obtained by the sublingual administration of 25 μg GTN (Ghiadoni et

al., 2001). Brachial artery diameter measurements were performed by computerized edge detection system **(Figure 6)** (Gemignani et al., 2008). FMD and response to GTN were calculated as the maximal percent increase in diameter above baseline (mean of measures obtained during the first min).

Volume blood flow was calculated by multiplying duplex flow velocity (corrected for the angle) by heart rate and vessel cross-sectional area (πr^2). Mean blood flow velocity in the brachial artery was determined by pulsed Doppler signal at 70°, with the range gate in the center of the artery, and continuously acquired throughout all the study. Resting and hyperemic shear rate (SR) were calculated according to the following equation: 8 *mean flow velocity / brachial artery diameter. Reactive hyperemia was calculated as the maximum percent increase in flow after cuff release as compared to baseline flow.

4.4 Renal vasodilating capacity

Renal scans were performed by a single trained operator using an ultrasound machine (MyLab 25, ESAOTE Florence, Italy) with a high-resolution multifrequency Convex probe (2.5 - 4.5 MHz). Three velocimetric measurements of the interlobar renal arteries adjacent to medullary pyramids were obtained by a translumbar or anterior approach **(Figure 8)**. Renal resistive index (RI) was calculated in both kidneys according to the formula: (systolic peak velocity – end diastolic velocity)/systolic peak velocity. RI measurement was obtained at baseline and 5 min after pharmacological stimulus with a low-dose (25 μg) administration of sublingual GTN. Renal vasodilating capacity was calculated as absolute percent changes from baseline in response to GTN (Bruno et al., 2011).

Figure 7. Carotid PWV assessment

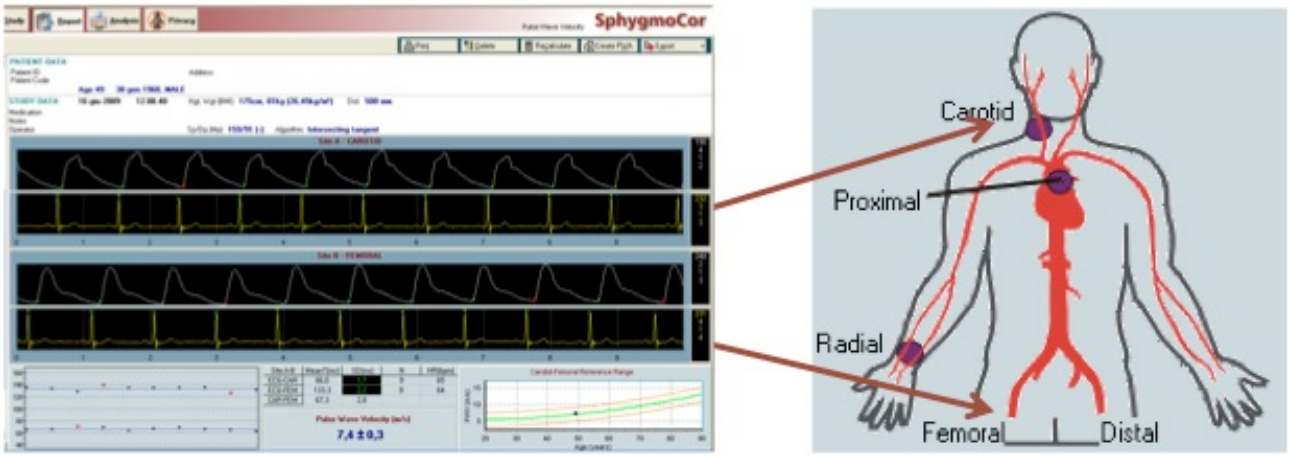
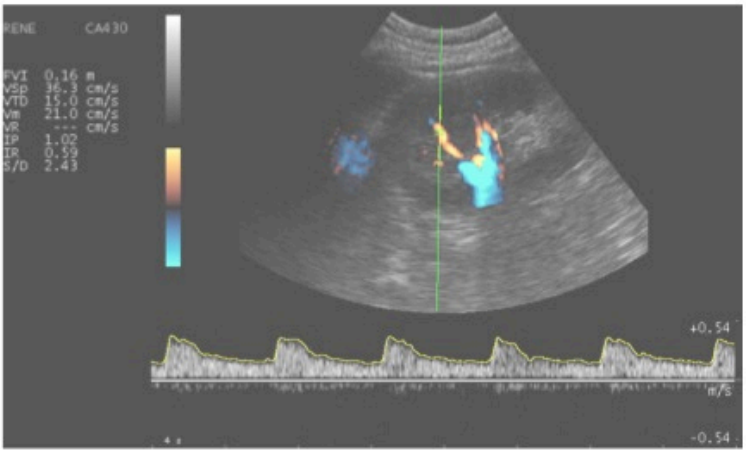


Figure 8. Methodology for renal vasodilating capacity assessment

- High resolution duplex ultrasound
- Automated velocity flow curve detection
- 3 consecutive measurements for each kidney

→ At baseline and 5' after sublingual glyceril trinitrate (GTN) administration (25 µg)



$$\text{Renal resistive index (RI)} = \frac{\text{peak systolic velocity– end diastolic velocity}}{\text{peak systolic velocity}}$$

$$\text{RI response to GTN (\%)} = -\left(\frac{\text{RI after GTN – baseline RI}}{\text{Baseline RI}} \times 100\right)$$

Chapter 5. Does vascular aging has a "functional" component?

The aim of this study was to investigate whether a "functional" component of arterial stiffness exists, based on endothelium-mediated mechanisms, in patients with arterial hypertension, and whether the concomitant presence of metabolic alterations such as type 2 diabetes mellitus might influence this relationship. The results of this research have been recently published (Bruno et al., 2012a).

5.1 Experimental protocol

Study population – A total of 341 individuals (259 patients and 82 age- and sex- matched healthy subjects) were enrolled at the Diabetes Outpatient Clinic of the Department of Endocrinology and Metabolism and the Hypertension Outpatient Clinic of the Department of Clinical and Experimental Medicine of the University Hospital of Pisa, Italy. For the patients' group inclusion criteria were: diagnosis of essential hypertension and / or type 2 diabetes according to current guidelines (Mancia et al., 2007), or current treatment with antihypertensive or antidiabetic drugs. Exclusion criteria were: chronic kidney disease KDOQI stage 4 and 5, current insulin therapy, prior CV events, major co-morbidities (malignancies, chronic and acute inflammatory diseases, chronic heart failure and liver insufficiency, atrial fibrillation or frequent ectopic beats), non-CV medications interfering with vascular function (i.e. hormonal therapy, steroidal and non-steroidal anti-inflammatory drugs). Patients were divided in two groups according to the presence (n=84) or absence (n=175) of type 2 diabetes mellitus. All pharmacologically treated patients were on a stable therapeutic regimen for at least 3 months. The study conformed to the Declaration of Helsinki, was approved by the local Ethical Committee and all patients provided written informed consent prior to entering the study.

Experimental session – The volunteers were requested to refer to the local Diabetes Unit after an overnight fasting for collection of medical history, anthropometric parameters (body weight, height, and waist circumference) as well as blood and urine samples. On the following day, BP measurement and vascular assessment (FMD and carotid-femoral PWV) were determined at the Hypertension Unit. All measurements were performed in the morning after an overnight fasting, in a quiet air-conditioned room (22 to 24°C). For the duration of the study patients were kept on their usual pharmacologic treatment.

Statistical analysis - All statistical analyses were performed using NCSS 2004 (NCSS: Kaysville, Utah, USA). For normally distributed data, results are expressed as mean ± SD, whereas median value and 25%-75% interquartile range is used for non-normally distributed data. Differences in means among groups were analyzed using ANOVA and Bonferroni post-hoc analysis for normally distributed variables, or Kruskal-Wallis Z Test for not normally distributed variables; categorical variables were analyzed by χ^2 test. Analysis of covariance was used to compare aortic PWV and FMD in different subgroups, as appropriate. Spearman's rank was used to explore correlations among variables. Multiple linear regression analysis was performed including parameters with significant correlation with the dependent variable and building different models. The study was powered (90%) to detect a 1.3% difference in FMD, a 0.8 m/s difference in aortic PWV, and a difference of 0.20 in the slopes of regression lines, with a Type I error probability of 0.05.

5.2 Results

Clinical characteristics - Clinical characteristics of the study population are shown in **Table 1**. Diabetic and non-diabetic hypertensive patients were comparable for age, sex, duration of hypertension, percentage of smokers, renal function, and UACR. Diabetic hypertensive patients showed a higher prevalence of isolated systolic hypertension (78.8% vs. 63.1%, p=0.012) in comparison to non-diabetic hypertensive patients, in spite of similar systolic BP and lower diastolic

BP. Diabetic patients had also higher body mass index (BMI), waist circumference, blood glucose and HbA_1c values. Due to the higher use of statins (35 vs. 6%, $p<0.001$), diabetic hypertensive patients had lower total- and LDL-cholesterol, and a higher prevalence of hypercholesterolemia (defined according ATP-III criteria or by current lipid-lowering therapy, 82% vs. 63%). HDL-cholesterol was lower and triglycerides higher in diabetic than in non-diabetic hypertensive patients. Diabetic hypertensive patients showed also higher levels of hsCRP. Obesity (defined as BMI $\geq$ 30 kg/m^2) and metabolic syndrome, defined according to ATP-III criteria (Grundy et al., 2005), were significantly more prevalent among diabetic hypertensive patients than in normoglycaemic hypertensive patients (66 vs. 18%; 91 vs. 40%; $p<0.001$ for both). The majority of patients in both groups were on antihypertensive treatment (65% vs. 59%, p=ns). Details on CV drug therapy are shown in **Table 2.**

Vascular variables - Brachial artery diameter and hyperemic shear stress did not significantly differ between diabetic and non-diabetic hypertensive patients, although in both groups they were significantly higher than in healthy subjects **(Table 1).** FMD was lower in non-diabetic hypertensive patients than in healthy subjects, and further reduced in diabetic patients (Figure 1). When brachial artery diameter and hyperemic shear stress were considered as covariates, FMD was still significantly different among the three groups studied ($p<0.001$). On the contrary, GTN response was not different between diabetic and non-diabetic hypertensive patients, though similarly reduced as compared to healthy subjects **(Table 1),** even after adjustment for brachial artery diameter.

PWV was higher in non-diabetic hypertensive patients than in healthy subjects, and further increased in diabetic hypertensive patients **(Figure 9).** The significance was not affected after considering mean BP, age, and BMI as covariates ($p<0.001$). Central systolic BP and PP were similarly increased in diabetic and non-diabetic hypertensive patients, while mean BP was higher in the latter group **(Table 1).**

Relationship between PWV and FMD: role of type 2 diabetes mellitus - In the univariate analysis performed on the whole study population, aortic PWV was significantly and negatively correlated with FMD (r =-0.272; p<0.001). Among the other vascular parameters, aortic PWV was also correlated with hyperemic shear stress and brachial artery diameter **(Table 3)**. However FMD remained a significant determinant of PWV even after adjustment for these two confounders, as showed by multiple regression analysis (Model 1, **Table 4**). Among clinical features, PWV significantly correlated in the univariate analysis with age, brachial and central BP values, heart rate, BMI and waist circumference, cholesterol levels, triglycerides, fasting blood glucose and HBA_{1c}, and hs-PCR. In the multiple regression analysis including variables in Model 1 plus age, mean BP, heart rate, total and HDL cholesterol, triglycerides and hs-PCR, (Model 2, **Table 4**), FMD remained a significant predictor of PWV. After adding blood fasting glucose to the model, the relationship between PWV and FMD was no longer significant (Model 3, **Table 4**). In Model 3, independent predictors of PWV were: age, sex, BMI, heart rate, mean BP and blood fasting glucose **(Table 4).**

Univariate correlation analysis performed separately in the three groups showed that FMD was significantly correlated to PWV only in diabetic hypertensive patients, while the relationship was not significant either in healthy subjects or in normoglycaemic hypertensive patients (**Table 3**, **Figure 10**). The slope of the relationship between FMD and PWV in diabetic hypertensive patients was steeper than in the other two groups (p<0.05). Multivariate regression analysis performed in the diabetic group showed that FMD was an independent predictor of aortic PWV in a model adjusted for age, sex, mean BP, BMI and fasting blood glucose, (β =-0.348, p=0.003, 95%CI -0.577 to -0.120), explaining 8.3% of the variance of PWV.

Subgroup analysis in non-diabetic hypertensive patients - Among non-diabetic hypertensive patients, the relationship between FMD and PWV was investigated comparing patients with

obesity (n=33) or metabolic syndrome (n=69) to their counterparts. PWV was higher in obese hypertensive patients but not in those with metabolic syndrome, whereas FMD was not affected by the two conditions. FMD and PWV were not correlated neither in obese nor in lean hypertensive patients (r=0.167, p=0.360 and r=-0.118. p=0.167). Results were also non significant in hypertensive patients with (r=-0.006, p=0.961) or without metabolic syndrome (r=-0.106 p=0.305).

The role of medications known to influence vascular function was also investigated. PWV was lower on patients on antihypertensive treatment, but not affected by use of statins. Among BP-lowering drug-treated patients, PWV was lower, but not significantly, in patients on renin-angiotensin system blockers. FMD was not significantly affected by any of the above-mentioned treatments (see **Table 5**). The relationship between FMD and PWV was not influenced by the presence or not of antihypertensive treatment (r=0.027, p=0.800 vs. r=-0.130, p=0.232) and by current therapy with renin-angiotensin system blockers (r=-0.029, p=0.821 vs. r=-0.110, p=0.249) or statins (r=-0.021, p=0.950 vs. r=-0.069, p=0.384).

5.3 Discussion

The present study explored the role of endothelial function in the peripheral conduit arteries, measured as FMD, as a predictor of arterial stiffness in hypertensive patients with and without type 2 diabetes mellitus, in the attempt to establish the role of endothelial dysfunction in the pathophysiology of arterial stiffening. The main finding of this study is that FMD is related to aortic stiffness in hypertensive patients with, but not in those without type 2 diabetes mellitus. These results suggest that diabetes-related metabolic alterations on top of hypertension might contribute, through reduced endothelial function, to increase wall stiffness of large arteries independently of other confounders.

An element of novelty of the present paper is the demonstration of an additive effect of hypertension and type 2 diabetes in causing an impairment of conduit artery endothelial function. Hypertensive diabetic patients had lower FMD compared to non-diabetic hypertensive patients, in the presence of similar baseline brachial artery diameter, peak shear stress and endothelium-independent dilation, highlighting a specific deleterious effect of diabetes on NO-dependent vasodilatation of conduit arteries. These results are in contrast with those obtained in small resistance arteries, showing that the concomitance of the two risk factors did not cause a further impairment of endothelial function (Rizzoni et al., 2001;Schofield et al., 2002). However this discrepancy should not be surprising, given the different district studied and the known heterogeneity of the vascular properties in different vascular beds (Deanfield et al., 2005). On the other hand, our results confirm the presence of endothelial dysfunction and increased arterial stiffness in hypertensive patients as compared to healthy subjects(Taddei et al., 2000;Ghiadoni et al., 2009), as well as the further increased PWV in diabetic hypertensive patients compared to normoglycaemic hypertensive patients (Tedesco et al., 2004).

Brachial artery FMD and aortic PWV were negatively correlated in the whole population. This finding is in agreement with previous studies performed in mixed populations comprising healthy individuals and patients with different CV risk factors or established coronary artery disease (Nigam et al., 2003;Kobayashi et al., 2004;Wallace et al., 2007), including also diabetic patients (Ravikumar et al., 2002;Jadhav and Kadam, 2005;Gunarathne et al., 2009). Multiple regression analysis, including vascular determinants of FMD such as baseline brachial artery diameter and hyperaemic shear stress, showed that endothelium-dependent vasodilation remained *per se* an independent predictor of aortic PWV. Furthermore, FMD resulted to be a predictor of arterial stiffening independently from age and BP, which are known to be the main determinants of arterial stiffness (Cecelja and Chowienczyk, 2009), and other classical CV risk factors. Only the inclusion of blood fasting glucose caused loss of significance of the relationship between FMD and PWV in

the whole population. This result suggests a specific role of glucose abnormalities in determining the association between endothelial dysfunction and increased arterial stiffness.

This hypothesis is further supported by subgroup analysis. Interestingly, FMD was a predictor of aortic PWV only among diabetic hypertensive patients. In this subgroup the relationship remained significant after taking into account age, BP values, BMI, and fasting blood glucose. Thus the greater aortic stiffening in diabetic hypertensive patients than in patients with hypertension alone, is determined, at least in part, by an independent, detrimental effect of type 2 diabetes mellitus on endothelial function. Despite the present study was not designed to investigate the pathophysiological mechanisms underlying the development of arterial damage, several factors related to type 2 diabetes mellitus may be involved, including obesity, insulin resistance and hyperinsulinemia, low-grade inflammation, increased oxidative stress, deposition of advanced glycation end products (Stehouwer et al., 2008). All these factors may concur in accelerating and worsening endothelial dysfunction, favouring arterial stiffness, although the present study suggests that nor obesity neither low-grade inflammation seem to play a major role. Moreover, we cannot rule out that, rather than a single mechanism, it is the complexity of the diabetic condition that sustains a greater and more prolonged alteration of endothelial function, leading to an increased PWV.

Because of the cross-sectional design of the study, it is not possible to establish whether the association between endothelial function and arterial stiffness in diabetes depends on common causing factors or on a cause-effect relationship. Nonetheless, growing evidence suggests that endothelial dysfunction can lead to arterial stiffness. In keeping with this hypothesis is the notion that NO, whose reduced bioavailability is the molecular basis of endothelial dysfunction, is involved in the regulation of arterial distensibility (Wilkinson et al., 2004). Diseases characterized by endothelial dysfunction such as diabetes (Ravikumar et al., 2002) and arterial hypertension (Virdis et al., 2008) also show increased arterial stiffness (Laurent et al., 2001;Stehouwer et al., 2008). Moreover, therapeutic approaches, such as blockade of the renin-angiotensin system,

improve both vascular alterations (Taddei et al., 2002;Ghiadoni et al., 2009). Conversely, the hypothesis that structural alterations associated with arterial stiffness could impair vasodilation is unlikely, since the response to GTN was not different between hypertensive patients with and without type 2 diabetes mellitus, though impaired as compared to healthy controls. However, we cannot completely exclude that increased arterial stiffness, altering pulsatile haemodynamics, blood flow pattern and shear stress, could lead to decreased NO bioavailability(Thacher et al., 2007).

In the present study, endothelial dysfunction was not a determinant of aortic stiffness either in healthy subjects or in non-diabetic hypertensive patients. As far as healthy subjects are concerned, our results are in agreement with a recent study carried out in a cohort of 1754 adults, aged 30-45 years, with a very low prevalence of CV risk factors, suggesting that in young individuals PWV and FMD might reflect different aspects of CV damage (Koivistoinen et al., 2011). In contrast McEniery et al (McEniery et al., 2006) found a significant correlation between endothelial function and PWV. This conflicting result might be attributable to biological difference of subjects enrolled (wider age range, younger population as compared to our own), as well as different methodology for the assessment of endothelial function (pulse wave analysis after albuterol inhalation, with FMD performed only in a subgroup). On the other hand, to our knowledge this is the first study aimed, and adequately powered, to directly address the role of endothelial function in determination of arterial stiffening in essential hypertension, since previous studies included in pooled analysis healthy people and did not exclude diabetic subjects (Ravikumar et al., 2002;Nigam et al., 2003;Kobayashi et al., 2004;Jadhav and Kadam, 2005;Wallace et al., 2007;Gunarathne et al., 2009). Our results suggest that in essential hypertension the “functional” component of PWV is negligible in comparison to hemodynamic load and structural wall alterations associated to BP increase, while only the presence of type 2 diabetes mellitus is able to cause endothelium-dependent stiffening of large arteries.

We also investigated the possible role of metabolic factors on top of high BP in hypertensive, normoglycaemic patients. Obesity was demonstrated to further worsen PWV but not FMD, and the

relationship between the two parameters was not significant either in the presence or in the absence of obesity. This observation suggests that the deleterious effects on arterial stiffness of obesity on top of hypertension are unlikely to be mediated by endothelium-related mechanisms. Furthermore, it indirectly confirms that differences found between hypertensive patients with or without type 2 diabetes mellitus were not secondary to the greater prevalence of obesity in the former group. On the other hand, our results support the hypothesis, not universally accepted in the literature, of blood-pressure dependence of vascular alterations in metabolic syndrome (Czernichow et al., 2005;Schillaci et al., 2005;Plantinga et al., 2008).

Finally, it is well known that antihypertensive and lipid-lowering treatments can affect vascular function (Ghiadoni et al., 2003;Ghiadoni et al., 2009). Therefore a further analysis was performed, in order to exclude that the lack of relationship between FMD and PWV in hypertensive patients could be due to the confounding effect of chronic therapies. Although chronic antihypertensive treatment was associated with a lower PWV, it did not affect the relationship between FMD and PWV.

We should acknowledge some limitations in this study. The cross-sectional design of the study does not allow to determine whether the association between endothelial function and arterial stiffness we found in diabetic patients was due to common causing factors or really reflected a cause-effect relationship. Also, our data do not allow investigating the pathophysiological mechanisms underlying the development of arterial damage. Further prospective and mechanistic studies are required to confirm the present findings and to answer these crucial questions.

Some methodological limitations of our study are worth commenting. Stimulus for FMD was evaluated as peak hyperemic shear stress. Although this parameter was chosen because a correlation with clinical risk factors was demonstrated in large cohort studies (Mitchell et al., 2004), emerging evidence suggest that shear rate area under the curve could be a more accurate estimation of the hyperemic stimulus (Thijssen et al., 2011a). Moreover, estimation of central BP was derived by radial artery waveform through a transfer function, upon calibration with brachial BP. Though

validity of transfer function in resting conditions was demonstrated in comparison to invasive central BP recordings (Pauca et al., 2001), PP amplification over the brachial-to-radial arterial path can be a critical source of error (Verbeke et al., 2005). Nonetheless, the method is widely accepted because is a simple, non-invasive way to obtain a highly predictive parameter in CV disease (Ghiadoni et al., 2009).

In conclusion, our results suggest that endothelial dysfunction can contribute to arterial stiffness in diabetic hypertensive individuals independently of common confounders. Conversely, this mechanism does not seem to play a major role in patients with essential hypertension in the absence of type 2 diabetes mellitus. The greater impairment in nitric oxide availability brought about by type 2 diabetes may accelerate and worsen vessel wall distensibility, critically worsening CV prognosis inherent to this condition.

Table 1: Clinical and vascular parameters in hypertensive patients without or with type 2 diabetes mellitus and in healthy subjects

Variables	**Healthy subjects (82)**	**Hypertensive patients without type 2 diabetes mellitus (175)**	**Hypertensive patients with type 2 diabetes mellitus (n=84)**
Clinical variables			
Age (years)	54.8±6.6	54.4±6.5	56.7±7.6
Male sex (n,%)	47 (57%)	105 (60%)	49 (58%)
Smokers (n,%)	15 (18%)	32 (18%)	10 (12%)
Duration of hypertension (years)	-	9(6-14)	10(7-16)
Duration of diabetes (years)	-	-	8(5-13)
Brachial systolic BP (mmHg)	128.7±9.6	144.6±13.2†	143.9±14.3†
Brachial diastolic BP (mmHg)	78.9±7.7	85.6±9.3†	81.4±10.1*
Brachial PP (mmHg)	49.8±6.9	58.0±10.6†	62.5±13.1*†
Heart rate (bpm)	67.5±9.9	66.8±10.9	75.0±12.6*†
Body mass index (kg/m^2)	25.3 (23.2-27.4)	26.9 (24.5-29.3)†	32.8 (28.7-36.0) *†
Waist circumference (cm)	90.5±6.6	98.3±10.1†	112.1±11.6*†
Blood glucose (mmol/L)	5.2 (4.8-5.5)	5.2 (4.8-5.7)	8.9 (7.5-10.9) *
HbA_1c (%, mmol/mol)	5.5±0.6, 36.3±5.6	5.6±0.5, 37.4±4.2	8.0±1.0, 64.4±9.3*
Total cholesterol (mmol/L)	5.6±0.9	5.7±0.9	4.9±1.1*
HDL cholesterol (mmol/L)	1.6±0.5	1.4±0.4†	1.3±0.3*†
LDL cholesterol (mmol/L)	3.4±0.7	3.5±0.8	2.9±1.0*
Triacylglycerols (mmol/L)	1.0 (0.8-1.4)	1.3 (1.0-2.0) †	1.6 (1.2-2.2) *†
Plasma creatinine (µmol/L)	75 (63-88)	74 (61-94)	73 (63-87)
Estimated GFR (ml/min)	91.7±18.8	89.1±15.1	91.7±18.8
UACR (mg/g)	0.2 (0-2.5)	0.3 (0-4.9)	0.6 (0.3-2.4)
hsCRP (mg/L)	0.5 (0.1-1.5)	0.8 (0.3-2.7)	2.3 (1.3-6.0)*†
Vascular variables			
Central systolic BP (mmHg)	118.3±23.5	128.4±25.1†	130.5±14.5†
Central PP (mmHg)	39.6±7.8	49.8±12.8†	49.1±13.1†
Mean BP (mmHg)	101.5±12.4	109.3±18.3†	103.6±10.8*
Aortic PWV (m/s)	7.9±1.6	8.6±1.4†	10.1±2.3*†
Baseline brachial artery diameter (mm)	3.77±0.88	4.15±0.96†	4.33±0.77†
Maximum brachial artery diameter after reactive hyperaemia (mm)	4.01±0.97	4.36±0.87†	4.48±0.78†
Peak shear stress (dyne/cm^2)	5.6±2.8	4.7±2.2†	4.5±2.0†
FMD (%)	6.68±3.97	5.16±2.96†	3.51±2.07*†
Maximum brachial artery diameter after GTN (mm)	4.06±1.01	4.44±0.86†	4.58±0.78†
Response to GTN (%)	7.93±4.09	6.87±3.78†	6.11±2.92†

BP: blood pressure; HDL: high density lipoprotein; LDL: low density lipoprotein. UACR: urinary albumin to creatinine ratio; hsCRP: high sensitivity C-reactive protein; FMD: flow-mediated dilation, GTN: glyceryl trinitrate, PWV: pulse-wave velocity. Values are mean±standard deviation or median value (25-75% interquartile range), if not otherwise specified. * $p<0.05$ vs hypertensive patients without diabetes; † $p<0.05$ vs healthy subjects.

Table 2. Current CV drugs treatment at the time of the examination in hypertensive patients without or with type 2 diabetes mellitus

Drugs (n, %)	**Hypertensive patients without type 2 diabetes mellitus (n=175)**	**Hypertensive patients with type 2 diabetes mellitus (n=84)**
BP-lowering drugs	88 (50%)	50 (59%)
ACE-inhibitors	34 (19%)	21 (25%)
Angiotensin receptor blockers	39 (22%)	26 (31%)
β-blockers	9 (5%)	6 (7%)
Calcium channel blockers	30 (17%)	23 (27%)
Diuretics	32 (18%)	20 (24%)
Others	2 (1%)	3 (4%)
Glucose-lowering drugs	-	66 (79%)
Metformin	-	60 (71%)
Sulphonylureas	-	25 (30%)
Thiazolidinediones	-	6 (7%)
Others	-	2 (2%)
Other drugs		
Statins	11 (6%)	29 (35%)*
Fibrates	-	2 (2%)
Antiplatelet drugs	2 (1%)	20 (24%)*
Nitrates	-	1 (1%)

* $p<0.05$ vs hypertensive patients without type 2 diabetes mellitus.

Table 3. Univariate correlations between PWV and clinical and vascular variables in the overall population and in different subgroups

Variables	Overall population (341)		Hypertensive patients without type 2 diabetes mellitus (175)		Hypertensive patients with type 2 diabetes mellitus (84)		Healthy subjects (82)	
	r	p	r	p	r	p	r	p
Clinical variables								
Age	0.217	<0.001	0.108	0.157	0.249	0.028	0.195	0.092
Duration of hypertension	-	-	0.057	0.864	0.197	0.175	-	-
Duration of diabetes	-	-	-	-	-0.056	0.676	-	-
Brachial systolic BP	0.405	<0.001	0.238	0.002	0.454	<0.001	0.513	<0.001
Brachial diastolic BP	0.196	<0.001	0.140	0.067	0.187	0.103	0.476	<0.001
PP	0.352	<0.001	0.198	0.009	0.326	0.004	0.231	0.038
Heart rate	0.261	<0.001	0.153	0.047	0.134	0.246	0.307	0.006
Body mass index	0.286	<0.001	0.057	0.459	0.299	0.008	0.061	0.592
Waist circumference	0.242	<0.001	-0.058	0.566	0.186	0.145	-0.031	0.862
Blood glucose	0.426	<0.001	0.065	0.401	0.267	0.020	0.131	0.282
HbA_1c	0.310	<0.001	0.240	0.268	-0.068	0.590	-0.282	0.373
Total cholesterol	-0.156	0.006	0.057	0.465	-0.116	0.319	-0.102	0.409
HDL cholesterol	-0.115	0.041	-0.020	0.793	-0.017	0.885	-0.112	0.379
LDL cholesterol	-0.135	0.021	0.081	0.308	-0.128	0.291	0.005	0.965
Triacylglycerols	0.224	<0.001	0.144	0.062	0.183	0.113	0.072	0.560
Serum creatinine	0.013	0.818	0.018	0.815	0.015	0.897	0.184	0.155
Creatinine clearance	0.077	0.183	0.042	0.589	-0.037	0.750	0.110	0.396
UACR	0.103	0.180	0.061	0.570	0.274	0.026	-0.077	0.776
hsPCR	0.297	0.006	-0.132	0.651	0.023	0.863	-0.260	0.439
Vascular variables								
Central systolic BP	0.355	<0.001	0.255	<0.001	0.433	<0.001	0.453	<0.001
Central diastolic BP	0.132	0.016	0.028	0.712	0.247	0.030	0.399	<0.001
Central PP	0.263	<0.001	0.119	0.242	0.230	0.049	0.033	0.817
Mean BP	0.171	0.002	0.057	0.453	0.375	<0.001	0.359	0.001
Brachial artery diameter	0.198	<0.001	0.091	0.236	0.071	0.540	0.189	0.100
Hyperemic shear stress	-0.267	<0.001	-0.206	0.016	-0.037	0.761	-0.432	<0.001
FMD	-0.272	<0.001	-0.088	0.248	-0.456	<0.001	0.008	0.946
Response to GTN	-0.041	0.466	0.028	0.716	-0.093	0.427	0.197	0.089

BP: blood pressure; HDL: high density lipoprotein; LDL: low density lipoprotein. UACR: urinary albumin to creatinine ratio; hsCRP: high sensitivity C-reactive protein; FMD: flow-mediated dilation, PWV: pulse-wave velocity.

Table 4. Multiple regression analysis in the whole study population, considering PWV as dependent variable and FMD as independent variable.

	r^2	p	β	95%CI
FMD (unadjusted)	**0.061**	**<0.001**	**-0.135**	**-0.193 to -0.078**
Model 1				
FMD	**0.047**	**<0.001**	**-0.132**	**-0.197 to -0.066**
brachial artery diameter	0.038	0.984	0.003	-0.314 to 0.320
hyperemic shear stress	0.022	0.009	-0.141	-0.245 to -0.036
Model 2				
FMD	**0.019**	**0.010**	**-0.092**	**-0.161 to -0.022**
brachial artery diameter	0.001	0.394	-0.169	-0.557 to 0.219
hyperemic shear stress	0.007	0.134	-0.084	-0.192 to -0.025
age	0.033	0.008	0.041	0.010 to 0.071
sex	0.034	0.020	0.680	0.110 to 1.249
BMI	0.140	<0.001	0.113	0.069 to 0.158
Heart rate	0.042	<0.001	0.031	0.013 to 0.049
Total cholesterol	<0.001	0.762	-0.0009	-0.007 to 0.0050
HDL cholesterol	<0.001	0.966	0.0004	-0.016 to 0.017
Triglycerides	<0.001	0.979	-0.00003	-0.002 to 0.002
Mean BP	0.025	0.007	0.018	0.005 to 0.031
hsPCR	0.002	0.311	0.024	-0.024 to 0.072
Model 3				
FMD	**0.009**	**0.074**	**-0.064**	**-0.134 to 0.006**
brachial artery diameter	<0.001	0.348	-0.182	-0.563 to 0.198
hyperemic shear stress	0.008	0.096	-0.084	-0.192 to -0.025
age	0.033	0.010	0.039	0.010 to 0.069
sex	0.034	0.012	0.717	0.159 to 1.276
BMI	0.140	<0.001	0.091	0.045 to 0.136
Heart rate	0.042	0.019	0.022	0.004 to 0.040
Total cholesterol	<0.001	0.920	0.0003	-0.005 to 0.006
HDL cholesterol	0.002	0.645	0.004	-0.013 to 0.021
Triglycerides	<0.001	0.856	-0.0002	-0.002 to 0.002
Mean BP	0.028	0.003	0.019	0.006 to 0.032
hsPCR	<0.001	0.426	0.019	-0.029 to 0.068
blood fasting glucose	0.036	0.001	0.010	0.004 to 0.016

CI: confidence interval; BP: blood pressure; HDL: high density lipoprotein; LDL: low density lipoprotein. hsCRP: high sensitivity C-reactive protein; FMD: flow-mediated dilation, PWV: pulse-wave velocity.

Table 5. Subgroup analysis in hypertensive patients without diabetes: Behavior of PWV and FMD in the presence or absence of different conditions and treatments

Condition / treatment	PWV if present (m/s)	PWV if absent (m/s)	FMD if present (%)	FMD if absent (%)
Obesity	8.95±1.70*	8.42±1.28	4.73±2.95	5.25±2.97
Metabolic syndrome	8.65±1.41	8.56±1.44	5.12±3.08	5.17±2.88
BP-lowering treatment	8.38±1.35*	8.73±1.42	4.96±2.88	5.36±3.09
RAS-blockers use	8.00±1.35	8.53±1.36	4.73±2.29	4.80±2.70
Statins use	8.54±1.41	8.56±1.39	5.61±3.71	5.13±2.90

* $p<0.05$, analysis of variance with age and mean BP as covariates. FMD: flow-mediated dilation, PWV: pulse-wave velocity, RAS: renin-angiotensin system blockers.

Figure 9: Dot plots showing aortic pulse wave velocity (PWV, figure 9a) and brachial artery flow mediated dilation (FMD, figure 9b) in hypertensive patients with (black circles) and without (grey circles) type 2 diabetes mellitus, and in healthy subjects (white circles).

a) b)

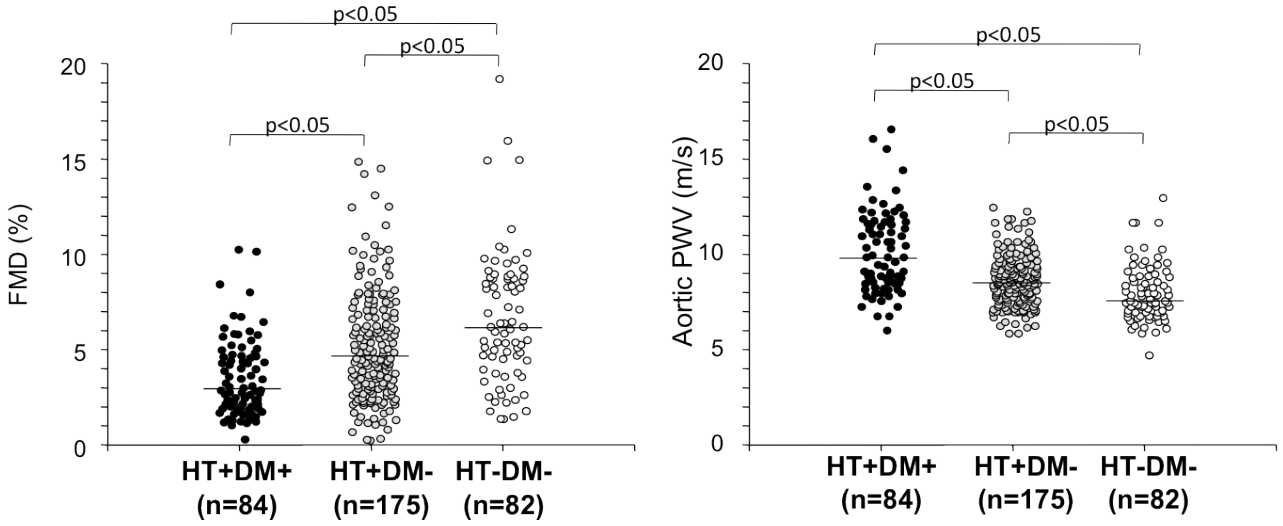
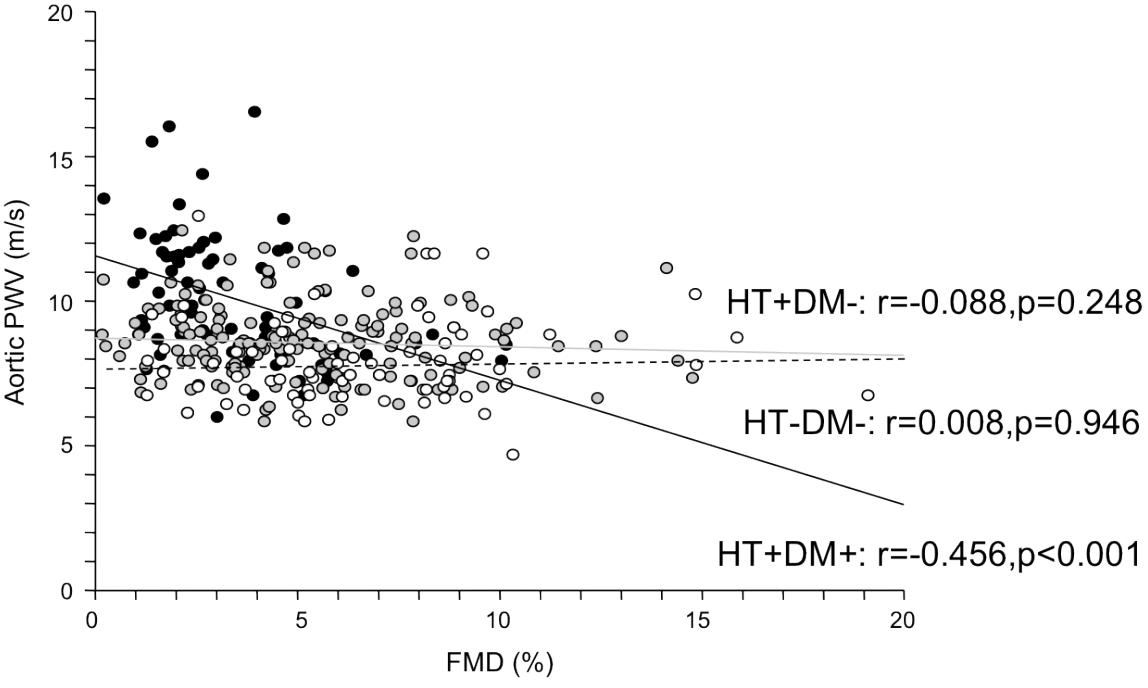


Figure 10: Scatterplot and regression lines of the correlation between aortic PWV (PWV) and brachial artery flow mediated dilation (FMD) in hypertensive patients with (HT+DM+, black circles and line) and without (HT+DM-, grey circles, grey line) type 2 diabetes mellitus, and in healthy subjects (HT-DM-, white circles, dotted line).



Chapter 6. Is vascular aging similar in different districts? Is it related to cardio-renal damage ?

The aim of this study was to investigate whether vascular aging is similar in the carotid and arterial district and whether CV risk factors might differentially influence carotid and aortic stiffness on top of hypertension. Furthermore, the relationship between carotid and aortic stiffness and cardio-renal damage in hypertensive patients has been explored. This research has been accepted as an oral presentation at the 2011 High Blood Pressure Research AHA Scientific Sessions and is now under revision for publication as an article. Data collected for this research were also used for definition of Reference values for C-IMT measured by echotracking (Engelen et al., 2012).

6.1 Experimental protocol

Study population – 314 essential hypertensive patients consecutively referring to the Hypertension Outpatient Clinic of the Department of Clinical and Experimental Medicine of the University Hospital of Pisa, Italy, were enrolled. Inclusion criteria were: diagnosis of essential hypertension according to current guidelines (Mancia et al., 2007) or current treatment with antihypertensive drugs. Exclusion criteria were: end-stage renal disease and any other major co-morbidities. A group of 110 age- and sex- matched healthy subjects were also recruited as controls. The study was approved by the local Ethical Committee and all patients provided written informed consent before entering the study.

Experimental session – Subjects were requested to refer to the Hypertension Outpatient Clinic in the morning after an overnight fasting for collection of medical history, anthropometric parameters (body weight, height, and waist circumference) as well as blood and urine samples, including serum blood glucose, total and HDL-cholesterol, triglycerides, serum creatinine, urinary albumin-to-

creatinine ratio (UACR) on a spot urine sample, assessed by standard techniques. Measurements of arterial stiffness were then performed in a quiet air-conditioned room (22 to 24°C) according to current guidelines (Laurent 2006). Patients under pharmacological treatment were asked to assume their medications as usual on the day of the experimental session. Brachial BP was measured with the patients resting in a supine position for at least 10 minutes under quiet environmental conditions. BP measurement was repeated three times at 2-minute intervals by a trained physician by using an automatic oscillometric device (OMRON-705IT). Average BP was then calculated on the last two measurements. Arterial tonometry was obtaining carotid-femoral PWV (PWV) and central BP values from radial artery waveform through validated transfer function. Carotid systolic BP and PP were then obtained from carotid pressure waveform, using central diastolic and mean BP for calibration. Common carotid artery scans were obtained for the calculation of carotid diameter, carotid intima-media thickness, carotid distensibility parameters.

Statistical analysis - All statistical analyses were performed using NCSS 2004 (NCSS; Kaysville, Utah, USA). For normally distributed data, results are expressed as mean ± standard deviation (SD), whereas median value and 25%-75% interquartile range is used for not normally distributed data. Differences in means between hypertensive and normotensive were analyzed using ANOVA for normally distributed variables, or Kruskal-Wallis Z Test for not normally distributed variables; categorical variables were analyzed by χ^2 test. Analysis of variance was used to compare aortic PWV and CS in the presence or absence of classical CV risk factors; for aortic PWV, mean BP was considered as covariate. Logistic regression analysis was performed in order to identify risk factors associated with an increased PWV and CS.

6.2 Results

Clinical and vascular parameters in healthy subjects and hypertensive patients – Clinical characteristics of the study population are listed in **Table 6**. The two groups were similar for age, gender and percentage of smokers, as well as for renal function and albumin excretion. Among other clinical characteristics, hypertensive patients showed higher brachial and central BP values, as well as higher BMI, blood glucose, and triglycerides and lower HDL-cholesterol. Going to vascular parameters **(Table 7, Figure 11),** hypertensive patients had higher PWV than healthy subjects, while timing of the reflected wave and Aix where not different. Carotid diameter and PP were greater in the hypertensive group, while stroke change in diameter was similar. As a consequence, CC and CS were significantly greater in hypertensive patients as compared to healthy subjects **(Table 7, Figure 11)**. Also common carotid C-IMT was significantly increased in the patients' group.

Within the hypertensive population, PWV and CS were considered increased when they trespassed the 90^{th} percentile of the distribution obtained in healthy subjects, corresponding respectively to 9.3 m/s and 7.6 m/s. Using this arbitrary cutoff, we found that 42.1% of the hypertensive population had increased PWV and 26.6% had increased CS. For PWV we also considered another classification, defining increased PWV as above the predicted value considering the reference values according to age and BP categories, as recently published by the Reference Values for Arterial Stiffness Collaboration (2010); according to this classification, increased PWV was found in 27.9% of hypertensive patients.

CV risk factors and aortic stiffness in the hypertensive population - Hypertensive patients with previous CV events, family history of premature CV disease, diabetes mellitus, obesity, hypertriglyceridemia, metabolic syndrome, and chronic kidney disease, had higher PWV as compared with patients who did not had these risk factors (**Table 8**). Hypertensive patients on

antihypertensive treatment showed higher PWV as compared to untreated patients (9.6±2.2 vs 8.8±2.1, $p<0.05$), but the difference disappeared upon adjustment for age and mean BP.

Multiple logistic regression, including all the above-mentioned factors, and adjusted for age, mean BP, gender and number of antihypertensive drugs, demonstrated that diabetes mellitus (OR 3.8, CL95% 1.6-9.1), obesity (OR 2.7, CL95% 1.1-6.4) and chronic kidney disease (OR 5.2, CL95% 1.3-21.7) are independently associated to an increased PWV, defined as greater than the 90^{th} percentile of the distribution obtained in healthy subjects. The full model explained 29.0% of the variance of PWV. The same analysis repeated considering increased PWV as above the predicted value considering the reference values according to age and BP categories (2010), showed similar results, with diabetes mellitus (OR 5.3, CL95% 2.2-12.8), chronic kidney disease (OR 3.3, CL95% 1.0-10.7), and obesity (OR 2.8, CL95% 1.3-6.4) being independently associated with increased PWV.

Cardiovascular risk factors and carotid stiffness in the hypertensive population - Hypertensive patients with diabetes mellitus, metabolic syndrome, and chronic renal failure had higher CS as compared with patients who did not had these risk factors (**Table 8**). Moreover, CS was significantly higher in females as compared to males. Multiple logistic regression, including the above-mentioned factors, and adjusted for age, mean BP, and mean carotid diameter, gender and number of antihypertensive drugs as discrete variables, demonstrated that only diabetes mellitus is independently associated to an increased CS (OR 2.4, CL95% 1.1-5.5). The full model explained 27.4% of the variance of PWV.

Relationship with target organ damage in the hypertensive population - Population was divided in four groups according to the presence or not of increased carotid and/or aortic stiffness, in order to establish if the presence of both aortic and carotid stiffness was associated to a worse organ damage, evaluated at the vascular level as C-IMT, at the renal level as the presence of eGFR<60

and/or UACR>21, and at the cardiac level as LVMI. Both the presence of isolated increased PWV or CS was associated to an increased LVMI. Hypertensive patients with both increased PWV and CS showed a further increased LVMI **(Figure 12).** Conversely, only the presence of increased PWV, but not of increased CS, was associated to a greater C-IMT and a lower eGFR (**Figure 12**). UACR was not significantly different among the four groups.

6.3 Discussion

The main finding of this study is that in a population of hypertensive individuals recruited in a Hypertension Outpatient Clinic, type 2 diabetes mellitus, obesity and chronic kidney disease are able to further worsen carotid-femoral PWV, even independent of each other. On the other side, carotid stiffness, which is also increased in hypertensive individuals in comparison to the normotensive ones, is further increased only in the presence of diabetes mellitus. These findings suggest that in the patients with high CV risk for the presence of multiple risk factors, PWV might be a better integrated biomarker of vascular damage than carotid stiffness. Furthermore, arterial stiffness is related to cardiac organ damage, with carotid and aortic stiffness having an additive effect on left ventricular mass, suggesting that multidistrict arterial stiffness might correspond to an increased hemodynamic load to the heart, and possibly to an increased risk of CV events.

The measurement of aortic stiffness as PWV by arterial tonometry is generally accepted as the most simple, non-invasive, robust, and reproducible method to determine arterial stiffness (Laurent et al., 2006). However, carotid-femoral PWV is not a direct measurement, since it is based on the acceptance of a propagative model of the arterial system. On the other hand, the measurement of local carotid stiffness may also provide important prognostic information, as above discussed.

Our study confirmed that aging and BP are the main determinant of stiffness both in the carotid artery and in the aorta. Paini and coauthors suggest that carotid and arterial stiffness are strictly correlated in the healthy population, while the correlation becomes weaker as soon as the number of

CV risk factors increases (Paini et al., 2006). Therefore, although carotid-femoral PWV and carotid stiffness provide similar information on the aging impact on the stiffness of large arteries in healthy subjects, this is not the same for patients with CV risk factors, in whom the aorta seems to stiffen more than the carotid artery (Paini et al., 2006).

Our data suggest that type 2 diabetes is able to negatively influence stiffness either in the carotid artery or in the aorta: a diabetic hypertensive patient has a 3.8-fold higher probability of having increased PWV and 2.4-fold higher probability of having increased CS, independent of confounders. Thus type 2 diabetes mellitus resulted to be the only risk factor able to affect carotid stiffness in the hypertensive population, even though with a smaller effect than on aorta. Several causative mechanisms for this phenomenon can be hypothesized. The strong association between hypertension and diabetes mellitus can be explained, in part, by the presence of a maladaptive interaction of factors, such as insulin resistance, chronic activation of the renin–angiotensin–aldosterone system, the sympathetic nervous system, and abnormalities of innate immunity, inflammation, and oxidative stress, together with the epidemic of obesity and sedentary lifestyle, and the aging of the population worldwide (Sowers, 2013): all these factors can act synergistically to negatively impact arterial stiffness at multiple levels. Vascular damage that can be found in the diabetic patients is characterized by a broad extension, ranging from large vessels, to arterioles to the microcirculation (Sowers, 2013). Alterations in small arteries and microcirculation have been also recently found to be associated with an increased incidence of diabetes (Muris et al., 2012), suggesting that they are an early phenomenon, related also to pre-diabetic states, and that they can possibly favor diabetes development. In this framework, it is not surprising that type 2 diabetes mellitus is associated with increased stiffness of a musculo-elastic artery such as the carotid artery. Future studies should investigate the prognostic role of carotid stiffness in type 2 diabetic patients.

An interesting finding if this study is the independent worsening effect of obesity, diabetes and chronic kidney disease on PWV. Indeed, it is well established that all these factors are highly interrelated. Obesity, impaired glucose tolerance, and diabetes mellitus are associated with a

substantially increased prevalence of hypertension, CV disease, and chronic renal disease (Sowers, 2013). The prevalence of hypertension in patients who have type 2 diabetes mellitus is up to 3 times higher than in patients without diabetes mellitus, while the prevalence of type 2 diabetes in the hypertensive population in Italy is at least two-fold as compared to the general population (Lonati et al., 2008). Further, the coexistence of hypertension in diabetic patients greatly enhances their likelihood of developing CV disease and chronic kidney disease, as well as presence of hypertension in diabetic patients is a risk factor for CV disease (Stamler et al., 1993;Chen et al., 2011). These findings suggest that, when studying patients at high CV risk for the presence of multiple risk factors, PWV might be a better integrated biomarker of vascular damage than carotid stiffness.

Surprisingly, in our population chronic kidney disease was not independently associated with carotid stiffness. This was demonstrated even considering separately estimated GFR and microalbuminuria. Carotid mechanical and structural properties have been extensively studied in cohorts with chronic kidney disease, showing also a predictive value for CV events and renal disease progression at follow up (Blacher et al., 1998;Briet et al., 2011). However those results were obtained on cohort with end-stage renal disease or with eGFR<60 ml/min and they cannot be translated to population with a broader range of eGFR. Furthermore, in our cohort chronic kidney disease was conceivably due only to diabetic and hypertensive nephropathy, since there were no patients with primitive tubulo-interstitial or glomerular renal disease, at variance with the abovementioned studies. Future studies should address the role of arterial stiffness in nephropathies of different etiology.

A novel finding of this study concerns the relationship between cardiac organ damage, evaluated as left ventricular mass index, and arterial stiffness. In our cohort, aortic and carotid stiffness is associated with higher left ventricular mass, and the presence of both has an even greater negative effect. On the basis of this result, it is possible to hypothesize that large artery stiffness in multiple districts might correspond to an increased hemodynamic load to the heart in comparison to stiffness

of one arterial district. Multidistrict arteriosclerotic burden might convey a greater prognostic significance than the single-district one, in parallel to what has been demonstrated for multidistrict atherosclerosis. The relationship found with left ventricular mass, which is a powerful predictor of CV events in hypertensive patients (Verdecchia et al., 2001), confirms the prognostic relevance of arterial stiffness in this population.

Table 6. Clinical characteristics of the study population

Parameter	**Hypertensive patients (n=314)**	**Normotensive subjects (n=110)**
Age (years)	57.9±16.1	56.4±10.4
Male sex (n)	191 (60.8%)	71 (64.5%)
Smokers (n)	47 (15.0%)	24 (21.8%)
Brachial systolic BP (mmHg)	143.5±16.3	121.7±10.7*
Brachial diastolic BP (mmHg)	82.0±10.0	70.7±8.7*
Brachial PP (mmHg)	61.5±14.3	51.0±8.8*
Heart rate (bpm)	68.5±11.8	66.0±8.8
Body mass index (kg/m^2)	27.8 (25.5-31.2)	23.4 (22.1-25.7)*
Waist circumference (cm)	103.3±13.0	94.0±11.2
Blood glucose (mmol/L)	98.0 (86.0-120.5)	91 (85-97)*
Total cholesterol (mmol/L)	205.2±36.5	218.3±34.7
HDL cholesterol (mmol/L)	52.2±16.8	67.2±16.3*
LDL cholesterol (mmol/L)	123.6±34.6	131.5±31.2
Triglycerides (mmol/L)	128.0 (93.0-178.8)	91 (69.3-111.5)*
Plasma creatinine (μmol/L)	0.93±0.25	0.80±0.29
Estimated GFR (ml/min $1.73m^2$)	79.1±17.7	83.1±19.4
UACR (mg/g)	4.0 (0.2-12)	2.8 (0.5-4.9)

BP: blood pressure; PP: pulse pressure; HDL: high-density lipoprotein; LDL: low-density lipoprotein. GFR: glomerular filtration rate; UACR: urinary albumin-creatinine ratio. *: $p<0.05$.

Table 7. Vascular parameters in the study population

Parameter	**Hypertensive patients (n=314)**	**Normotensive subjects (n=110)**
Central systolic BP (mmHg)	131.5±16.9	113.8±10.4*
Central diastolic BP (mmHg)	83.0±10.5	72.7±8.6*
Central PP (mmHg)	48.5±14.0	41.1±8.6*
Carotid PP (mmHg)	52.7±14.7	45.5±9.8*
Mean BP (mmHg)	104.0±11.7	90.7±8.5*
Timing of reflected wave (ms)	139.1±17.0	134.8±15.3
Aortic PWV (m/s)	9.36±2.13	7.37±1.76*
AIx (%)	27.8±11.0	31.0±12.8
Aix@75 (%)	25.0±11.9	27.1±12.8
C-IMT (mm)	0.74±0.16	0.68±0.12 *
Mean diameter (mm)	7.48±1.13	6.86±1.14*
Diastolic diameter (mm)	7.21±1.12	6.57±1.45*
ΔD (mm)	0.53±0.18	0.52±0.14
Distensibility Coefficient (kPa^{-1})	23.7±9.3	28.1±7.7*
Carotid Stiffness (m/s)	6.91±1.34	6.19±1.13*

BP: blood pressure; PP: pulse pressure; PWV: pulse wave velocity; AIx: augmentation index; AIx@75: augmentation index normalized at 75 bpm; C-IMT: carotid intima-media thickness; ΔD: carotid distension. *: $p<0.05$.

Table 8. Prevalence of classical risk factors among the hypertensive population and behavior of aortic and carotid stiffness in their presence or absence

Risk factors	**Prevalence (%)**	**PWV if absent (m/s)**	**PWV if present (m/s)**	**CS if absent (m/s)**	**CS if present (m/s)**
Male sex	60.8	9.3±2.2	9.5±2.2	6.7±1.3	7.2±1.5*
Type 2 diabetes mellitus	22.6	8.9±2.1	10.7±2.2*	6.8±1.4	7.4±1.3*
Obesity	33.1	9.1±2.1	9.8±2.4*	6.9±1.5	7.0±1.2
Current smoking	15.0	9.4±2.2	9.3±2.2	6.9±1.4	6.8±1.4
Previous CV events	7.3	9.2±2.1	10.7±3.0*	6.9±1.4	7.2±1.8
Family history of premature CV disease	24.2	9.2±2.0	9.8±2.2*	6.9±1.4	7.0±1.3
Hypercholesterolemia	76.7	8.9±2.1	9.5±2.2	6.6±1.3	7.0±1.4
Low HDL	21.4	9.3±2.1	9.3±2.2	7.0±1.4	7.0±1.4
Hypertriglyceridemia	34.2	9.2±2.1	9.6±2.1*	6.9±1.3	7.1±1.4
Metabolic syndrome	54.8	8.7±1.8	9.8±2.3*	6.8±1.4	7.2±1.3*
Chronic kidney disease	11.7	9.2±2.0	10.9±2.5*	6.9±1.4	7.2±1.5*

PWV: pulse wave velocity; CS: carotid stiffness; CV: cardiovascular: HDL: high-density lipoprotein. *: $p<0.05$.

Figure 11. Dot plots showing aortic pulse wave velocity (PWV, on the left) and carotid stiffness (on the right) in healthy volunteers (empty circles) and hypertensive patients (grey circles). The dotted line corresponds to the 90th percentile calculated the healthy population.

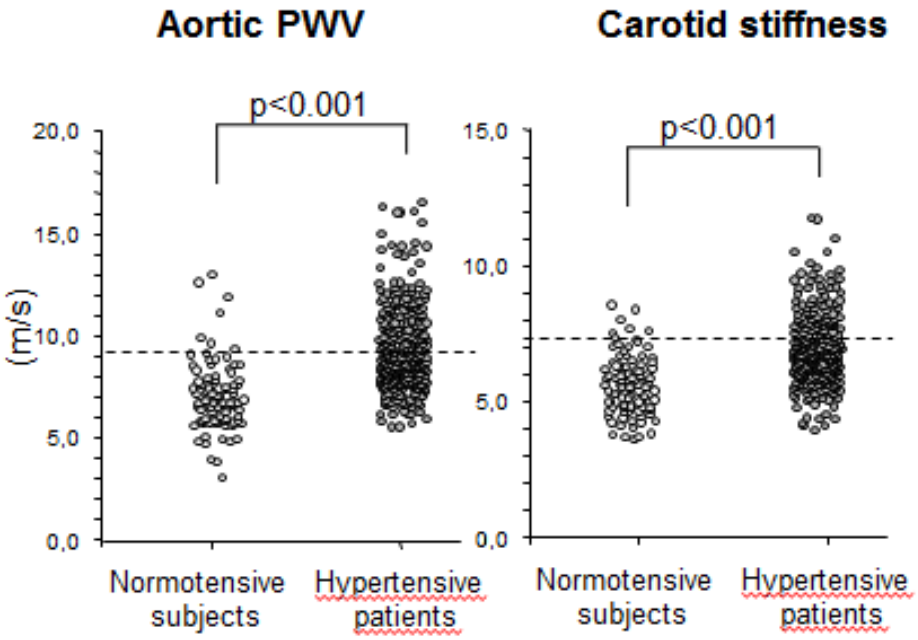
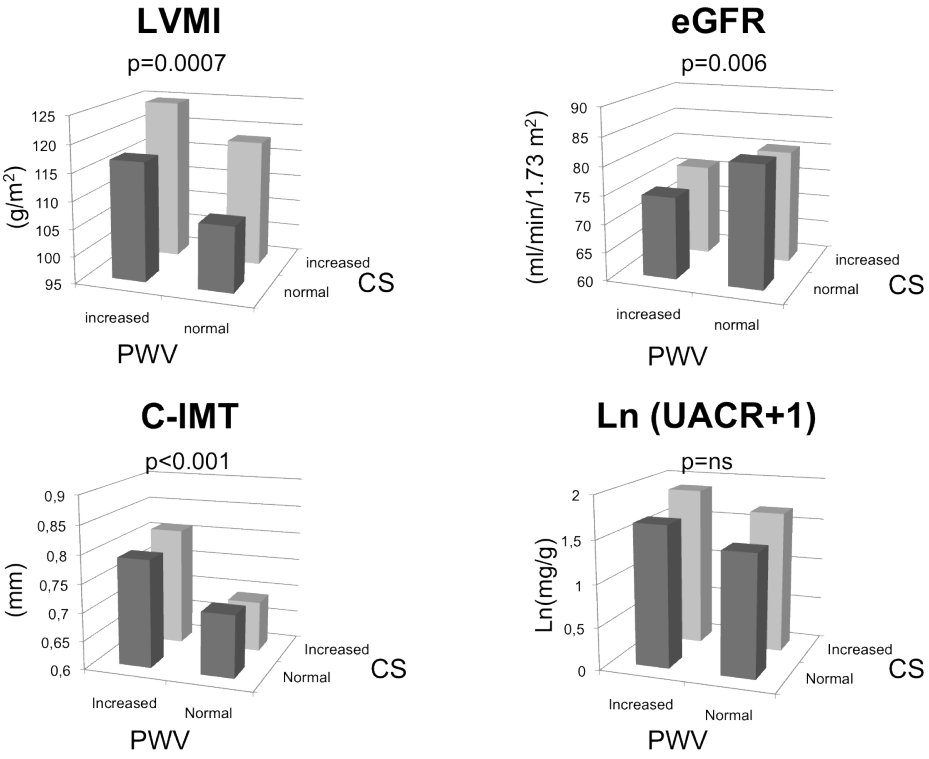


Figure 12. Histograms showing target organ damage in the hypertensive population according to the presence of increased or normal pulse wave velocity (PWV) and carotid stiffness (CS).



LVMI: left ventricular mass index; eGFR: estimated glomerular filtration rate; C-IMT: carotid intima-media thickness; UACR: urinary albumin-creatinin ratio.

Chapter 7. How to evaluate renal vascular damage?

The aim of this study was to evaluate RI in basal conditions and under pharmacological vasodilatory stimulus in newly diagnosed, treatment-naive type 2 diabetic patients with normal urinary albumin excretion and glomerular filtration rate, comparing them to a group of essential hypertensive patients, in order to investigate whether a reduced vasodilating capacity could be a specific feature of diabetes. As a secondary aim, this study investigated the possible association of DRIN with established indexes of systemic vascular damage, such as endothelial function and arterial stiffness, and increased oxidative stress. This research was published in 2011(Bruno et al., 2011) and was dedicated a commentary (Jerums and MacIsaac, 2011).

7.1 Experimental protocol

Study population - In the present study 108 subjects have been included: 32 newly diagnosed (< 3 months), treatment-naive type 2 diabetic patients (T2DM), defined according to the American Diabetes Association criteria (2008a); 49 never treated essential hypertensive patients (EH); and 27 healthy subjects, serving as controls (C). T2DM and EH were recruited consecutively from January to June 2009 in the Metabolism and Hypertension outpatient clinic at the University Hospital, Pisa, while controls were enrolled on a voluntary basis. Inclusion criteria were: age between 40 and 70 years; written informed consent; diagnosis of essential hypertension (for the EH group) or type 2 diabetes (for the T2DM group) within the previous 6 months, according to current guidelines (Mancia et al., 2007). Criteria of exclusion were: previous or current chronic treatment with antihypertensive or antidiabetic medication; reduced renal function (estimated GFR<60 ml/min/1.73 m^2); micro- or macroalbuminuria in any measurement in the previous six months preceding the study; clinical or laboratory signs of inflammatory diseases or other major comorbidities; history of CV disease; secondary hypertension. Type 2 diabetic patients were all free

of diabetic retinopathy. In accordance with institutional guidelines, the protocol was approved by the local ethical committee and all patients gave a written consent.

Experimental session - Patients were asked not to eat, and to avoid caffeine-containing beverages, alcohol, strenuous exercise, and smoking for the 12 hours prior the experiment. Measurements were performed in the morning with subjects supine, at rest, in a quiet air-conditioned room (22-24°C). Blood was collected from all patients to measure, by standard techniques, fasting glucose and HbA_{1c}, serum creatinine, total and HDL cholesterol, and triglycerides. GFR was estimated using the CKD-EPI formula (Levey et al., 2009). Urinary albumin excretion was evaluated as urine albumin to creatinine ratio (UACR) in spot morning urine samples collected in three different days during the month preceding the study. Plasma nitrotyrosine was analyzed by using the Nitrotyrosine ELISA Test Kit (Cell Sciences, Canton, MA, USA) according to the manufacturer's instructions (sensitivity=2 nmol/l). Clinic BP (mean of at least two measurements in 5 minutes by automatic sphygmomanometer – OMRON M4) and heart rate were measured at the beginning and at the end of the study. The following imaging biomarkers were performed: baseline and dynamic renal resistive index (RI and DRIN respectively); endothelium-dependent and independent vasodilation in the brachial artery (FMD and response to GTN respectively); carotid-femoral PWV, central BP and augmentation index. The timeline of the study protocol is illustrated in **Figure 13**.

Statistical analysis - Statistical analysis was performed using NCSS 2004 (NCSS, Kaysville, Utah) The results were expressed as mean ±SD. Differences among groups were analyzed using ANOVA and Fisher LSD post-hoc analysis for normally distributed variables, or Kruskal-Wallis Z Test for not normally distributed variables; categorical variables were analyzed by χ^2 test. Multiple linear regression was applied to build a model to identify the determinants of DRIN and RI. Among clinical factors, age, creatinine, fasting glucose, and PP were included, then adding those vascular parameters that resulted to be significant in the univariate analysis. For both DRIN and RI two

models were performed: Model 1, including brachial PP, and Model 2, including aortic PP. Not normally distributed variables were log-transformed for this analysis. A $p<0.05$ was considered significant.

7.2 Results

Clinical parameters of the study groups are summarized in **Table 9**. T2DM patients had significantly higher BMI as compared to the other groups. UACR, even within the normal range, was higher in T2DM as compared to C and EH, while serum creatinine, uric acid and estimated GFR were not significantly different among all groups **(Table 9)**. HDL-cholesterol and triglycerides were respectively lower and higher in EH and T2DM compared to C. Nor systolic neither diastolic BP or heart rate were significantly modified by GTN administration in the overall population (-0.6±3.0%, -0.8±5.8% and -0.9±7.9% respectively). RI values were significantly higher in T2DM as compared to C and EH (0.65±0.06, 0.59±0.05 and 0.58±0.05 respectively), while EH showed values superimposable to C (**Figure 14**). Only a minority of subjects showed RI > 0.70 (8 out of 108). DRIN was significantly reduced in both groups of patients (T2DM 7.1±6.1%, EH 9.0±5.2%) as compared to C (11.1±6.9%); noteworthy, T2DM showed a reduced DRIN also compared to EH (**Figure 14**). Systemic vascular parameters in the three study groups are summarized in **Table 10**. FMD was reduced in EH and in T2DM patients as compared with C, while the endothelium-independent brachial vasodilation was not significantly different among the three groups. Reactive hyperemia was reduced in T2DM as compared to C and EH. Aortic PWV was higher in EH and in T2DM patients than in C, whereas timing of the reflected wave was significantly shortened. AIx did not significantly differ within the three groups. To test whether these differences might be related to an increased degree of oxidative stress in T2DM, we measured circulating levels of nitrotyrosine, which were significantly increased in T2DM and EH (0.56±0.10 and 0.43±0.06 μmol/L respectively) as compared to C (0.39±0.12 μmol/L), and also significantly higher in T2DM than in

EH ($p<0.05$). Either RI and DRIN were not affected by smoking status and gender (p=ns for both). A univariate correlation analysis (**Table 11**) and a multiple regression analysis (**Table 12**) were then performed in order to identify the independent predictors of DRIN and RI. In the univariate analysis on the overall population, DRIN was significantly correlated to both clinical (age, fasting glucose, brachial and aortic SBP and PP) and vascular factors (baseline RI, AIx, PWV, brachial response to GTN, reactive hyperemia), as illustrated in **Table 11**. In the multiple regression analysis (**Table 12**). DRIN was independently related to fasting glucose, PWV, and reactive hyperemia in both models 1 and 2, including respectively brachial (full model r^2 =0.41) and aortic PP (r^2 =0.39). RI was significantly correlated to both clinical factors (age, fasting glucose, creatinine, brachial PP, aortic SBP and PP) and vascular factors (timing of the reflected wave, PWV, FMD, reactive hyperemia) **(Table 11).** However, when multiple regression analysis was performed **(Table 12),** RI remained independently related only to fasting glucose, creatinine and aortic PP in Model 2. This relation was less tight in Model 1, including brachial instead of aortic PP.

When univariate analysis was performed in the T2DM group, DRIN resulted to be correlated with aortic PP (r=-0.54, $p<0.01$), aortic SBP (r=-0.40, p=0.003), and HbA_{1c} (r=-0.47, $p<0.01$), while for RI the correlation was present with brachial (r=0.44, p=0.02) and aortic PP (r=0.52. p=0.004) and brachial SBP (r=0.38, p=0.04). DRIN was not related to any of the other parameters considered, including baseline RI and UACR. In the EH group, DRIN correlated with brachial (r=-0.43, p=0.004) and aortic SBP (r=-0.50, $p<0.001$), brachial (r=-0.31, p=0.04) and aortic PP (r=-0.54, $p<0.001$), PWV (r=-0.40, $p<0.01$), timing of the reflected wave (p=0.32, r=0.04), AIx (r=-0.51, $p<0.001$); RI was related to age (r=0.31, p=0.04), brachial (r=0.40, p=0.007) and aortic SBP (r=0.44, p=0.004), aortic PP (r=0.42, p=0.008), timing of the reflected wave (r=0.34, p=0.03).

In the overall population, nitrotyrosine was significantly related both to RI and DRIN **(Table 11)**. However significance was lost for both parameters after inclusion of clinical factors (age, PP, creatinine, fasting glucose) in the multiple regression model.

7.3 Discussion

The present study demonstrates that drug-induced renal vasodilation is impaired in neo-diagnosed, never treated, type 2 diabetic and hypertensive patients without clinically evident nephropathy. Moreover, it suggests that the estimation of the dynamic renal resistive index (DRIN) by Duplex ultrasonography, a relatively simple and low cost technique, is able to identify subtle alterations in intrarenal vasculature even before albumin excretion has trespassed the cutoff for the definition of microalbuminuria. DRIN correlates with parameters of vascular function and arterial stiffness, beyond the effect of classical CV risk factors, suggesting a parallel damage progression in different vascular target districts. In particular, DRIN is related with metabolic and BP control in the diabetic subgroup, and with arterial stiffness and wave reflections in hypertensive individuals, confirming that different mechanisms are involved in the development of renal damage in the early stages of the two diseases.

Clinical markers of renal dysfunction, such as GFR and urine albumin excretion, are extensively used as indicators of renal damage in hypertensive and diabetic patients (Jerums et al., 2009); however, indicators of early impairment of kidney function during the clinical course of chronic nephropathies are required, in particular to distinguish parenchymal from microvascular abnormalities. In this view, duplex evaluation of RI has been recently implemented in order to better detect early renal vascular alterations. A mean renal RI of around 0.60 in subjects without preexisting renal diseases (Keogan et al., 1996) and a cut-off value of 0.70 for the upper threshold of normality (Platt et al., 1991a;b;Platt et al., 1992) have been suggested. An elevated RI was found to correlate with left ventricular hypertrophy and carotid intima-media thickening (Ishimura et al., 1997;Pontremoli et al., 1999;Florczak et al., 2009), suggesting this index as marker of systemic structural organ damage. However in the present study only a small percentage of patients had RI values above the 0.70 cutoff. Moreover, in hypertensive patients RI values were not different compared to normal subjects, suggesting that this duplex parameter does not discriminate initial

renal vascular effects of hypertension, despite the presence of a systemic vascular dysfunction, namely reduced FMD and increased PWV.

The evaluation of renal vasodilation to GTN by DRIN is able not only to confirm the impairment in renal microvasculature in type 2 diabetes, as already shown by RI, but also to detect a difference between hypertensive patients and both type 2 diabetic patients and controls. Therefore, the implementation of RI assessment by DRIN adds discriminating power to resting duplex evaluation of renal vascular resistances. We found no relationship between RI and DRIN in the group of type 2 diabetic patients, suggesting that a high baseline RI does not necessarily imply a reduced renal vasodilatory response, possibly reflecting functional, rather than structural alterations. In patients with overt diabetic nephropathy, a reduced renal vasodilation was already demonstrated using a high dose of GTN, able to influence systemic hemodynamics (Frauchiger et al., 2000). In comparison to that report, our work presents two important elements of novelty: first, it suggests that DRIN could detect early renal impairment, even before the development of microalbuminuria; second, these results were obtained using subpressor doses of GTN, taking as advantages a better tolerability and the avoidance of systemic BP variation that may represent a relevant counfounding factor in interpreting the results.

In contrast to DRIN, brachial artery response to GTN was similar in the three study groups. This apparent discrepancy is at least in part justified by the different characteristics of the two districts. Indeed, it is commonly accepted that renal RI, though measured at the level of the interlobar renal arteries, is an index of downstream renal microvascular impedance (Krumme, 2006); although a comparison between nitrate-induced changes in renal RI and other districts has never been performed, it was demonstrated that in type 2 diabetic patients skin microvascular reactivity (Lim et al., 1999), as well as forearm blood flow changes (McVeigh et al., 1992) to sodium nitroprusside administration, are reduced, whereas brachial artery vasodilation to GTN is preserved (Henry et al., 2004). Thus, it is conceivable that renal RI behavior could be different from that of large arteries and similar to microcirculation.

To elucidate the determinants of DRIN and comparing them to RI, in order to comprehend the clinical significance of this new parameter, a regression analysis was performed in the whole study group. First of all, both parameters are more tightly related to aortic than to brachial BP values, confirming that central BP reflects more accurately loading conditions of target organs (Roman et al., 2007). In the univariate analysis, besides classical CV risk factors, baseline RI was related to indexes of systemic vascular damage. However, this relationship was lost in the multiple regression analysis. The main determinants of RI were, as expected, PP, since RI is an intrinsic parameter of pulsatility, accompanied by serum glucose and creatinine. Thus the link between RI and systemic vascular alterations appears to be mediated by the classical markers of CV and renal disease. At variance to RI, the independent predictors of DRIN were reactive hyperemia and PWV, together with HbA_{1c}. A reduced vascular smooth muscle cells response or the presence of vascular remodeling in the microcirculation, as previously demonstrated in the forearm (McVeigh et al., 1992), could account for the observed reduction of DRIN in hypertensive and, especially, in type 2 diabetic patients. Arterial stiffness could be related to renal vasodilating capacity by two different mechanisms: both alterations could be manifestations of atherosclerosis in different districts (Ohta et al., 2005); otherwise, high PWV might lead to a heavier hemodynamic burden on the renal microcirculation, causing vascular remodeling and consequently reducing the vasodilating capacity (Safar and Lacolley, 2007). The present study reinforces the latter hypothesis, which was conceived referring to heart, applying it to another target organ of vascular diseases such as the kidney.

The mechanisms responsible for the onset of renal damage are conceivably different in the presence of hypertension or type 2 diabetes. PP is a strong predictor of DRIN in both conditions. Moreover, we show here that DRIN is independently related with arterial stiffness and wave reflection in hypertensive patients, suggesting a possible major role of hemodynamic load in determining early renal microvascular alterations in essential hypertension. On the contrary, in type 2 diabetic patients, DRIN is significantly related with HbA_{1c} and systolic BP. These observations support the importance of glucose and BP control in contrasting the development and progression

of diabetic microvascular complications (Zoungas et al., 2009), and suggest that renal vasculature might be compromised even in presence of more subtle glucose metabolism impairment, such as in the pre-diabetic condition, where systemic vascular dysfunction and increased arterial stiffness are already present (Ghiadoni et al., 2008a).

In the present study, performed in normoalbuminuric individuals, both RI and DRIN do not correlate with UACR. Previous studies, including normo-, micro- and macro-albuminuric hypertensive patients, found a significant relationship between resting RI and microalbuminuria (Leoncini et al., 2002;Derchi et al., 2005). Similarly, a study in type 2 diabetic patients showed that RI was associated with UACR only in albuminuric patients (Hamano et al., 2008). This could be related to the fact that increase of both RI and UACR could be the expression of a more advanced phase of renal disease. It has also to be noticed that, though still within the normoalbuminuric range, UACR levels are significantly higher in type 2 diabetic subjects than in the other two groups, and this is clinically relevant since the higher is the albumin excretion, the worse is the CV outcome (Arnlov et al., 2005). Despite that, we found no correlation between UACR and DRIN or RI, neither in the overall population, nor in the diabetic subgroup. This observation suggests, although only at a merely speculative level, that albuminuria and DRIN could be hallmarks of different mechanisms of renal damage, the former being more related to an altered glomerular permeability, while the latter to a more typical vascular damage.

Finally, a plausible common mechanism for impaired systemic and renal vascular function is represented by oxidative stress. In both groups of patients, the present study confirms the presence of such alteration, since nitrotyrosine levels were higher in hypertensive patients, and even more in T2DM, than in controls. Moreover, this systemic marker of oxidative stress correlates with RI, with the impairment of endothelium-dependent vasodilation and with aortic stiffness, although not independently affecting renal vascular parameters in multivariate analysis.

In conclusion, the present study, although limited by the relatively small cohort of patients, supports the role of DRIN, a novel parameter obtained by a widely available, low cost technique, as

an early detector of renal vascular alteration in the presence of type 2 diabetes and hypertension, irrespectively of the underlying involved mechanisms. The use of a vasodilator to unmask renal subclinical alterations is driven by the widespread use of challenges in other vascular districts and organs applied to different techniques, such as FMD for conduit large arteries and stress echocardiography for the myocardium. Further longitudinal studies are required in order to assess the clinical and prognostic value of an impaired drug-induced renal vasodilation; however, the demonstration of an impaired DRIN in patients with recent onset of the two main causes of chronic renal disease, along with the significant correlation of DRIN with other systemic markers of vascular dysfunction, supports its potential use as a clinical tool.

Table 9. Clinical characteristics of the study population.

Variables	Controls (n=27)	EH (n=49)	T2DM (n=32)	p value
Age (years)	51.0±7.1	51.8±8.8	55.3±9.6	0.12
Males (n,%)	15 (56%)	34 (69%)	19 (59%)	0.43
Smokers (n,%)	5 (19%)	11 (22%)	6 (19%)	0.89
Body mass index (Kg/m^2)	26.1±4.1†	27.2±4.3†	31.0±6.7*‡	0.003
Heart rate (bpm)	67.8±11.4	66.6±10.5	72.0±12.9	0.12
Fasting glucose (mmol/L)	5.1 (4.4-5.4)†	4.9 (4.8-5.4)†	7.1 (7.0-10.2)*‡	<0.001
HbA_{1c} (%)	5.7±0.8†	5.6±0.6†	7.3±1.6*‡	<0.001
Serum creatinine (μmol/L)	79.6±14.1	78.7±16.8	78.7±15.1	0.98
Estimated GFR ($ml/min/1.73m^2$)	89.4±9.8	92.1±14.6	86.5±16.1	0.36
UACR (mg/g)	1.5 (0.3-3.0)†	1.5 (0.5-7.2)†	7.8 (2.9-10.8) *‡	0.01
Total cholesterol (mmol/L)	5.7±0.9	5.5±1.1	5.9±1.4	0.65
LDL-cholesterol (mmol/L)	3.6±0.6	3.6±1.0	3.4±1.5	0.80
HDL-cholesterol (mmol/L)	1.5±0.5†‡	1.2±0.3*	1.2±0.3*	<0.001
Triglycerides (mmol/L)	0.9 (0.8-1.3)†‡	1.8 (1.2-2.4)*	2.3 (1.5-2.9)*	<0.001
Uric acid (μmol/L)	280±54	333±101	297±71	0.138
Brachial systolic BP (mmHg)	130.3±8.0†‡	145.6±10.3*†	137.5±12.6*‡	<0.001
Brachial diastolic BP (mmHg)	78.5±6.1†‡	86.7±9.6*†	78.0±8.3‡	<0.001
Brachial PP (mmHg)	51.8±7.6†‡	58.9±13.1*	59.9±14.5 *	0.03
Mean BP (mmHg)	97.2±6.9‡	103.4±16.7*†	99.2±9.8‡	<0.001
Aortic systolic BP (mmHg)	120.5±9.1‡	133.3±12.4*†	125.2±15.6‡	<0.001
Aortic PP (mmHg)	41.5±8.1†‡	49.0±15.6*	47.6±15.5*	<0.001

EH: essential hypertensive patients; T2DM: type 2 diabetic patients; GFR: glomerular filtration rate; UACR: urinary albumin-creatinine ratio; LDL: low-density lipoprotein; HDL: high-density lipoprotein; BP: blood pressure; PP: pulse pressure.
Data are expressed as mean ± SD or median (25-75%); p values are for trend (ANOVA) or χ^2.
$^{*}p<0.05$ vs C; $^{\dagger}p<0.05$ vs T2DM; $^{\ddagger}p<0.05$ vs EH.

Table 10. Systemic vascular parameters in the three study groups

Variables	Controls (n=27)	EH (n=49)	T2DM (n=32)	p value
Brachial artery diameter (mm)	4.2±0.9	4.2±0.9	4.4±0.6	0.64
Reactive hyperemia (%)	674±290†	532±254†	362±223*‡	0.001
FMD (%)	6.7±3.3†‡	4.9±2.4*	3.9±1.7*	<0.001
Brachial artery response to GTN (%)	6.1±3.7	6.4±3.0	5.8±2.3	0.65
Augmentation Index	21.1±12.5	27.0±22.1	24.6±14.1	0.33
Timing of the reflected wave (ms)	147±23†	138±18	130±18*	<0.001
Aortic PWV (m/s)	7.5±1.1†‡	8.2±1.7*	8.6±1.8*	0.004

EH: essential hypertensive patients; T2DM: type 2 diabetic patients; FMD: flow-mediated dilation; GTN: glyceril trinitrate; PWV: pulse wave velocity. Data are expressed as mean ± SD; p values are for trend (ANOVA). $^{*}p<0.05$ vs C; $^{\dagger}p<0.05$ vs T2DM; $^{\ddagger}p<0.05$ vs EH.

Table 11. Clinical and vascular variables affecting dynamic (DRIN) and baseline resistive index (RI) in the overall population – univariate analysis.

Variables	DRIN		RI	
	r	p	r	p
Clinical variables				
Age	-0.351	<0.001	0.386	<0.001
Ln (BMI)	0.023	0.819	0.024	0.811
Ln (fasting glucose)	-0.122	0.296	0.420	<0.001
Ln (HbA_{1c})	-0.040	0.849	0.173	0.408
Serum creatinine	0.043	0.724	-0.308	0.008
Estimated GFR	0.191	0.114	0.053	0.657
Ln (UACR)	0.192	-0.168	0.227	0.083
Total cholesterol	0.010	0.928	0.081	0.499
LDL-cholesterol	-0.160	0.197	0.001	0.990
HDL-cholesterol	-0.058	0.635	-0.019	0.871
Ln (triglycerides)	-0.039	0.744	0.087	0.467
Uric acid	0.051	0.729	-0.154	0.292
Heart rate	0.017	0.867	-0.036	0.726
Brachial systolic BP	-0.208	0.042	0,167	0.099
Brachial diastolic BP	0.047	0.646	-0,181	0.074
Brachial PP	-0.246	0.016	0.388	<0.001
Mean BP	-0.107	0.312	-0.025	0.805
Aortic systolic BP	-0.357	<0.001	0.219	0.033
Aortic PP	-0.490	<0.001	0.419	<0.001
Vascular variables				
Brachial diameter at baseline	-0.064	0.553	-0.035	0.739
Reactive hyperemia	0.318	0.009	-0.276	0.025
Flow-mediated dilation	0.173	0.104	-0.298	0.004
Brachial response to GTN	0.242	0.023	0.024	0.819
Augmentation Index	-0.357	<0.001	0.174	0.096
Timing of the reflected wave	0.163	0.121	-0.253	0.013
PWV	-0.373	<0.001	0.260	0.018
Nitrotyrosine	-0.241	0.020	0.421	<0.001
RI	-0.279	0.005	-	-

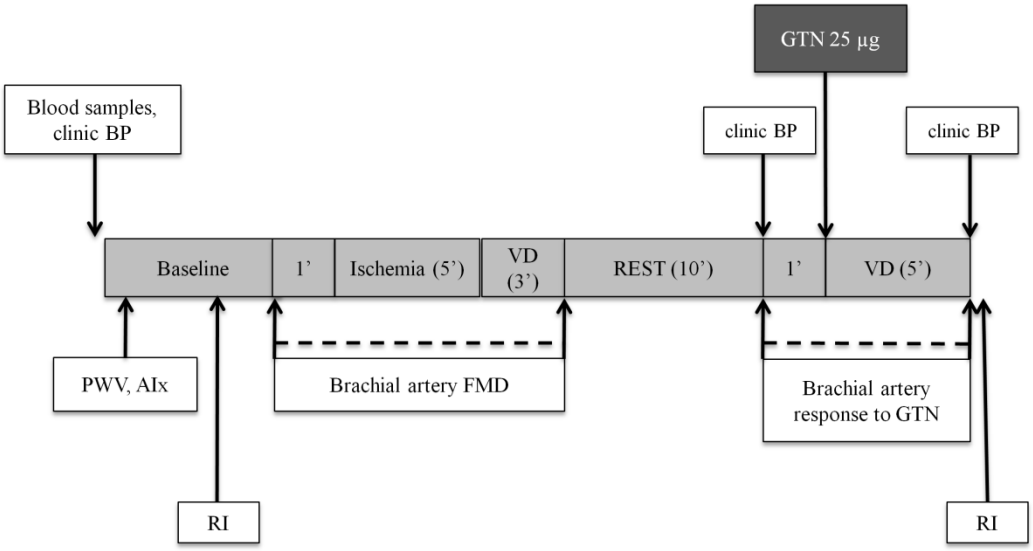
RI: renal resistive index; DRIN: dynamic resistive index; BMI: body mass index; GFR: glomerular filtration rate; UACR: urinary albumin-creatinine ratio; LDL: low-density lipoprotein; HDL: high-density lipoprotein; BP: blood pressure; PP: pulse pressure; GTN: glyceril trinitrate; PWV: pulse wave velocity.

Table 12. Clinical and vascular variables affecting dynamic dynamic (DRIN) and baseline resistive index (RI) in the overall population – multivariate analysis.

	DRIN			
	Model 1		**Model 2**	
	r^2	p	r	p
Clinical variables				
Age	0.026	0.111	0.026	0.165
Serum creatinine	0.025	0.079	0.025	0.107
Ln (serum glucose)	0.124	0.004	0.103	0.009
Brachial PP	0.007	0.321	-	-
Aortic PP	-	-	0.042	0.810
Vascular variables				
Reactive hyperemia	0.097	0.031	0.090	0.040
Brachial response to GTN	0.022	0.742	0.016	0.809
Augmentation Index	0.027	0.828	0.028	0.948
PWV	0.070	0.006	0.045	0.018
RI	0.006	0.224	0.010	0.462
	RI			
	Model 1		**Model 2**	
	r^2	p	r	p
Clinical variables				
Age	0.065	0.310	0.063	0.463
Serum creatinine	0.182	0.045	0.180	0.048
Ln (serum glucose)	0.061	0.088	0.097	0.025
Brachial PP	0.107	0.053	-	-
Aortic PP	-	-	0.076	0.036
Vascular variables				
Reactive hyperemia	<0.001	0.911	<0.001	0.973
Flow-mediated dilation	<0.001	0.812	0.008	0.869
Timing of the reflected wave	0.003	0.855	0.001	0.673
PWV	<0.001	0.730	0.003	0.704

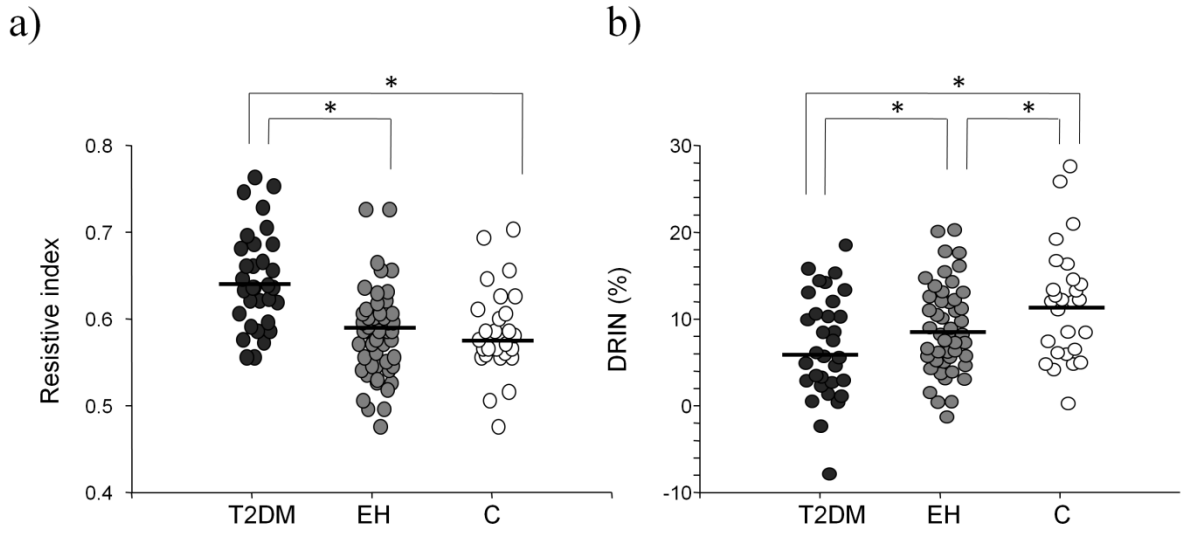
RI: renal resistive index; DRIN: dynamic resistive index; BP: blood pressure; PP: pulse pressure; GTN: glyceril trinitrate; PWV: pulse wave velocity.

Figure 13. Time-line of the study protocol.



BP: blood pressure; PWV: pulse wave velocity; AIx: augmentation index; RI: renal resistive index; FMD: flow-mediated dilation; VD: vasodilation; GTN: glyceril trinitrate.

Figure 14. Dot-plots representing baseline (a) and dynamic (b) renal resistive index in the three study groups. *: $p<0.05$



EH: essential hypertensive patients; T2DM: type 2 diabetic patients; DRIN: dynamic resistive index.

Chapter 8. Vascular consequences of environmental and therapeutic radiation

Radiation exposure gives an excess risk of cancer, whereas CV effects are less defined. Aim of this study is to assess the pro-atherosclerotic effects of environmental and iatrogenic radiation exposure in young survivors of the Chernobyl disaster, treated for thyroid cancer with radioiodine ablation. This research has been recently published as research letter (Bruno et al., 2013b)

8.1 Rationale

The Chernobyl disaster, which took place on April 26th 1986 at the nuclear plant in northern Ukraine, represents the largest nuclear accident ever happened. As a result of the accident, about five million people were exposed to radioactive contamination in Belarus, the Russian Federation and Ukraine (2000). The effective dose estimates accumulated over the 20 years following the accident ranged from a few mSv to some hundred mSv, depending on location, age and lifestyle factors, such as diet, or time spent outdoor (2000). The main health effect of radiation observed is an increase in the incidence of thyroid cancer in persons exposed as young people (Baverstock et al., 1992;Tuttle et al., 2011). Over the last 20 years, nearly 5000 cases of differentiated thyroid cancer have been diagnosed and treated in young people previously exposed to the Chernobyl radioactive fallout during childhood (Baverstock et al., 1992;Tuttle et al., 2011). An increased incidence of other types of malignancies, such as leukaemia, breast cancer, cancers of the bladder and kidney, has been also reported (Cardis and Hatch, 2011). A number of non-cancer end points have been reported in populations exposed to radiations from the Chernobyl accident (Cardis and Hatch, 2011). Recently, studies on the atomic bomb survivors in Japan demonstrated that moderate doses of ionizing radiations may contribute to excess CV disease risk (Preston et al., 2003;Shimizu et al., 2010). However, these effects are still largely unexplored and there is great uncertainty regarding the effects of low-moderate doses on CV disease (2000;2006). CV imaging endpoints such as endothelial function measured by brachial artery flow-mediated dilation (FMD), C-IMT and

arterial stiffness, can be used as preclinical biomarkers to assess early signs of atherosclerosis and predict CV risk (Inaba et al., 2010;Vlachopoulos et al., 2010;Bianchini et al., 2013). Circulating endothelial progenitor cells (EPCs), derived from CD34+ hematopoietic stem cells, are emerging as a rescue squad for endothelial damage and as independent predictors of CV outcome (Shantsila et al., 2007). The aim of the present study was to assess the pro-atherosclerotic effects of radiation exposure in survivors of Chernobyl disaster, who were children at the time of the nuclear blast, subsequently diagnosed with papillary thyroid cancer and treated with radioiodine ablation.

8.2 Experimental protocol

Study population - The study population comprised 23 subjects who were children (≤ 8 years old) living in the Chernobyl area in Belarus at the time of the nuclear blast in 1986 (Group E-exposed), subsequently diagnosed and treated with radioiodine ablation for papillary thyroid cancer according to current Guidelines (Cooper et al., 2009). Twenty-three non exposed (NE) healthy subjects, matched with E for age, gender and CV risk factors, and without any evidence of coronary artery disease (CAD) by history and physical examination, served as controls. Informed consent was obtained from all patients before testing, and the study protocol was approved by the institutional ethics committee.

Biomarkers of vascular function and structure - All subjects underwent C-IMT assessment, aortic and carotid stiffness measurement, and peripheral blood testing for EPCs evaluation. In a subset of 28 subjects (14 subjects from each group), endothelial function was assessed by brachial artery flow mediated dilation (FMD).

Endothelial progenitor cells (EPCs) - A volume of 100 μL peripheral blood was immunostained with 10 μL of PERcP-conjugated human anti-CD34 monoclonal antibody (mAb) (BD Biosciences),

10 μL fluorescein-conjugated anti-human kinase insert domain receptor (KDR) mAb (R&D Systems, Minneapolis, MN, USA), 10μL of PE-conjugated human anti-CD133 monoclonal antibody (mAb) (Miltenyi Biotec) or isotype control and incubated for 30 minutes in the dark at 4°C. Red cells were lysed with 1x BD FACS Lysis solution (BD Pharmigen, UK) and incubated for 5 minutes at room temperature. Samples were analysed by a BD FACS Calibur flow cytometer with CellSystems® software (Becton Dickinson, UK). The frequency of peripheral blood cells positive for above reagents was assessed by a two-dimensional side scatter fluorescence dot plot analysis, after appropriate gating and staining with the different reagents: CD34+ peripheral blood cells were initially gated in the mononuclear cell fraction and the resulting population was examined the resulting population for expression of KDR and CD133. Validation of the assay was performed by the ISHAGE method (Gratama et al., 2003). For FACS analysis, a minimum of 5×10^5 cells were acquired and scored using a FACS Calibur analyzer (BD Biosciences); cell counts were then expressed as cells per 10^6 cytometric events. The operators were trained in flow cytometry, trained for rare event analysis and blinded to the clinical status of participants.

Statistical analysis - Statistical analysis was performed with SPSS (version 16.0, SPSS Inc, Chicago, IL). Data were expressed as mean ± SD or frequency and percentage, as appropriate. ANOVA and Bonferroni post-hoc analysis was used for normally distributed variables, and Kruskal-Wallis Z Test for not normally distributed variables; when mentioned, other variables were also considered as covariates. Furthermore, we performed an ANCOVA-based allometric approach in order to adjust for the influence of baseline diameter on FMD (Atkinson and Batterham, 2013). Briefly, the slope of the regression between the logarithmically transformed baseline and peak BA diameter and allometric scaling were computed, in order to take into account of the non-linear relation between baseline BA diameter and BA hyperemic dilation. The difference between logarithmically transformed peak and baseline BA diameter was analyzed by ANCOVA, considering logarithmically transformed baseline BA diameter as a covariate. Corrected FMD was

then calculated by the following formula: [exponential (difference between logarithmically transformed peak and baseline BA diameter) -1]*100. Categorical variables were analyzed by χ^2 test. All tests were 2-sided, and $p<0.05$ was considered statistically significant.

8.3 Results

Clinical characteristics of the study population - The baseline characteristics of the study participants are given in Table 13. E and NE were superimposable for age, gender, BP values and prevalence of traditional CV risk factors **(Table 13).** In E mean total radiation dose was 47.3±16.1 mCi (range 30-200 mCi). E underwent initial treatment included a near-total thyroidectomy followed by radioiodine (131I) ablation (30mCi) of postsurgical thyroid remnants. Patients referred after initial surgery were submitted to completion thyroidectomy if the first operation had been less than a near-total thyroidectomy. Patients with metastatic disease underwent additional treatments consisting of 131I therapeutic doses (100–200 mCi), until a negative post-therapeutic whole-body scan was found. Whenever necessary, 131I treatments were repeated at interval times not shorter than 6 months. All E subjects were treated with L-thyroxin: their median TSH was 0.018 μg/mL (25-75 percentile: 0.007-0.462).

Endothelial progenitor cells - Subpopulations of putative EPCs (CD34+KDR+, CD34+CD133+, CD34+CD133+KDR+) expressed as cell numbers per 106 cytometric events, were significantly decreased in E group when compared to NE group **(Table 14, Figure 15).**

Aortic and carotid stiffness and carotid intima-media thickness - E and NE presented similar carotid diameter and CIMT **(Table 14).** The presence of carotid plaques was detected in one subject from Group E. Carotid PP was significantly lower in E than in NE, in spite of similar mean BP and brachial PP. Accordingly, PP amplification from carotid to brachial site was significantly higher in

E than in NE even after considering age, height and heart rate as covariates (p=0.04). Carotid AIx was also higher in E than in NE **(Table 14).** A reduced carotid PP corresponded to a reduced stroke change in carotid diameter, leading to similar values for carotid compliance, distensibility and stiffness **(Table 14).** Carotid-femoral PWV was not significantly different between E and NE, even after considering mean BP as a covariate (p=0.15).

Endothelium-dependent and -independent dilation in the brachial artery - E and NE showed similar brachial artery diameter, absolute and percent hyperemic change in diameter **(Table 15).** Baseline and hyperemic SR were also similar in E and NE. In E group, brachial artery dilation in response to GTN was significantly greater than in NE **(Table 15).**

The slope of the regression between the logarithmically transformed baseline and peak BA diameter was 0.988 (CI 0.869-0.976). The difference between logarithmically transformed peak and baseline BA diameter, analyzed by ANCOVA and considering logarithmically transformed baseline BA diameter as a covariate, resulted to be similar in E and NE (p=0.39). Corrected FMD in the 2 groups, calculated by the formula [exponential (difference between logarithmically transformed peak and baseline BA diameter) -1]*100, is reported in **Table 15.** The addition in the model of the difference between peak and baseline mean flow velocity did not change the significance of the test (p=0.60).

8.4 Discussion

The main result of this study is that in a group of young survivors of the Chernobyl nuclear disaster, subsequently treated with radioiodine ablation for thyroid cancer, the number of endothelial progenitor cells was significantly reduced in comparison to healthy volunteers, suggesting a reduced endothelial regeneration and repair capacity. This selective alteration, which seems not to influence flow-mediated dilation, might however impair endothelial response to vascular injuries occurring with aging, suggesting that E subjects are at higher risk of developing CV complications.

Epidemiology and mechanisms of radiation-induced cardiovascular damage - Cancer has been the main radiation-induced late effect considered for radiation protection after low dose protracted exposure. At higher, therapeutic doses, an increase in CV diseases has been demonstrated (Darby et al., 2003). More recently, a meta-analysis suggested that moderate doses of radiations could also contribute to excess CV disease risk (Little et al., 2010). A recent prospective cohort study with more than 50 years of follow up and 86000 participants, the Life Span Study of Japanese atomic bomb survivors, showed that doses above 0.5 Gy are associated with elevated risk of both stroke and heart disease, though the risk at lower doses is unclear (Shimizu et al., 2010). Evidence concerning CV consequences of radiation exposure after the Chernobyl blast is limited (Cardis and Hatch, 2011). In a study on a Russian cohort of 60000 subjects exposed to an average dose of 109 mGy and followed-up from 1986 to 2000, an increased for ischemic heart disease was found, though conventional CV risk factors were not weighed in the analysis (Ivanov et al., 2006;Cardis and Hatch, 2011).

The mechanisms by which relative low radiation doses (<2 Gy) may cause ischemic heart disease remain unsettled. Several mechanisms have been hypothesized such as pro-inflammatory responses to radiation, endothelial cellular loss or functional changes, or microvascular damage (Basavaraju and Easterly, 2002). Associations between dose of radiation and long-term levels of inflammatory

cytokines and biomarkers have been documented among atomic bomb survivors (Hayashi et al., 2003). Evidence suggests that, at least for high doses, radiation may cause microvascular disease, with a decrease in capillary density causing chronic ischemic heart disease, as well as macrovascular disease via accelerated vascular aging in the coronary arteries (Fajardo, 1999).

Endothelial parameters - In the present study, endothelial pathology was evaluated by means of two different and complementary approaches: EPCs assessment, exploring endothelial repair capacity, and brachial artery FMD, exploring conduit artery endothelial function. EPCs may be used to improve risk stratification and identify patients at high risk for major adverse CV events beyond the commonly used risk markers (Shantsila et al., 2007). Several studies have demonstrated reduced availability and impaired function of EPCs in the presence of both established CV disease and associated comorbid risk factors (Shantsila et al., 2007). Interestingly, low-dose irradiation has been recently found to cause EPCs depletion in a murine model and in isolated human EPCs (Lee et al., 2012). On the other hand, FMD is an early step in the development of atherosclerosis and is increasingly being recognized as a long-term predictor of CV events (Inaba et al., 2010;Bianchini et al., 2013). To our knowledge, this is the first study demonstrating that young adults exposed during childhood to environmental and iatrogenic radiation showed impaired endothelial regeneration capacity, mirrored by low levels of EPCs. Interestingly, EPCs reduction is not associated with brachial artery endothelial function. EPCs are quickly mobilized, and their circulating pool increased, after acute exposure to a pathogen, leading to restoration of endothelial integrity and function, whereas prolonged exposure induces EPCs depletion (Heiss et al., 2005). The lack of repair reserve in the endothelium might have no functional effects in young subjects, but might become critical during aging, predisposing this population to increased CV risk.

Vascular structural alterations - In the present study imaging biomarkers, such as C-IMT, carotid and aortic stiffness, measured in a group of thyroid cancer survivors treated with radioiodine

ablation, are not significantly different as compared to non-exposed age and sex-matched controls. A population study performed among 1804 survivors of the atomic bombing in Hiroshima demonstrated that, after correction for confounders, C-IMT was not related to radiation exposure, while aortic arch calcification was (Yamada et al., 2005). It is conceivable that the young age of the studied population, as well as the low sample size, made not possible to appreciate significant structural changes in large arteries.

On the other side, exposed subjects showed an increased endothelium-independent vasodilation in the brachial artery. Despite mechanisms underlying this finding are unknown at the moment, an increased smooth muscle cells sensitivity to exogenous NO can be suggested, which may act as a compensatory mechanism settled to maintain a preserved vascular function in the presence of reduced endogenous NO generation. A dysregulated autonomic control of smooth muscle tone might also be hypothesized. An increased endothelium-independent vasodilation has been associated to exaggerated vagal responses, such as subjects with neurally-mediated syncope (Santini et al., 2012), and both suppressive doses of L-thyroxine (Casu et al., 2005), and exposure to radiation (Niagu and Zazimko, 1995) have been associated to vegetative dysfunction. Smooth muscle cell dysfunction is suggested also by the presence of an increased AIx. In the presence of a similar PWV, the differences in AIx, which is an integrated marker of amplitude and timing of the global reflected wave, might be attributed to different sites of reflection between E and NE, which are mainly determined by the vasomotor state of microcirculation (Laurent et al., 2006). This observation is of clinical interest, since a recent meta-analysis showed that a 10% absolute increase in central AIx was independently associated with a +32% relative risk of CV events (Vlachopoulos et al., 2010). Future studies should address the clinical significance and mechanisms underlying radiation-induced smooth muscle cell dysfunction.

Strengths and limitations - Strengths of this study consist in the recruitment of an almost unique, homogeneous population, exposed to environmental and iatrogenic radiation, in which vascular

function, structure and regenerative potential was extensively studied with a multiparametric approach. Several limitations should also be acknowledged. First, the recruited sample had a small size and may not be representative of the whole population of Chernobyl thyroid cancer survivors. Moreover, only a subgroup of exposed subjects underwent FMD assessment. The control group consisted of healthy, age and gender matched, non-exposed controls living in a different country (Italy); therefore differences in genetic and lifestyle factors, as well as the absence of thyroid disease and L-thyroxin treatment, may have introduced a potential bias. Both environmental and therapeutic radiation dose history of the E group was reconstituted retrospectively, leading to a potential underestimation of the calculated risk. Finally, the cross-sectional design of the study cannot provide information about the predictive role of the alterations found for the development of symptomatic CV disease.

Conclusions - Young adults exposed during childhood to environmental (Chernobyl disaster) and iatrogenic (radioiodine ablation) radiation showed decreased endothelial progenitor cells number, in the presence of preserved conduit artery endothelium-mediated dilation. Furthermore, they exhibited altered wave reflection and smooth muscle cell reactivity. The impact of radiation exposure in the low-to-moderate dose range on CV risk is an emerging, but still largely unexplored, public health concern, mainly due to the exponentially increasing use of nuclear and X-ray based imaging modalities in medicine (Picano, 2004;2006). The preliminary results of this study depict an early, peculiar pattern of pre-clinical vascular involvement associated with exposure to radiation and, if confirmed by prospective studies on larger populations, support the hypothesis that low-moderate doses of ionizing radiation can contribute to excess CV disease risk.

Table 13. Clinical characteristics of the study population

Clinical characteristics	**Group E** n= 23	**Group NE** n= 23	**P value**
Age (yrs)	25.7±2.8	27.0 ± 4.9	0.35
Men	10 (44.4%)	10 (44.4%)	1.00
Systolic BP (mmHg)	125.4 ± 13.8	125.4 ± 10.0	0.99
Diastolic BP (mmHg)	79.2 ± 11.1	75.5 ± 11.2	0.27
Mean BP (mmHg)	96.9 ± 11.4	94.5 ± 9.9	0.48
Pulse pressure (mmHg)	46.2 ± 8.7	49.8 ± 8.0	0.17
Heart rate (bpm)	72.4 ± 9.8	67.2 ± 10.5	0.11
Height (m)	1.71 ± 0.07	1.71 ± 0.07	0.92
BMI (kg/m²)	22.9 ± 4.1	23.7 ± 3.4	0.34
Hypercholesterolemia (n, %)	0 (0%)	0 (0%)	1.00
Current smoking (n, %)	13 (56.5%)	11 (47.8%)	0.55
Hypertension (n, %)	1 (4.3%)	1 (4.3%)	1.00
Diabetes mellitus (n, %)	1 (4.3%)	0 (0%)	0.31

E: exposed individuals; NE: non-exposed individuals; BP: blood pressure; BMI: body mass index.

Table 14. Carotid intima-media thickness, aortic and carotid stiffness and endothelial progenitor cells.

Variables	**Group E** n= 23	**Group NE** n= 23	**p value**
C-IMT (μm)	521 ± 62	532 ± 77	0.59
Carotid diastolic diameter (mm)	6.24 ± 0.49	6.23 ± 0.47	0.95
Carotid distension (mm)	0.59 ± 0.11	0.69 ± 0.16	0.03
Carotid compliance coefficient (m^2*kPa^{-1})	1.25 ± 0.37	1.16 ± 0.56	0.38
Carotid distensibility coefficient (kPa^{-1})	41.8 ± 12.4	38.1 ± 9.2	0.29
Carotid stiffness (m/s)	5.1 ± 1.0	5.2 ± 0.9	0.58
Carotid-femoral PWV (m/s)	5.8 ± 1.4	6.3 ± 1.7	0.15
Carotid PP (mmHg)	37.9 ± 6.8	45.8 ± 10.8	0.008
Carotid AIx (%)	-5.5 ± 12.4	-16.8 ± 13.8	0.02
Carotid-brachial PP amplification	1.21 ± 0.08	1.09 ± 0.12	0.0008
n° of EPCs/ 10^6 cytometric events			
$CD34^+KDR^+$	11.3±8.8	83.6±39.7	< 0.0001
$CD34^+CD133^+$	15.8±19.1	222.8±154.2	< 0.0001
$CD34^+CD133^+KDR^+$	7.1±9	61.2±25.5	< 0.0001

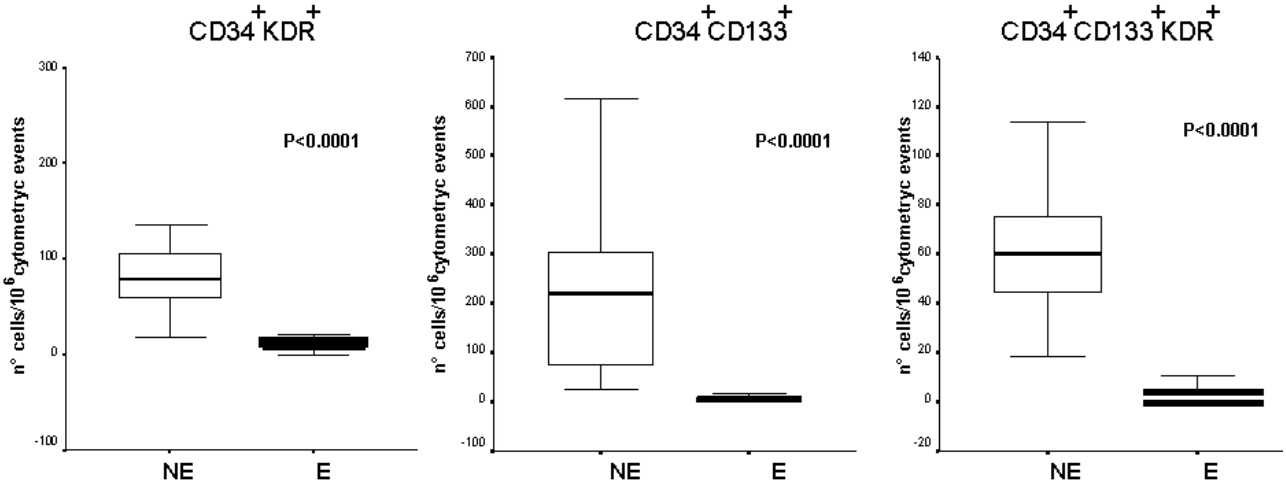
E: exposed individuals; NE: non exposed individuals; C-IMT: carotid intima-media thickness; PWV: pulse wave velocity; PP: pulse pressure; Aix: augmentation index normalized at 75 bpm; n° of EPCs: number of endothelial progenitor cells. P value for Carotid-femoral PWV is obtained with mean BP as covariate.

Table 15. Endothelium-dependent and –independent dilation in the brachial artery

Variables	**Group E** n= 14	**Group NE** n= 14	**P value**
Baseline brachial artery diameter (mm)	3.50 ± 0.96	3.22 ± 0.80	0.25
Maximum brachial artery diameter (mm)	3.80 ± 0.91	3.47 ± 0.50	0.24
FMD (%)	8.04 ± 4.22	7.72 ± 3.44	0.67
Corrected FMD (%)	8.33 ± 1.03	7.25 ± 1.03	0.39
Baseline SR (s^{-1})	177 ± 90	161 ± 96	0.59
Hyperemic SR (s^{-1})	891 ± 292	739 ± 321	0.19
Response to GTN (%)	10.5 ± 4.1	7.1 ± 2.5	0.01

E: exposed individuals; NE: non-exposed individuals; FMD: flow-mediated dilation; SR: shear rate; GTN: glyceryl trinitrate.

Figure 15: Subpopulations of putative endothelial progenitor cells (EPCs) ($CD34^+KDR^+$, $CD34^+CD133^+$, $CD34^+CD133^+KDR^+$) are significantly lower in the exposed (E) than in non-exposed individuals ($p<0.0001$ for all).



Chapter 9. Vascular function in isolated obstructive sleep apnea syndrome

Patients with obstructive sleep apnea syndrome (OSAS) exhibit accelerated vascular aging and renal damage. Aim of the study was to investigate whether vascular dysfunction is a feature of obstructive sleep apnea syndrome per se or instead related to the presence of traditional CV risk factors. The study was supported by a grant "Research project for assistant professors of the Faculty of Medicine of the University of Pisa" and has been recently published (Bruno et al., 2013a).

9.1 Rationale

OSAS is associated with increased incidence of CV morbidity and mortality (Somers et al., 2008), but causative mechanisms remain uncertain. The greater prevalence of traditional CV risk factors such as obesity, hypertension, and diabetes mellitus, is known to play a role (Somers et al., 2008), though recent studies suggest that the increased risk of coronary and cerebrovascular events in OSAS is independent of age, obesity, cholesterol and BP values (Gottlieb et al., 2010;Redline et al., 2010). Patients with OSAS exhibit an accelerated vascular aging, expressed by endothelial dysfunction (Ip et al., 2004;Jelic et al., 2008;Jelic et al., 2010;Butt et al., 2011) and arterial stiffness (Drager et al., 2007;Phillips et al., 2013), which can represent the integrated effect of traditional CV risk factors on vasculature, but also confers an independent, additive risk (Inaba et al., 2010;Vlachopoulos et al., 2010).

Recent studies suggested that endothelial dysfunction occurs in OSAS patients regardless of the presence of hypertension or obesity (Jelic et al., 2008;Jelic et al., 2010). Increased oxidative stress, reduced endothelial nitric oxide synthase (eNOS) expression, and inflammation have been suggested as potential mechanisms, although not univocally (Svatikova et al., 2004;Selmi et al., 2007;Grabska-Kobylecka et al., 2008;Jelic et al., 2008;Jelic et al., 2010;Mancuso et al., 2012). Furthermore, contrasting literature exists regarding behavior of biomarkers of endothelial activation

(Ohga et al., 1999;Dyugovskaya et al., 2002;El-Solh et al., 2002), damage and repair (Jelic et al., 2008;Martin et al., 2008;Jelic et al., 2010;Butt et al., 2011) in this population. Arterial stiffness has been reported to be either increased (Drager et al., 2007) or normal (Butt et al., 2011) in normotensive OSAS patients, while the role of obesity has never been excluded. A relationship between OSAS and chronic kidney disease has been also suggested (Adeseun and Rosas, 2010;Buchner et al., 2011), although these preliminary studies did not take into account the role of traditional CV risk factors that often accompany OSAS.

The aim of this study was to investigate whether vascular dysfunction is related to OSAS *per se*. Accordingly, a comprehensive vascular evaluation was conducted by different techniques in a carefully selected population of patients with OSAS, free of traditional CV risk factors (in particular obesity and hypertension) or established CV disease. Moreover, several potential mechanisms of vascular dysfunction were also explored.

9.2 Experimental protocol

Study population - Twenty patients with newly diagnosed moderate-severe OSAS (Apnea-Hypopnea Index, AHI, >15/h), selected for absence of traditional CV risk factors and established CV or renal disease, were recruited by the Sleep Medicine Unit of the University of Pisa. Exclusion criteria were: obesity (body mass index > 30 kg/mq); history of arterial hypertension (BP > 140/90 mmHg on repeated occasions or current antihypertensive treatment), diabetes mellitus (fasting blood glucose>126 mg/dl on repeated occasions or current antidiabetic treatment) or severe hypercholesterolemia (LDL-cholesterol > 200 mg/dl or current lipid-lowering treatment); smoking more than 5 cigarettes per day; previous CV events, including coronary artery disease, heart failure, cerebrovascular disease; chronic kidney disease, defined as estimated estimated glomerular filtration rate (GFR) < 60 ml/min, or albuminuria (urinary albumin/creatinine ratio ≥ 22 mg/g for males, ≥ 31 mg/g for women in a single sample of morning urine); any other disease or current

treatment possibly interfering with vascular function evaluation, according to clinical judgment. Two different control groups, matched for age, gender and BP, were enrolled: 20 patients presenting moderate-severe OSAS and traditional CV risk factors, but free of established CV disease or chronic kidney disease, and 20 healthy subjects, in which OSAS was excluded by polysomnography. The study was approved by the local ethical committee. All patients gave written informed consent before entering the study.

Experimental session - In-lab polysomnography (Planet 200-Sistema Galileo, Esaote Biomedica, Florence, Italy) was performed according to standard guidelines. Sleep recordings were scored according to international criteria (Iber et al., 2007). The experimental session took place no more than 1 month after polysomnography, in the morning after an overnight fasting. Patients were asked to maintain their usual pharmacological treatment. Medical history, body weight and height, as well as blood and urine samples were collected. Lipid profile, plasma glucose, serum creatinine, and urinary albumin were determined according to standard laboratory procedures. For serum isolation and RNA purification, blood samples were drawn and immediately stored at 4 °C, and processed within 1 h. Serum and RNA samples were then stored at -80 °C until their use in experimental tests. Vascular function tests were performed in a quiet air-conditioned room (22-24 °C). Brachial BP was measured three times at 2-min intervals by automatic oscillometric device (OMRON-705IT), with the patients resting in a supine position for at least 10 min. The following tests for vascular structure and function were performed: endothelium-dependent and -independent dilation in the brachial artery, renal vasodilating capacity, carotid-femoral PWV, carotid stiffness, C-IMT.

RNA extraction, cDNA production, Real-time RT-PCR - Nucleated blood cell RNA was extracted and amplified by real-time reverse transcription polymerase chain reaction (RT-PCR) technique. RNA expression of the following adhesion molecules were analyzed: integrin α-L (CD11A), integrin α-M (CD11B), integrin α-X (CD11C), L-selectin, glutathione peroxidase-1

(GPX1) and catalase. In detail, 2 ml of blood were added to 28 ml of erythrocyte lysis solution (168 mM NH_4Cl; 10mM $KHCO_3$; 100 µM EDTA) and incubated for 8 min at room temperature. After centrifugation the white blood cell pellet was washed with phosphate-buffered saline (PBS) and resuspended in 250 µl of PBS. RNA was then extracted using Trizol LS reagent (Invitrogen) following manufacturer's instructions. RNA was quantified using Nanodrop spectrophotometer and 200 ng were retro-transcribed into cDNA using hexanukleotide-random-primers and Superscript II reverse transcriptase (Invitrogen) following manufacturer's instruction. Real-time PCR analysis was performed using Maxima SYBR green qPCR master mix (Fermentas), specific primers and amplification conditions as listed in Table 16. Analysis was carried out in the Rotor-gene-6000 system (Corbett Life Science). Relative quantification and statistical data analysis were performed according to the delta-Ct method using beta-actin as internal reference. Two independent amplification experiments were performed for each gene. For absolute quantification of transcript copy number, external standard were obtained by amplifying cDNA of CD11A, CD11B, CD11C, L-selectin, GPX1 and catalase. Circulating endothelial progenitors were estimated by a real-time RT-PCR-based approach, evaluating the RNA expression of the specific marker CD34 in nucleated blood cells (Steurer et al., 2008). Amplification products were purified and quantified by measuring their absorbance at 260 nm. Serial dilutions of the amplified fragments containing 10^8, 10^6, 10^4, and 10^3 cDNA copies were used in the amplification experiments as calibration curves. These curves were used to extrapolate copy numbers per µg total RNA.

Measurement of serum hydroperoxide, malondialdehyde and E-selectin concentrations - Hydrogen peroxide was assayed using the xylenol orange technique (Jiang et al., 1990). H_2O_2 oxidizes iron (II) to iron (III) in the presence of sorbitol, which acts as a catalyst. Iron (III) then forms a purple complex with xylenol orange. Briefly, 1:10 dilutions of patient serum were incubated with an equal volume of detection solution (0.5 mM ammonium ferrous sulphate/0.2 mM Xylenol orange/50 mM H2SO4/ 100 mM sorbitol) for 45 min at room temperature. Absorbance at

560 nm was determined using the UV 340 plate reader (ASYS) and compared with a hydrogen peroxide standard curve. Each sample was analyzed in triplicate. Malondialdehyde (MDA) was also assayed by spectrophotometric assay (Bioxitech LPO-586. OXIS International Inc., CA, USA) (Esterbauer and Cheeseman, 1990) Serum concentration of E-selectin was determined using the E-selectin (Human) ELISA Kit (Abnova) following manufacturer's instructions. Each sample was analyzed in duplicate.

Evaluation of DNA damage - We evaluated DNA integrity in blood samples by the use of alkaline single-cell gel electrophoresis or comet assay, according to Singh et al. (Singh et al., 1988), with minor modifications(Lenzi et al., 2003). Electrophoretic DNA migration is proportional to the level of DNA damage producing comet-like images under a fluorescence microscope (magnification 200×). We used an image analyzer (Komet, version 4; Kinetic Imaging Ltd., Bromborough, UK) to quantify the percentage of DNA migrated in the tail of at least 50 cells per sample. Slides were coded and scored blindly to avoid risk of bias. Two parallel tests were performed per sample and the mean was calculated. For statistical analysis we used the software Statgraphics Plus for Windows (version 2.1; Microsoft Corp., Redmond, WA, USA).

Statistical analysis - Statistical analysis was performed using NCSS 2004 (NCSS, Kaysville, Utah). The results were expressed as mean ± SD. ANOVA and Bonferroni post-hoc analysis was used for normally distributed variables, and Kruskal-Wallis Z Test for not normally distributed variables; when mentioned, other variables were also considered as covariates. Categorical variables were analyzed by χ^2 test. Linear regression analysis (Spearman's rank) was also performed in patients with OSAS (n = 40). A p value < 0.05 was considered significant.

9.3 Results

C**linical characteristics of the study population -** Patients with OSAS and no traditional CV risk factors were comparable to healthy controls except for greater AHI, lower minimum O_2 saturation, and greater time spent below 90% O_2 saturation, and comparable to OSAS patients with traditional CV risk factors, except for higher BMI and plasma glucose **(Table 17).** Of OSAS patients with traditional CV risk factors (15 hypertension, 18 obesity, 4 smoking, 6 hypercholesterolemia, 2 diabetes), 10 were on antihypertensive and 6 on lipid-lowering treatment, leading to BP and cholesterol values superimposable to the other groups.

Endothelium-dependent and -independent dilation in the brachial artery - Patients with OSAS with or without traditional CV risk factors showed a greater brachial artery diameter compared to controls. Reactive hyperemia was similar to controls in patients with OSAS and no traditional CV risk factors, and reduced in the presence of both OSAS and CV risk factors. FMD was similarly reduced in both groups of OSAS patients compared to healthy subjects **(Table 18, Figure 16).** In contrast, brachial artery vasodilatation to GTN was similar in all groups. Among OSAS patients, FMD was related with age ($r = -0.54$, $p = 0.003$) and brachial artery diameter ($r = -0.67$, $p < .0001$), but not with OSAS severity, arterial stiffness or renal parameters.

Renal vasodilating capacity - No differences were found between right and left RI in the whole population (0.60 ± 0.05 vs 0.60 ± 0.05, p = ns), nor within each group. Patients with OSAS and no traditional CV risk factors had resting RI comparable to healthy subjects, whereas OSAS patients with traditional CV risk factors had increased RI **(Figure 17, Table 19).** Percent change in RI induced by GTN administration was reduced in both groups of OSAS patients as compared to controls **(Figure 17).** Among OSAS patients, neither resting RI nor RI response to GTN were related to OSAS severity indices or endothelial parameters. Renal vasodilating capacity (but not

resting RI) was inversely related to central PP (r = -0.41, p = 0.03), carotid-femoral PWV (r = -0.43, p = 0.03), and serum hydroperoxide (r = -0.53, p = 0.04).

Aortic and carotid stiffness - Carotid-femoral PWV was comparable to healthy subjects in patients with isolated OSAS, while it was significantly increased in OSAS patients with traditional CV risk factors **(Table 18).** This difference remained significant even after adjustment for mean BP. No differences in central BP values, carotid-radial PWV and AIx were found between the groups (Table 18). Mean common carotid diameter and distension were similar among the groups. Distensibility coefficient was reduced, whereas carotid stiffness and IMT were increased, only in patients with OSAS and CV risk factors compared to healthy volunteers **(Table 18).** Carotid-femoral PWV was related to age (r = 0.49, p = 0.01), AHI (r = 0.40, p = 0.03), mean BP (r = 0.52, p = 0.005), distensibility coefficient (r = -0.48, p = 0.01), carotid stiffness (r = 0.45, p = 0.02), renal vasodilating capacity (r = -0.43, p = 0.03), but not with other endothelial variables. Carotid distensibility coefficient, as well as carotid stiffness (data not shown), was related to age (r = -0.67, p < 0.001), mean BP (r = -0.53, p = 0.004), PWV (r = -0.48, p = 0.01), but not to OSAS severity or endothelial variables.

Endothelial and oxidative stress biomarkers - As presented in Table 19, eNOS transcripts were significantly and similarly reduced in patients with OSAS without and with CV risk factors. Among OSAS patients, linear regression analysis showed a significant direct correlation between eNOS expression and FMD (r = 0.58, p = 0.03).

Serum E-selectin was significantly higher in OSAS patients with and without traditional CV risk factors than in controls (63.8 ± 16.2, 49.8 ± 11.5, and 38.9 ± 17.9 ng/ml respectively; p < 0.05, **Figure 16).** No significant differences in the expression level of leukocyte adhesion molecules (CD11A, CD11B, CD11C, L-selectin) were appreciated by both relative **(Table 19)** and absolute

(data not shown) real-time RT-PCR analysis. A consistent (1.8-fold) and significant reduction of CD34 transcripts was observed only in OSAS patients with traditional CV risk factors **(Table 19).**

Serum hydroperoxide was not different in OSAS patients with or without traditional CV risk factors as compared to controls (271 ± 95, 349 ± 169, and 289 ± 124 ng/ml respectively; p = ns), as well as serum malondialdehyde levels (2.7 ± 0.7, 2.7 ± 0.8, and 2.1 ± 0.6 μmol/ml respectively; p = ns).

The expression level of GPX1 and catalase, quantified by relative and absolute real-time RT-PCR, do not show significant variation between the three groups **(Table 19).**

Patients with OSAS with and without traditional CV risk factors showed similar DNA damage in nucleated blood cells as compared to controls (18.8 ± 11.8, 15.9 ± 3.4, and 15.0 ± 5.3 tail DNA (%) respectively, p = ns).

9.4 Discussion

This study aimed to detect the specific effect of OSAS on vascular function regardless of the presence of traditional CV risk factors frequently associated with this condition. The main result is that early vascular alterations, namely endothelial dysfunction and reduced renal vasodilating capacity, were present in a group of patients with isolated OSAS carefully selected for the absence of traditional CV risk factors, including obesity and hypertension. Early vascular dysfunction is associated with reduced eNOS expression, which appears to be a possible causative mechanism.

Brachial artery FMD, a non-invasive approach to test endothelial function, was reduced in patients with OSAS free of traditional CV risk factors, in agreement with previous observations (Jelic et al., 2010). Interestingly, the presence of CV risk factors does not appear to further reduce FMD. Indices of OSAS severity are not related to FMD, at variance with other studies, which however enrolled patients with mild OSAS (Kraiczi et al., 2001), or also included in the correlation analysis subjects with normal AHI (Ip et al., 2004;Bayram et al., 2009). Endothelial dysfunction is increasingly accepted as the first step of the atherosclerotic process and a common trait of essentially all CV risk

factors, including obesity and hypertension (Brunner et al., 2005;Bianchini et al., 2012), and is a predictor of coronary events in both high-risk and low-risk populations (Inaba et al., 2010). Furthermore, improvement of FMD in hypertensive women after pharmacological treatment is associated with a better CV outcome (Modena et al., 2002). In OSAS, brachial artery FMD was associated with cardiac organ damage, i.e., abnormal myocardial perfusion (Butt et al., 2011), and was corrected by CPAP therapy (Bayram et al., 2009), though the prognostic role of endothelial dysfunction in OSAS patients has never been explored by prospective studies. Thus, current evidence suggests that endothelial dysfunction characterizes OSAS per se, might represent an intermediate, reversible, causative step toward established CV disease even in this condition.

Another element of novelty in the present study is the demonstration of subtle early renal vascular damage in OSAS patients regardless of the presence of CV risk factors and renal impairment. We previously demonstrated that in newly diagnosed essential hypertensive and type 2 diabetic patients the impairment of nitrate-induced renal vasodilation precedes the development of microalbuminuria and renal function decline (Bruno et al., 2011). This new index is correlated to macrovascular and microvascular systemic alterations (Bruno et al., 2011), representing an intriguing marker of the effects of CV risk factors on renal vasculature. Interestingly, the alteration in renal vasodilation in OSAS occurs in the presence of normal resting renal RI, thus preceding nephrosclerosis. Given the relevance of renal hemodynamics as well as of endothelium-derived factors in development of hypertension (Singh et al., 2010), it also conceivable that early vascular alterations at the renal level might favor hypertension onset in OSAS patients later on. However, at the moment this hypothesis is highly speculative and should be confirmed by prospective studies.

This study is the first to comprehensively assess arterial stiffness in multiple arterial districts and in a lean, normotensive population affected by OSAS. This is of relevance, since risk factors may affect elastic or musculo-elastic areas differentially (Paini et al., 2006). Our data suggest that OSAS *per se* is not associated with large artery stiffness, either at the carotid and the aortic level. Discrepant results found by Drager et al. (Drager et al., 2007), showing an increased aortic PWV in

normotensive subjects with OSAS, can be explained by inclusion of obese subjects in the population.

Given the possible prognostic implications of endothelial function in OSAS patients, much effort has been made to identify its causative mechanisms. This study showed decreased eNOS transcripts in nucleated blood cells of patients with isolated OSAS, a pool also including circulating endothelial progenitors, in agreement with previous studies (Jelic et al., 2008;Jelic et al., 2010). Furthermore, eNOS expression is significantly correlated with endothelium-dependent vasodilation. Taken together, these data suggest that the main and peculiar mechanism inducing endothelial dysfunction in isolated OSAS is reduced eNOS expression. Indeed, in other conditions characterized by endothelial dysfunction, such as hypertension and other traditional CV risk factors, eNOS expression is unchanged or paradoxically increased, with endothelial dysfunction being induced mainly by increased NO degradation by reactive oxygen species excess (Munzel et al., 2010).

Several mechanisms can reduce eNOS expression especially at post-transcriptional level, by reducing eNOS RNA stability. Hypoxia *per se* might be sufficient to elicit eNOS mRNA degradation (McQuillan et al., 1994;Takemoto et al., 2002). Another possible cause of reduction of eNOS transcripts is increased oxidative stress, but this was not confirmed by the present results. This point is highly controversial in literature (Yamauchi et al., 2005;Jordan et al., 2006;Grabska-Kobylecka et al., 2008;Lee et al., 2011). OSAS might cause a selective increase in intracellular endothelial oxidative stress (Butt et al., 2011), not detectable with the techniques used in present study, but nevertheless capable of causing eNOS mRNA degradation. A compensative potentiation of antioxidant defenses is not supported by our data, in agreement with previous studies (Jordan et al., 2006;Lee et al., 2011). High levels of pro-inflammatory cytokines, which have been described in OSAS pathology (Jelic et al., 2008;Jelic et al., 2010), have been also associated with reduced eNOS expression (de et al., 2001). NO is also an important homeostatic regulator of leukocyte adhesion (Kubes et al., 1991;May et al., 1991;Akimitsu et al., 1995;Hossain et al., 2012), both at the

endothelial level, mediated by increased P- and E-selectin expression (Hossain et al., 2012), and at the leukocyte level, via modulation of leukocyte-specific integrin alpha expression, sub-cellular localization or activity (Kubes et al., 1991;Mitchell et al., 1998;de et al., 2001;Canalli et al., 2008;Saluja et al., 2011). Accordingly, in the present study serum E-selectin was increased. In other diseases characterized by endothelial dysfunction, such as essential hypertension, E-selectin is not associated with endothelial-dependent vasodilation, again suggesting differential patterns and mechanisms of vascular alteration between these two conditions (De Caterina et al., 2001). On the other hand, our expression analysis at the transcriptional level did not reveal any difference in the abundance of leukocyte adhesion molecules between the compared groups, at variance with other studies (Dyugovskaya et al., 2002), whereas using a flow cytometry approach, an increased number of cells expressing the CD11C antigen on the cell surface after leukocyte activation has been demonstrated (Ohga et al., 1999). These findings might suggest that despite a normal cellular CD11C amount, a different sub-cellular localization might occur in OSAS patients. Similarly, while the percentage of T cells bearing L-selectin (Dyugovskaya et al., 2003) and serum L-selectin levels have been found to be increased (Ohga et al., 1999) or unchanged (El-Solh et al., 2002) in OSAS patients, we found normal L-selectin gene transcriptional activity, suggesting possible post-transcriptional modifications or differential sub-cellular localization.

In our study we also evaluated the expression of CD34, a marker of endothelial progenitor cells, circulating bone marrow-derived cells whose physiological function is to maintain vascular integrity, a crucial aspect in the pathogenesis of various condition comprehending vascular insult as a mechanisms of disease (George et al., 2011). Results of our study indicate that a reduction in CD34 expression occurs only in OSAS patients with traditional CV risk factors, confirming other studies (Martin et al., 2008;Butt et al., 2011) and suggesting that the impairment of endothelial repair capacity is a consequence of the atherosclerotic process.

The careful selection of the patients, representing a particular phenotype of moderate-severe, isolated OSAS, not associated with traditional CV risk factors or established CV and renal disease,

and the multi-parametric assessment of vascular function, as well as the inclusion of both a negative and a positive control group, are the strengths of this study. The relatively small sample size, due to the strict exclusion criteria, the presence of higher (though not significant) indices of OSAS severity in the positive control group, as well as the concomitant anti-hypertensive and lipid-lowering treatment in patients with CV risk factors, are limitations of the study.

In conclusion, patients with OSAS, even in the absence of CV risk factors, show endothelial dysfunction and activation and impaired renal vasodilating capacity. This is accompanied by decreased eNOS levels, suggesting that this enzyme might be one of the earliest targets of repetitive episodes of hypoxia characterizing OSAS. These alterations might be at least partly responsible for the increased incidence of CV events and renal function decline in patients with OSAS. Atherosclerotic and arteriosclerotic phenomena appear to be related to comorbidity with traditional CV risk factors.

Table 16. Real-time PCR primers and amplification conditions

Gene name	Gi number	Forward primer	Reverse primer	Annealing temperature
CD11A	167466214	CCCAAGATCCACCAAGTCA	CCCAACCACAGCCTCCAG	60°C
CD11B	224831238	AGCACACGGGATCGGCTAA	GTGTGCTGTTCTTTGTCTCATT	58°C
CD11C	34452172	ACAATCTCGGCATCTCCTTC	GGCGCTGTCACATGTCAGG	62°C
L-selectin	262206314	AGAATGTGTAGAAATCATCAATAA	TTCCCAAAGGGTGAGTACAG	60°C
GPX1	41406083	GGTACTACTTATCGAGAATGTG	CCACCACCAGGCCGGAC	58°C
Catalase	260436906	GCCTTCGACCCAAGCAACA	GGCGGTGAGTGTCAGGATA	59°C
eNOS	231571207	GAGACGCTGGTGCTGGTGGTAA	TCCGCCGCCAAGAGGACACC	62°C
CD34	68342037	TCCAGAGACAACCTTGAAGC	CTTCTTAAACTCCGCACAGC	52°C
Beta-actin	168480144	GCACTCTTCCAGCCTTCCTTCC	GAGCCGCCGATCCACACG	55°C

CD11A: integrin α-L; CD11B: integrin α-M; CD11C: integrin α-X; GPX1: glutathione peroxidase-1; eNOS: endothelial nitric oxide synthase; CD34: endothelial progenitors

Table 17. Clinical characteristics of the study population

Parameters	Healthy subjects	OSAS patients without CV risk factors	OSAS patients with CV risk factors
Age (years)	51.0±7.9	53.1±11.7	54.2±12.6
Males (n)	15	18	17
Smokers (n)	0	0	4
Body mass Index (Kg/m^2)	26.2 ±3.6	26.4±3.0	33.2±4.7*†
Brachial systolic BP (mmHg)	129.5±9.4	131.3±8.5	136.7±17.3
Brachial diastolic BP (mmHg)	79.1±7.6	77.9±6.5	79.7±7.5
Brachial PP (mmHg)	52.9±15.2	53.5±7.3	56.9±13.8
Serum glucose (mg/dl)	89.9±11.3	85.5±10.1	105.5±15.2 *†
Serum creatinine (mg/dl)	0.95±0.66	0.94±0.51	0.89±0.55
eGFR (ml/min/1.73 m^2)	84.1±11.3	86.7±5.7	91.9±6.3
UACR (mg/g)	0.6 (0-4.0)	1.6 (0-7.4)	2.7 (0-9.2)
Total cholesterol (mg/dl)	206±36	209±20	219±19
AHI (events/h)	3.5 ±1.4	35.2±10.6*	41.8±20.4*
Minimum SO_2 (%)	93.0±3.5	83.1±3.5*	79.5±6.7*
$SO_2 < 90\%$ (%)	0.2±0.2	9.5±10.7*	15.5±11.9*

OSAS: obstructive sleep apnea syndrome; CV: cardiovascular; BP: blood pressure; PP: pulse pressure; eGFR: estimated glomerular filtration rate; UACR: urinary albumin/creatinine ratio; AHI: apnea-hypopnea index; SO_2: O_2 saturation; $SO_2 < 90\%$: time spent below 90% O_2 saturation; *: $p < 0.05$ vs controls; †: $p < 0.05$ vs OSAS patients without CV risk factors.

Table 18. Vascular function parameters in the study population

Parameters	Healthy subjects	OSAS patients without CV risk factors	OSAS patients with CV risk factors
Brachial artery diameter (mm)	4.1±1.0	4.7±0.7*	4.9±1.1*
Flow-mediated dilation (%)	6.1±3.0	3.7±2.1*	3.7±2.6*
Reactive hyperemia (%)	558±352	404±256	224±144*†
Brachial artery response to GTN (%)	6.1±3.8	7.7±3.6	6.0±3.2
Mean BP (mmHg)	98.4±10.8	96.5±7.3	101.3±12.3
Aortic systolic BP (mmHg)	121.1±13.9	120.1±9.1	128.6±18.3
Aortic PP (mmHg)	40.8±8.8	41.6±8.3	49.3±14.3
Augmentation Index	19.0±14.3	17.8±9.8	21.6±11.7
Carotid-femoral PWV (m/s)	7.6±1.4	7.9±1.8	9.1±1.8*†
Carotid-radial PWV (m/s)	8.6±1.3	8.7±1.4	8.8±2.6
Carotid intima-media thickness (mm)	0.64±0.20	0.71±0.14	0.75±0.16*
Mean carotid diameter (mm)	7.19±0.89	7.23±0.55	7.58±0.95
Distension (mm)	0.58±0.17	0.51±0.17	0.49±0.16
Distensibility coefficient ()	29.4±12.9	30.1±14.1	20.1±8.9*†
Carotid stiffness (m/s)	6.2±1.4	6.1±1.1	7.5±1.5*†
Renal resistive index	0.58±0.04	0.59±0.04	0.65±0.05*†
Renal vasodilating capacity (%)	10.4±6.1	6.0±4.3*	4.3±4.9*

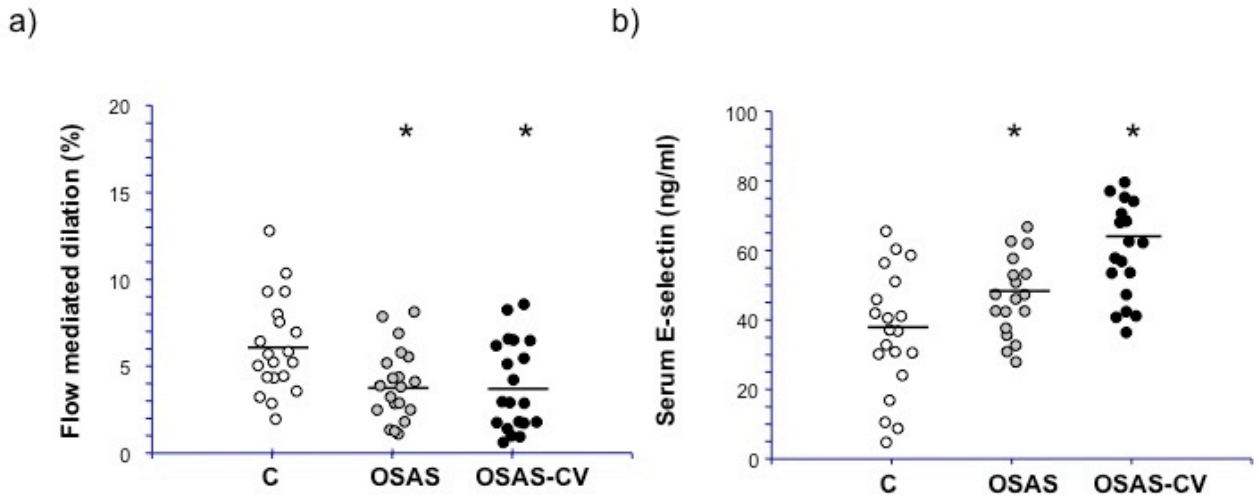
OSAS: obstructive sleep apnea syndrome; CV: cardiovascular; BP: blood pressure; PP: pulse pressure; GTN: glyceryl trinitrate; PWV: pulse wave velocity; *: $p < 0.05$ vs controls; †: $p < 0.05$ vs OSAS patients without CV risk factors.

Table 4. Relative expression at transcriptional level of leukocyte adhesion molecules, antioxidant enzymes, endothelial nitric oxide synthase and CD34

Gene name	Healthy subjects	OSAS patients without CV risk factors	OSAS patients with CV risk factors
CD11A (10^6 copies/μg RNA)	0.054	0.057	0.048
CD11B (10^6 copies/μg RNA)	2.331	2.877	2.266
CD11C (10^6 copies/μg RNA)	0.036	0.048	0.032
L-selectin (10^6 copies/μg RNA)	0.041	0.047	0.046
GPX1 (10^6 copies/μg RNA)	0.97	0.91	0.86
Catalase (10^6 copies/μg RNA)	0.82	0.78	0.80
eNOS (10^6 copies/μg RNA)	0.0221	0.0133*	0.0142*
CD34 (10^6 copies/μg RNA)	0.0202	0.0265	0.0114*

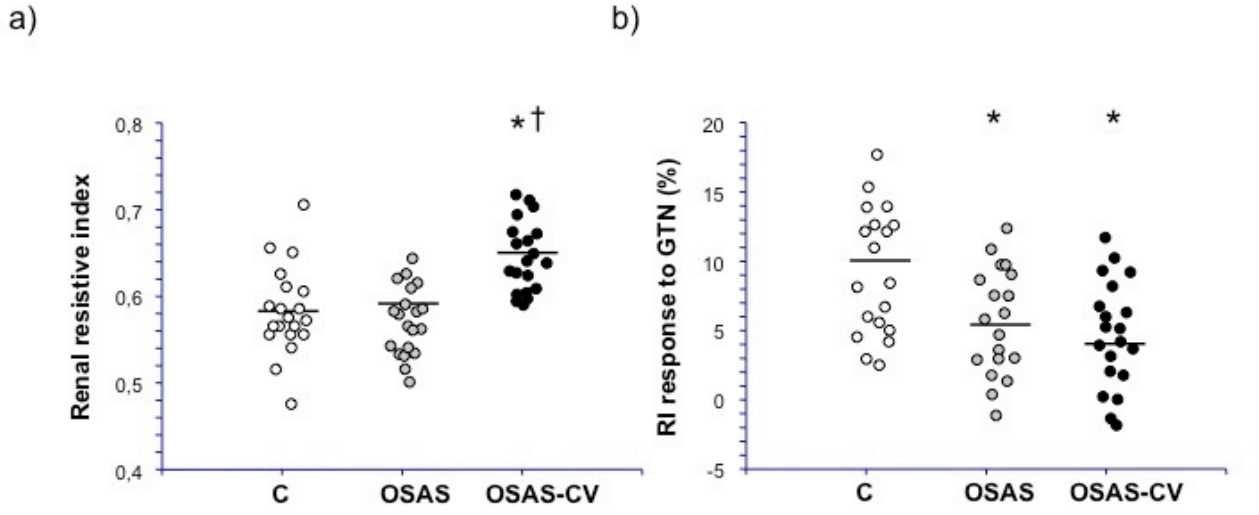
OSAS: obstructive sleep apnea syndrome; CV: cardiovascular; CD11A: integrin α-L; CD11B: integrin α-M; CD11C: integrin α-X; GPX1: glutathione peroxidase-1; eNOS: endothelial nitric oxide synthase; CD34: endothelial progenitors; *: $p < 0.05$ vs controls; †: $p < 0.05$ vs OSAS patients without CV risk factors.

Figure 16. Dot-plots representing flow-mediated dilation (a) and serum E-selectin (b) in the three study groups: healthy subjects (C, white circles), OSAS patients without (OSAS, grey circles) and with cardiovascular risk factors (OSAS-CV, black circles).



* $p < 0.05$ vs controls; † $p < 0.05$ vs OSAS patients without CV risk factors.

Figure 17. Dot-plots representing resting renal resistive index (a) and renal vasodilating capacity (b) in the three study groups: healthy subjects (C, white circles), OSAS patients without (OSAS, grey circles) and with cardiovascular risk factors (OSAS-CV, black circles).



* $p < 0.05$ vs controls; † $p < 0.05$ vs OSAS patients without CV risk factors.

Chapter 10.Vascular function in Healthy Himalayan High-Altitude Dwellers

Residents of the Himalayan valleys, chronically living at high altitude, have uniquely adapted to their hypoxic environment in terms of pulmonary vasculature, but their systemic vascular function is still largely unexplored. Thus the aim of the study was to investigate vascular function and structure in rural Sherpa population, permanently living in the Kumbu Valley (Nepal) (HA), in comparison with control Caucasian subjects (C) living at sea level. This research at the moment has been submitted for publication as an article.

10.1 Rationale

Many residents of the Tibetan Plateau live at high altitude, experiencing oxygen concentrations that are about 40% lower than those at sea level. Compared to other populations living at high altitude, such as Andean populations, they developed a favorable phenotype, characterized by decreased arterial oxygen content in the absence of pulmonary hypertension and polycythemia (Groves et al., 1993;Ge et al., 1994;Chen et al., 1997;Ge et al., 2002;Beall, 2007). In particular, oxygen delivery to the cells is suspected to be maintained by compensative modulation of vascular flow, probably due to tonically elevated circulating NO levels (Zhuang et al., 1993;Beall et al., 2001). NO is a key molecule in systemic and pulmonary vascular physiology, for its vasodilating, antihrombotic and antimitotic properties (Bruno and Taddei, 2011). Reduced NO availability in the systemic circulation, which is the main feature of endothelial dysfunction, has been recognized as the first step towards atherosclerosis development (Bruno and Taddei, 2011). Hypoxia can induce endothelial dysfunction and activation (Goerre et al., 1995;Berger et al., 2005) and diseases characterized by chronic hypoxia also present impaired NO-mediated vasodilation (Bruno et al., 2013a). However, vascular characteristics of populations chronically exposed to hypobaric hypoxia are still unknown. We hypothesized that chronic exposure to hypobaric hypoxia might impair endothelial function, thus favoring the development of CV damage.

Accordingly, the aim of the study was to investigate the presence of early markers of atherosclerosis in rural Sherpa population, permanently living in Kumbu Valley (Nepal), in comparison with lowlander Caucasian subjects. In particular we explored NO-mediated dilation at the brachial artery level, cardiac ultrasound, carotid geometry and stiffness, and aortic and peripheral PWV. Furthermore, the role of hypoxia on vascular features in this population was assessed by acute O_2 administration.

10.2 Experimental protocol

Study population - The study, which was part of the SHARE project (Stations at High Altitude for Research on the Environment), granted by EV K2 CNR, took place during two expeditions, in April and October 2011. The study population was constituted by 117 high-altitude dwellers, born and permanently living in the Khumbu Valley (Nepal) at high altitude (>2500 meters), enrolled by local advertising in three rural villages (2600, 3800 and 3800 meters respectively). Criteria of inclusion were age between 15 and 65 years, apparent good health status, and written informed consent. Criteria of exclusion were known established CV or renal disease, active infections or neoplasm, pregnancy. For the purposes of this study we further excluded those subjects with CV risk factors or taking CV medications, and those with pulmonary hypertension, assessed by echocardiography. We compared the vascular features of the selected 95 high altitude (HA) Himalayan healthy subjects with those of 60 Caucasian subjects (C), living and studied at the sea-level in Italy, recruited according to the same exclusion / inclusion criteria, and matched for age, sex, mean BP, and body mass index (BMI).

All the subjects enrolled were aware of the purposes of the study and gave written informed consent. The study was conducted with the approval of the Ethical Committee and of the Nepal Academy of Science and Technology (NAST) (Clinical Trials Gov Registration #NCT01329159).

Experimental session - All measurements were performed in the morning after an overnight fasting, in a quiet room. Women were studied during the follicular phase of the menstrual cycle. Since it was not possible to have controlled room temperature, room temperature was measured and accounted for as a confounding factor in the statistical analysis. Medical history was collected by Nepalese-speaking physicians (R.S. and K.T.). Brachial BP was measured with the patients resting in a supine position for at least 10 minutes under quiet environmental conditions. BP measurement was repeated three times at 2-minute intervals by a trained physician by using an automatic oscillometric device (OMRON-705IT, Omron Corporation: Kyoto, Japan). Average BP was then calculated on the last two measurements. Finger SO_2 (Pulse-oximeter Model Tuff-Sat, Datex-Ohmeda, General Electrics Healthcare Clinical System), weight and height were also taken. The following tests for vascular structure and function were performed: endothelium-dependent and -independent dilation in the brachial artery, carotid-femoral PWV, carotid stiffness, C-IMT. Static circumferential wall stress was also calculated, according to the formula: Mean BP · Mean internal carotid diameter)/(2 IMT) (Boutouyrie et al., 2004).

Echocardiography - Echocardiography was performed using a portable echo machine (Vivid I, General Electric Healthcare Clinical System) with a cardiac probe (2.5-3.5 MHz). Measurements of interventricular septum thickness, posterior wall thickness and left ventricular (LV) diastolic dimensions were taken at or just below the mitral valve tips, according to current guidelines, and used to calculated LV mass (Lang et al., 2005). LV end-systolic and end-diastolic volumes were measured and ejection fraction (EF) was calculated by the modified biplane Simpson's method (Lang et al., 2005). LV outflow tract diameter was measured in the parasternal long axis view, and its surface was calculated assuming circular geometry. Cardiac output was then obtained by multiplying LV outflow tract time-velocity integral by its cross-sectional area and heart rate (Quinones et al., 2002). After tricuspid regurgitation had been localized with Doppler color flow

imaging, the peak flow velocity of the transtricuspid jet was measured with the use of continuous-wave Doppler, and the pressure gradient between the right ventricle and the right atrium was calculated using the modified Bernoulli equation (Quinones et al., 2002). Systolic pulmonary artery pressure (PAP) was estimated from a trans-tricuspid gradient calculated from the maximal velocity of continuous Doppler tricuspid regurgitation as 4 x V2 + 5 mm Hg assigned to right atrial pressure (Yock and Popp, 1984). Left atrial pressure (LAP) was estimated from the ratio of Doppler mitral E flow-velocity wave and tissue Doppler mitral annulus flow e' early diastolic velocity (ie, LAP= 1.9+1.24 E/e') (Nagueh et al., 1997). Pulmonary vascular resistance (PVR) was calculated as (mean PAP-LAP)/CO (Argiento et al., 2012).

Effect of O_2 administration - In order to assess the role of hypoxia per se in inducing endothelial dysfunction, in 11 subjects with reduced FMD (<6%), the protocol was repeated after 100% O_2 administration for 1 hour, titrated to maintain SO_2 around 100%.

Statistical analysis - Statistical analysis was performed using NCSS 2008 (NCSS: Kaysville, Utah, USA). Results were expressed as mean ± SD. Differences in means among groups were analyzed using ANOVA for normally distributed variables, or Kruskal-Wallis Z Test for not normally distributed variables; categorical variables were analyzed by χ^2 test. Analysis of covariance was also used to compare vascular parameters, when indicated. Spearman's rank was used to explore correlations among variables. Multiple linear regression analysis was performed including parameters correlated with the dependent variable (FMD) with $p<0.10$.

10.3 Results

Clinical characteristics of the study population - 22 HA out of 117 were excluded because of the presence of CV risk factors (14 hypertension – 1 treated with beta-blockers, 5 smoking, 7 obesity). The remaining 95 HA were compared to 64 C. The clinical characteristics of the study population are listed in **Table 20**. As expected, HA had lower SO_2, higher heart rate and lower body size (in terms of height, weight and body surface area), but similar BMI, in comparison to C. Furthermore, in the presence of similar mean BP values, HA showed higher diastolic and lower systolic BP values, leading to a lower PP. Furthermore room temperature during the study was significantly lower in HA than in C.

Echocardiography - The rest echocardiographic parameters are reported in **Table 20**. LV diameters and wall thickness were significantly smaller in HA than in C: the difference was no longer significant after correction for body surface area, except for LD end-diastolic diameter and LV mass. EF and cardiac output, as well as E / e', were within normal limits and comparable in the two groups under investigation. Systolic and mean PAP and PVR were also within normal limits but were significantly higher in HA than in C **(Table 20)**.

Endothelial function in the brachial artery - BAD and FMD acquisition were successful in 57 C and 88 HA subjects, whereas SR calculation was available in 35 C and in 55 HA subjects. Baseline brachial artery diameter and SR were similar in the two groups; when considering body surface area as covariate, baseline BAD tended to be greater in HA than in C ($p=0.08$). HA presented reduced FMD, hyperemic SR, and FV-difference than C **(Table 21)**. A reduced endothelial function in HA was confirmed also by allometric analysis. Log-transformed difference between baseline and peak BAD (lnBAD-difference), considering log-transformed baseline BAD (ln-BAD) as covariate, was

significantly lower in HA as compared to C (corrected FMD 4.91 ± 0.51 vs 6.66 ± 0.67%, p=0.026). lnBAD-difference was significantly lower in HA than in C even when considering FV-difference as a covariate (p=0.031). Brachial artery response to GTN was increased in HA **(Table 21)**.

In C univariate analysis showed that FMD was significantly correlated with body surface area (r=-0.306, p=0.046), PP (r=-0.379, p=0.008), baseline BAD (r=-0.693, p<0.001, **Figure 18**), baseline SR (r= 0.462, p=0.005), and tended to correlate with age (r=-0.240, p=0.099). On the other hand, no significant correlation was found between FMD and hyperemic SR (r=0.230, p=0.189, **Figure 18**), FV-difference (r= -0.138, p=0.427), or response to GTN (r=0.035, p=0.847).

In HA univariate analysis showed that FMD was significantly correlated with PP (r= -0.225, p=0.036), baseline BAD (r= -0.216, p=0.043, **Figure 18**), hyperemic SR (r= 0.396, p=0.003), FV-difference (r=0.361, p=0.007, **Figure 18**) and tended to correlate with room temperature (r= -0.206, p=0.054), while no correlation was found with age (r= -0.134, p=0.213) and response to GTN (r=0.140, p= 0.198).

Multiple regression models were built in order to investigate independent determinants of FMD in the two populations **(Table 22)**. In Model 1, adjusted for vascular determinants of FMD (baseline BAD and FV-difference), BAD remained an independent predictor of FMD **(Table 22)** in Caucasian SL subjects. This was confirmed also by Model 2, considering as confounders the variables with correlated with FMD with p<0.10 at the univariate analysis. On the contrary in HA, multiple regression confirmed that FV-difference was significantly correlated with FMD, regardless of baseline BAD in Model 1 **(Table 22)**. In Model 2, FV-difference and room temperature remained independent predictors of FMD, after adjustment for baseline BAD and PP **(Table 22)**.

Superimposable results were obtained both in HA and SL in the allometric models, when lnBAD-difference was considered as dependent variable, with ln-BAD among confounding factors **(Table 22)**.

We also explored the relationship between endothelial function and variables connected to

adaptation to hypoxia or with established markers of cardiac and vascular damage. Among HA, FMD was not correlated to systolic and mean PAP (r=-0.150, p=0.162 for both) and PVR (r=-0.159, p=0.141), as well as to SO_2 (r=0.034, p=0.752) and heart rate (r=0.051, p=0.632). Similar results were obtained in the C group (data not shown). Conversely, in HA FMD correlated with LV mass index (r=-0.288, p=0.009) and tended to correlate with IMT (r=-0.177, p=0.099), with superimposable results in the C group (LV mass index: r=-0.431, p=0.036 and IMT r=-0.276, p=0.063).

Arterial stiffness and geometry - HA showed a significantly greater carotid diameter in comparison to C when body surface area was considered as covariate (p=0.004). Carotid IMT was significantly reduced in HA subjects (p=0.03, p=0.02 when adjusted for BSA). These features led to a reduced wall to lumen (W/ L ratio) and an increased static circumferential wall stress. Carotid compliance was significantly greater in HA than in C, whereas carotid-femoral and carotid-radial PWV were similar in HA and C **(Table 21)**. There were no differences in carotid-femoral PWV between the two groups even after inserting mean BP and age as a covariate (p=0.23). Augmented pressure and Augmentation index were similar in HA and C even after considering age, mean BP, height and heart rate as covariates (p=0.28 and p=0.80 respectively).

In HA IMT was related with SO_2 (r=-0.223, p=0.034), age (r=0.756, p<0.0001), BMI (r=0.328, p=0.002), mean BP (r=0.427, p<0.0001), carotid Augmentation index (r=0.311, p=0.003), carotid-femoral PWV (r=0.443, p<0.0001), carotid diameter (r=0.648, p<0.0001), distension (r=-0.390, p=0.0001) and distensibility (r=-0.502, p<0.0001), but not with FMD (r=-0.157, p=0.146). In a multiple regression model comprising age, BMI, mean BP, SO_2, augmentation index, carotid diameter and distension, age (p<0.0001), carotid diameter (p=0.005) and augmentation index (p=0.034) were directly and independently associated with IMT (full r2 0.678).

In C IMT was related with age (r=0.355, p=0.007), distension (r=-0.417, p=0.001) and distensibility

(r=-0.435, p=0.0008), tended to correlate with carotid-femoral PWV (r=0.220, p=0.095) and FMD (r=-0.274, p=0.063). In a multiple regression model comprising age, carotid-femoral PWV, carotid distensibility and PWV, only carotid distensibility (p=0.034) was inversely and independently associated with IMT (full r^2 0.199).

Effect of O_2 administration - In 11 Himalayan HA subjects, recruited in the same village (at 3800 m) and chosen because of low FMD levels (<6%), the same experimental protocol was repeated after 100% O2 administration for 1 hour. The individuals' baseline characteristics were superimposable to the remaining population of their village (n=37), except for body surface area (1.70 ± 0.14 vs 1.54 ± 0.13 m^2, p=0.02) and baseline FMD (3.10 ± 2.55 vs 5.61 ± 2.92%, p=0.01).

After O_2 administration, SO_2 rose from 89 ± 2 to 99 ± 1 % (p<0.0001). This was accompanied by a signficant reduction in heart rate (from 72 ± 14 to 55 ± 9 bpm, p=0.0003) and by unchanged BP (from 114±13 / 75±9 to 109±18 / 71±9 mmHg, p=ns). FMD increased significantly, in the presence of unchanged baseline BAD and FV-difference **(Figure 19)**. However this result was not confirmed by allometric scaling analysis (Corrected FMD from 2.6±0.7 to 2.5±0.7 %, p=0.84). An increase in augmentation index was observed (from 7.9±8.2 to 16.3±11.1, p=0.005), but was not significant when heart rate was considered as covariate (p=0.92). The remaining vascular parameters were not modified by O_2 administration.

10.4 Discussion

The main result of this study is the demonstration of a unique vascular phenotype in Himalayan healthy subjects born and permanently living at high altitude and free of traditional CV risk factors. This population presents an impaired NO-mediated dilation of the brachial artery, which was

independently associated with reduced hyperemic stimulus. Furthermore, impaired endothelial function might have clinical consequences in this population, since it is inversely associated with surrogate endpoints for CV events.

Physiological response to hypoxia in the systemic circulation is a balance between a direct vasodilating effect, which is prevalent during the first few hours of exposure (Thomson et al., 2006), and vasoconstriction induced by chemoreflex activation and increase in sympathetic traffic, which is prevalent after few days (Duplain et al., 1999;Bartsch and Gibbs, 2007;Parati et al., 2013). NO plays a key role in mediating acute hypoxic vasodilation in healthy humans in the resistance vessels (Blitzer et al., 1996). Furthermore, exogenous nitrite administration caused a greater vasodilation during hypoxia than during normoxia(Maher et al., 2008), possibly because an increased NO generation due to nitrite reductase activity of deoxyhemoglobin (Cosby et al., 2003).

High-altitude natives, such as Tibetans and Nepalese Sherpa, developed genetic adaptations to their hypoxic environment, as recently demonstrated (Simonson et al., 2010;Yi et al., 2010). In particular, O_2 delivery to the cells is suspected to be maintained by compensative modulation of vascular flow, probably due to elevated circulating NO levels, as well as increased ventilation (Zhuang et al., 1993;Beall et al., 2001;Beall, 2007) Concerning the microcirculation, an increased forearm blood flow has been demonstrated in Himalayan high-altitude dwellers (Erzurum et al., 2007), as well as in healthy volunteers not susceptible to high altitude pulmonary edema (HAPE) after 4-hour experimental hypoxia (Berger et al., 2005), but not in HAPE-prone individuals (Berger et al., 2005). In the present study we demonstrated an increased carotid and brachial artery diameter in HA in comparison to C, when adjusted for body surface area, supporting the hypothesis of a persistent systemic vasodilation in this population, which may act as a compensatory mechanism, aimed at maintaining tissue oxygenation.

While tonically increased NO production has been demonstrated in Himalayan dwellers, we have no information about stimulated NO release, which is the one associated with CV protection. After 4-hour experimental normobaric hypoxia, endothelial dysfunction in the microcirculation was

present only in HAPE-prone, but not in HAPE-resistant individuals (Berger et al., 2005). In patients with diseases associated with chronic hypoxemia at low altitude conduit artery endothelial function is altered even in the absence of traditional CV risk factors (Bruno et al., 2013a), possibly leading to increased CV morbidity and mortality. In Aymara healthy subjects, not affected by chronic mountain sickness but permanently living at high altitude, FMD seem to be preserved (Rimoldi et al., 2012). Our study demonstrated for the first time an impaired NO-mediated dilation in the brachial artery in Himalayan healthy subjects born and permanently living at high altitude, in the absence of classical CV risk factors. Brachial artery diameter is known to be the main determinant of FMD (Atkinson and Batterham, 2013): our study confirms this relationship in Caucasian lowlanders, but not in HA subjects. Reduced FMD in HA in comparison to C is also confirmed by allometric scaling analysis, which allows taking into account for differences in BAD between groups, as well of the non-linear relationship between baseline BAD and its flow-mediated increase (Atkinson and Batterham, 2013).

Hyperemic stimulus and FMD were not correlated in healthy Caucasian subjects, as previously demonstrated (Thijssen et al., 2009). On the contrary in HA FMD was significantly related to FV-difference, and the relationship was independent from confounders, with hyperemic stimulus explaining 8.7% of FMD variance. Measurement of hyperemic stimulus has become mandatory in FMD assessment, and some recent studies suggest that it could have an predictive role in CV disease that is even greater than FMD itself, being an index of microcirculatory dysfunction (Anderson et al., 2011). Pathophysiological mechanisms on the basis of this alteration in HA are unknown at the present. Impaired stimulated NO release can hardly be a consequence of tonically increased NO, inducing a chronically dilated macro- or microcirculatory district, given that in HA resting SR was not increased and the lack of dependence of FMD from BAD. The presence of structural alterations in macro- or microcirculation, hampering NO-mediated dilation, can be excluded since response to GTN is not reduced. Furthermore, hypoxia per se does not seem to play a relevant role, since O2 administration was not able to normalize vascular function. Although we

did not measure hemoglobin concentration in the study population, on the basis of current literature (Beall, 2007) we can hypothesize similar values in HA and C, thus excluding an increased blood viscosity in HA, possibly influencing shear stress and FMD. We suggest that the peculiar genetic background in Himalayan dwellers, protecting them from polycythemia and pulmonary hypertension, can negatively influence stimulated NO release. Mutations in the hypoxia-inducible factor (HIF) pathway were found by independent research groups in Tibetans and associated with their favorable phenotype (Simonson et al., 2010;Yi et al., 2010). Complex interrelationships between HIF and NO pathways have been documented (Ho et al., 2012), supporting this hypothesis, which however is highly speculative at the moment.

Which is the clinical significance of endothelial dysfunction in Himalayan HA dwellers? Lack of relationship between pulmonary pressure or SO_2 and FMD suggest that within this population a lower FMD is not an index of maladaptation to the challenging environment. Worth of note, in HA, as well as in C, a lower FMD is related to increased LVMI and carotid IMT, which are established surrogate intermediate endpoints of CV events. Thus, we hypothesize that impaired endothelial function might be associated with increased CV risk even in this population. Although to date the prevalence of CV risk factors and disease in this area is unknown, ischemic heart disease and diabetes represent the first cause of death in developing countries, with their health burden increasing over years (Abegunde et al., 2007).

Going to vascular structure and geometry results, we found no difference in aortic and peripheral PWV and in wave reflection between HA and C, excluding the presence of structural vascular alterations in these districts: however significant changes in PWV are expected over 50 years, thus this parameter could not be enough sensitive in the relatively young population studied (2010). On the other hand, large artery geometry was significantly altered: enlarged diameter, whose possible causes were already discussed, was accompanied by reduced IMT, leading to reduced W/L ratio and increased circumferential wall stress. A reduced IMT might indicate a lighter atherosclerotic burden in HA as compared to C, but might also represent a feature of smooth muscle relaxation (Thijssen et

al., 2011b). The failure to demonstrate a change in carotid diameter and IMT after O_2 administration might be due to the characteristic of the studied subgroup, which was small-sized and selected for reduced FMD and thus not representative of the overall population. Whatever is the mechanism inducing this unusual phenotype, further studies are necessary to ascertain the possible clinical consequences of increased carotid wall stress, which is of similar entity to that found in patients with Ehlers-Danlos syndrome (Boutouyrie et al., 2004).

In conclusion, Himalayan healthy subjects born and permanently living at high altitude are known for their particularly favorable adaptation to chronic hypoxia. This study demonstrated a unique vascular phenotype in this population, characterized by a mainly microcirculatory endothelial dysfunction and a maladaptive carotid remodeling as compared to Caucasian volunteers, studied at the sea level. These alterations occur even in the absence of classical CV risk factors, and might result from the combined effect of chronic exposure to hypobaric hypoxia and genetic background. Our study suggest that systemic CV burden of chronic hypoxia should not be overlooked in populations chronically living at high altitude as well as in lowlanders going to high altitude for recreational or working purposes, and in chronic diseases characterized by hypoxia.

Table 20. Clinical and echocardiographic characteristics of the study population

Variables	Caucasian controls - C (n=64)	Himalayan dwellers - HA(n=95)	p value
Men (%)	24, 37.5%	30, 31.6%	0.44
Age (years)	36.2±12.4	33.7±13.8	0.22
SO_2 (%)	98.2±0.91	90.6±2.6	<0.0001
Room temperature (°C)	23±1	18±2	0.02
Weight (kg)	65.0±12.3	57.5±8.3	<0.0001
Height (m)	1.69±0.09	1.57±0.08	<0.0001
Body surface area (m^2)	1.74±0.33	1.58±0.14	<0.0001
BMI (kg/m)	22.7±3.0	23.3±2.9	0.19
Systolic BP (mmHg)	119.7±11.5	113.2±11.6	0.0005
Diastolic BP (mmHg)	69.9±8.1	76.2±8.9	<0.0001
Mean BP (mmHg)	86.5±8.1	88.5±8.8	0.13
PP (mmHg)	49.9±9.9	36.8±9.8	<0.0001
Heart rate (bpm)	66.7±12.2	73.3±12.7	0.001
LV end-diastolic diameter (mm)	45±5	41±4	0.0008
LV end-sistolic diameter (mm)	26±5	25±5	0.11
Septum thickness (mm)	9.2±1.5	8.3±1.3	0.002
Posterior wall thickness (mm)	8.4±1.3	8.0±1.1	0.08
LV mass (g)	130±41	103±31	0.0002
LV mass index (g/m^2)	72±17	65±16	0.07
Cardiac output (l/min)	4.6±1.1	4.9±1.7	0.39
EF (%)	63.1±6.7	65.3±6.6	0.14
E / e' mean	5.71 ± 1.6	6,39 ± 2.1	0.09
LAP (mmHg)	8.3±1.7	9.3±2.6	0.02
Systolic PAP (mmHg)	23.6±4.8	29.4±5.5	<0.0001
Mean PAP (mmHg)	16.1±2.9	19.7±3.3	<0.0001
PVR (mmHg/min/L)	1.86±0.82	2.30±1.21	0.003

SO_2 :O_2 Saturation; BMI: body mass index; BP: blood pressure; PP pulse pressure; LV left ventricle; EF: ejection fraction; LAP: left atrial pressure; PAP: pulmonary artery pressure; PVR: pulmonary vascular resistances.

Table 21. Vascular characteristics of the study population

	Caucasian controls - C (n=64)	**Himalayan dwellers – HA (n=95)**	**P value**
Brachial artery diameter (mm)	3.57±0.77	3.64±0.74	0.47
FMD (%)	6.44±2.91	5.18±3.10	0.02
Baseline SR (1/s)	381±212	363±192	0.80
Hyperemic SR (1/s)	1724±746	1333±562	0.007
FV-difference (m/s)	0.60±0.25	0.45±0.20	0.002
Response to GTN (%)	6.90±2.47	8.21±3.11	0.04
Carotid-femoral PWV (m/s)	6.90±1.73	7.04±2.24	0.83
Carotid-radial PWV (m/s)	7.86±2.28	8.76±2.08	0.09
C-IMT (mm)	0.576±0.122	0.509±0.121	0.00008
Mean carotid diameter (mm)	6.81±0.85	6.98±1.07	0.48
Distension (mm)	0.57±0.13	0.53±0.10	0.06
Wall to lumen ratio	0.17±0.04	0.15±0.02	<0.0001
Circumferential wall stress	56.4±16.0	67.6±13.1	<0.0001
Carotid compliance (m^2*kPa^{-1})	1.07±0.68	1.24±0.63	0.04
Carotid distensibility (kPa^{-1})	33.2±12.3	38.2±17.9	0.07
Carotid PP (mmHg)	44.3±10.5	36.7±12.1	<0.0001
Augmented Pressure (mmHg)	5.7±3.2	6.5±5.4	0.92
Augmentation Index (%)	6.7±8.8	10.8±14.2	0.21
Young's elastic modulus (kPa)	0.31±0.09	0.35±0.10	0.62

FMD: flow-mediated dilation; SR: shear rate; FV: flow-velocity; GTN: glyceril trinitrate; PWV: pulse wave velocity; C-IMT: carotid intima-media thickness; PP: pulse pressure.

Table 22. Multiple regression models exploring determinants of endothelial function in Caucasian controls (a) and Himalayan high-altitude dwellers (b).

a)

		Variable	Standardized β	p value	r^2
Dependent variable FMD	Model 1 (0.516 full r^2)	Baseline BAD	-0.719	<0.0001	0.516
		FV-difference	0.002	0.989	<0.0001
	Model 2 (0.582 full r^2)	Baseline BAD	-0.832	<0.0001	0.472
		FV-difference	-0.060	0.690	0.003
		age	-0.064	0.661	0.014
		PP	-0.063	0.673	0.057
		BSA	0.223	0.210	0.035
Dependent variable lnBAD-diff	Model 1 (0.526 full r^2)	lnBAD-bas	-0.729	<0.0001	0.516
		FV-difference	0.023	0.854	0.010
	Model 2 (0.612 full r^2)	lnBAD-bas	-0.882	<0.0001	0.508
		FV-difference	-0.060	0.577	0.003
		age	-0.054	0.700	0.001
		PP	-0.040	0.779	0.057
		BSA	0.268	0.125	0.035

b)

		Variable	Standardized β	p value	r^2
Dependent variable FMD	Model 1 (0.142 full r^2)	Baseline BAD	-0.215	0.105	0.060
		FV-difference	0.288	0.031	0.082
	Model 2 (0.253 full r^2)	Baseline BAD	-0.832	0.151	0.048
		FV-difference	-0.060	0.022	0.087
		room temperature	-0.306	0.019	0.111
		PP	-0.092	0.471	0.008
Dependent variable lnBAD-diff	Model 1 (0,147 full r^2)	lnBAD-bas	-0.222	0.093	0.048
		FV-difference	0.293	0.029	0.099
	Model 2 (0,257 full r^2)	lnBAD-bas	-0.202	0.116	0.040
		FV-difference	0.300	0.020	0.103
		room temperature	-0.302	0.020	0.107
		PP	-0.084	0.507	0.007

FMD: flow-mediated dilation; BAD: brachial artery diameter; FV: flow velocity; PP: pulse pressure; BSA: body surface area.

Figure 18. Scatterplots showing the relationship between FMD and brachial artery diameter (on the left) and FVI-difference (on the right) in Himalayan high-altitude dwellers (black circles) and Caucasian controls (grey circles)

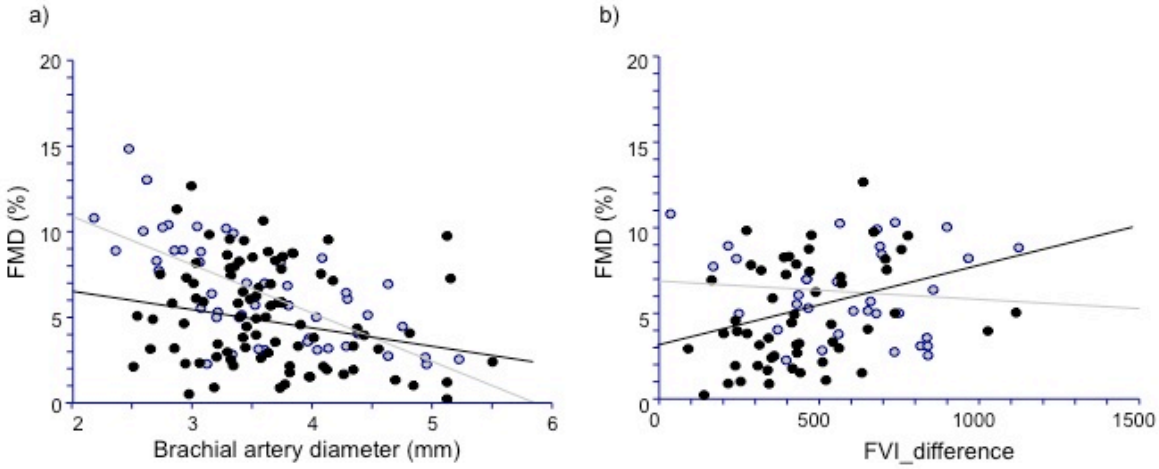
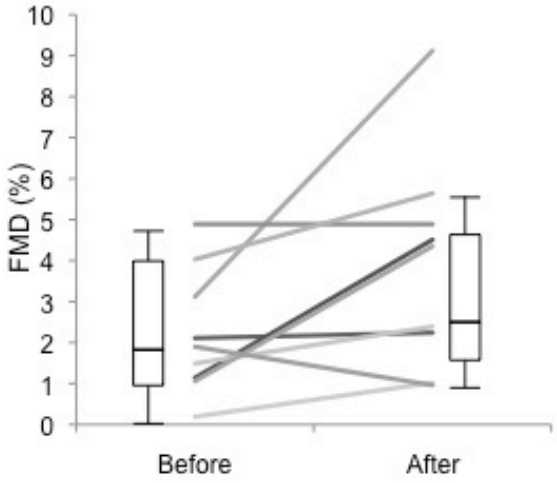


Figure 19. FMD behavior before and after 1-hour 100% O_2 administration in a subgroup of Himalayan high-altitude dwellers.



Chapter 11: Conclusions

CV disease remains a major cause of disability and mortality worldwide, with prevention as the best approach to the problem. In order to improve the accuracy of risk stratification beyond the evaluation of traditional CV risk factors, in the last decades the use of innovative non-invasive biomarkers has been proposed, able to identify subclinical CV disease and provide an integrated index of vascular damage exerted by different risk factors.

Some biomarkers are currently recommended by International scientific societies in order to improve stratification of CV risk, whereas other are considered useful only for research purposes (Mancia et al., 2007;Mancia et al., 2009;Greenland et al., 2010). Several questions in this field are still open, limiting the wide use of these tools in the clinical practice. Methodological as well as pathophysiological and prognostic aspects should be clarified before their widespread use for risk stratification in subjects with traditional risk factors as well as primarily non-CV conditions. In this PhD thesis I examined cross-sectionally a cohort of healthy subjects and patients with traditional and emerging CV risk factors in order to elucidate some of these aspects. My original contribution to the knowledge in this field consists in:

- the demonstration of a "functional" component in aortic stiffness, that is present only in diabetic patients and relies on endothelium-mediated mechanisms;
- the demonstration of a differential impact of different CV risk factors in the musculo-elastic and elastic arteries;
- the demonstration, in hypertensive patients, of an additive role of carotid and aortic stiffness on cardiac organ damage;
- the identification of a new marker of renal vascular damage.

Furthermore, I demonstrated the feasibility of a multiparametric approach, based on the use of several biomarkers of vascular function and structure, as a useful strategy to detect early vascular damage in non primarily CV diseases and conditions, with the double aim of elucidating the

pathophysiology of CV complications in non-CV diseases and, in long term, of proposing the most useful test to be used in the clinical practice for screening and follow-up.

My original contribution to the knowledge in this field consisted in:

- the demonstration of a selective reduction of circulating endothelial progenitor cells, in the presence of preserved vascular function and structure, in young adults exposed during childhood to environmental radiation doses after the Chernobyl disaster and to therapeutic radioiodine treatment after thyroid cancer
- the demonstration that conduit artery endothelial dysfunction and impaired renal vasodilating capacity are part of the vascular phenotype of OSAS per se, since they are present even in the absence of traditional CV risk factors, while structural alterations such as arterial stiffness and increased C-IMT characterized only obese and/or hypertensive OSAS patients
- the demonstration that Himalayan high altitude dwellers, chronically living above 2500 meters of altitude, are characterized by a mainly microcirculatory endothelial dysfunction and a maladaptive carotid remodeling even in the absence of traditional CV risk factors.

An important limitation that should be taken into account when interpreting these findings is represented by the cross-sectional design of the studies. More robust evidence will come from longitudinal studies, aimed at assessing: the natural history of vascular alterations in the presence of a variety of traditional and emerging CV risk factors; the influence of pharmacological and non-pharmacological interventions on different biomarkers of vascular function and structure; the predictive value and the prognostic significance of changes of these biomarkers for the individual in terms of development of organ damage and CV morbidity and mortality.

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