Imperial College London

# Effect of ageing on carotid artery morphology, hemodynamics, and the development of atherosclerosis.

by

**Claudio Carallo**

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Department of Clinical and Experimental Medicine, "Mater Domini" Hospital, "Magna Græcia" University, Catanzaro, Italy

Department of Chemical Engineering, Imperial College London

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# Declaration

I hereby declare that this Thesis and the work reported herein is the original work of the Author. Information derived from the unpublished work of others has been reported in the Acknowledgments Section. Information derived from the published work of others has been used under written permission and references are given in the list of sources and in the Appendix 3.

Claudio Carallo

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# Abstract

Cardiovascular diseases and ageing are two main challenges for health services. Cardiovascular disease is characterised by atherosclerosis, leading to heart attack and stroke. Atherosclerosis is a focal disease and occurs preferentially in regions of arterial bifurcation and curvature where complex flow features are observed.

The carotid arteries represent a region of significant involvement in atherosclerosis. Previous studies have shown that haemodynamic factors are important determinants of the local distribution of atherosclerosis. However, longitudinal studies are lacking. The aim of this study was to investigate age-related changes in carotid artery morphology and haemodynamics based on longitudinal data acquired from a group of middle-aged subjects recruited to a cardiovascular disease prevention programme in Italy. The longitudinal study started in 1996 and participants were examined twice 12 years apart.

All subjects underwent blood viscosity measurements and echo-Doppler examinations of the common carotid artery at baseline and follow-up. From the acquired ultrasound data, common carotid artery diameter, blood flow velocity, and intima-media thickness were measured, and wall shear stress, circumferential wall tension and Peterson elastic modulus were calculated.

It was found that with ageing, blood viscosity increased, common carotid artery diameter increased, mean blood velocity and wall shear stress decreased, while intima-media thickness, circumferential wall tension and arterial stiffness increased. Interrelationships of the data were also examined: reductions in common carotid wall shear stress were independently associated with intima-media thickening. Furthermore, ageing-associated wall shear stress reduction predicted the development of atherosclerotic plaques, independently of known cardiovascular risk factors. In addition, in

participants presenting shear stress reductions in only one side of the common carotid artery, development of atherosclerosis in the carotid tree was limited to the same body side.

In conclusion, this longitudinal study confirms the role of arterial wall shear stress as a mediator of the effects of ageing on atherosclerosis.

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# Chapter 1. Introduction

This chapter starts with a brief description of the main components of the study: large arteries, atherosclerotic process, and haemodynamics. This is followed by a summary of our current understanding of the role of arterial ageing in atherosclerosis, along with haemodynamic alterations. Finally, the aims of the present Thesis are specified.

## 1.1. Preamble

Cardiovascular diseases such as myocardial infarction, stroke and peripheral vascular disease are the most frequent causes of death and disability in developed countries. These diseases are frequently caused by commonly known risk factors such as hypertension, diabetes, obesity and smoking, but they are also associated with a number of biomechanical risk factors. In fact, it is recognised that fluid mechanics of the arterial circulation determines or modifies biomechanical factors that, in turn, regulate endothelial cell functions that are important in the pathophysiology of cardiovascular diseases. Consequently, the atherosclerotic process, which is the basis of cardiovascular diseases, is a systemic disease, but with focal manifestations strongly dependent on local haemodynamic factors. Arterial ageing is a common ground for establishing and understanding the interaction of all these known and emerging cardiovascular disease risk factors.

The present Thesis starts with a Literature review on the relevant topics. This is followed by detailed descriptions of the medical procedures and haemodynamic measurement techniques used. After this, the analysis of variations of blood viscosity and carotid haemodynamic factors with

ageing is presented. Furthermore, the impact of these variations on the development of atherosclerosis with ageing is analysed. Finally, a Perspective section explores possible improvements of some of the techniques used in data collection and analysis.

## 1.2. The structure and function of conductance arteries

The large conductance arteries are those most involved in the ageing and atherosclerosis processes. A frequently studied region is the carotid bifurcation, which is prone to atherosclerotic lesions that are responsible for strokes. Within this region, the common carotid artery (Figure 1.1) has been studied extensively, because it is easily accessible for ultrasound imaging. For the same reason, the common carotid artery was chosen to be the focus of the present Thesis. The common carotid artery runs on both sides of the trachea and larynx and passes deep to the sternocleidomastoid. It arises on the right from the brachiocephalic trunk and on the left directly from the aortic arch (Feneis et al. 2007).

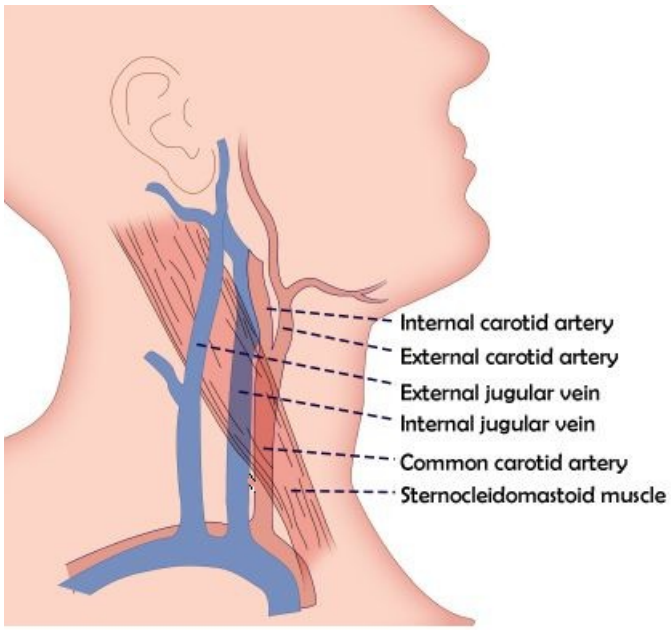



**Figure 1.1: The right common carotid artery.**

The morphological structure of this artery mirrors the complexity of its functions (Ross et al. 1976). The wall of large arteries consists of three functionally separate layers conventionally termed the intima, the media, and the adventitia. The intimal layer is composed of a monolayer of endothelial cells, in contact with circulating blood, which serves as a functional barrier regulating transport and metabolism, and modulating local and systemic vascular physiologic functions. The media consists of two subcomponents: 1) smooth muscle cells regulating vascular tone in response to local and humoral stimuli; 2) an extracellular matrix, mainly composed of collagen and elastin. The adventitia, meanwhile, harbours nutrient vessels (vasa vasorum), nerves, and dense fibroelastic tissue.

The endothelium is the inner most part of the vessel directly in contact with the flowing blood; it senses mechanical stimuli and modulates its function accordingly (Gokce et al. 1998). In endothelial cells (Figure 1.2), a pivotal role is played by a constitutive membrane-associated nitric oxide synthase that catalyses the conversion of amino acid L-arginine to nitric oxide and L-citrulline (Luiking et al. 2012). It has several agonists, both biochemical (acetylcholine, bradykinin, substance P, and platelet-derived serotonin), and mechanical (increased shear stress, the tangential stress of the blood flow on the endothelium) (Gokce et al. 1998). Nitric oxide modulates vascular tone exerting a relaxant effect on the vascular smooth muscle, and inhibits platelet activation (Luiking et al. 2012). Other actions of nitric oxide include inhibition of monocyte adhesion and smooth muscle cell proliferation. Individuals with coronary risk factors or atherosclerosis demonstrate impaired shear stress and agonist-induced endothelium-dependent vasodilation (Ando et al. 2011).

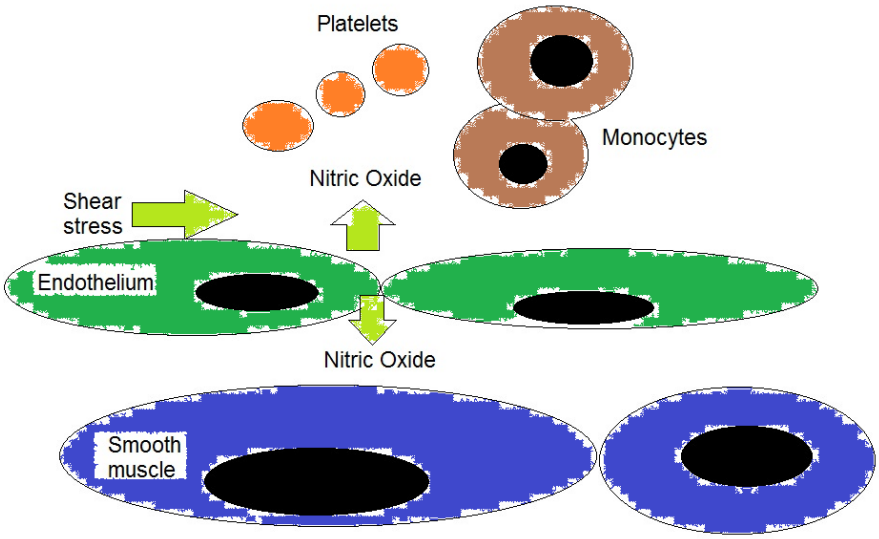



**Figure 1.2: Endothelium-derived nitric oxide synthesis and action.**

## *1.3. Basics in atherosclerosis*

In pathophysiology, the homeostasis described above is altered by various aetiologies (Ross 1993). In response to mechanical injury or exposure to atherogenic stimuli, such as hypercholesterolemia, diabetes mellitus, hyperhomocysteinemia, and cigarette smoking, endothelial cells express adhesion molecules and elaborate growth factors that lead to the recruitment of leukocytes in an inflammatory response. Leukocytes adhere and migrate into the vessel wall, localise subendothelially, and develop into lipid-laden macrophages (foam cells). These foam cells, in turn, release growth factors and cytokines that promote the recruitment of smooth muscle cells and stimulate neointimal proliferation, continue to accumulate lipid, and support endothelial cell dysfunction. Collectively, these events promote the development of a lipid-rich atheromatous lesion. Subsequent denudation of the endothelium exposes circulating platelets and coagulants to the underlying matrix, thereby initiating thrombosis, and triggering a cascade of events leading to a fibroproliferative lesion and luminal narrowing. The final event of this atherosclerotic process is

sometimes an arterial occlusion, and/or a plaque rupture with subsequent luminal thrombosis (Dobroski et al. 1998). A lipid-rich core (particularly in the shoulder regions of lesions), an abundance of inflammatory cells, a thin fibrous cap, and a dysfunctional overlying endothelium characterize the morphologic features of lesions which are prone to rupture. A dysfunctional endothelium may contribute to the propensity of plaque rupture owing to its proinflammatory, prothrombotic and vasoconstrictive properties which increase lesion composition, growth responses, vascular tone and local shear stress (Nilsson et al. 2017).

## 1.4. Haemodynamics in large arteries: an overview

### 1.4.1. Blood

#### *1.4.1.1. Introduction*

The interest for haemodynamics in cardiovascular medicine derives from the observations that the incidence of atherosclerosis is more frequent at arterial bends or branches, where haemodynamic disturbances occur (Zarins et al. 1983, Asakura et al. 1990). Human blood (Matrai et al. 1987) has physical properties mainly depending on its major constituents: viscous plasma and viscoelastic erythrocytes, whereas platelets and leucocytes play a minor role in the macrocirculation. The resulting mixture presents a physical behaviour quite similar to emulsions. Furthermore, other important rheological and biochemical phenomena occur at the interfaces between these different components.

#### *1.4.1.2. Plasma viscosity*

The viscosity of normal plasma (Brooks et al. 1987) is mainly determined by the macromolecules contained in the water medium, depending on their shape (elongated proteins as fibrinogen impact more on viscosity), molecular weight (pentameric immunoglobulin-M), and concentration (the small albumin is largely the most represented plasma protein).

*1.4.1.3. Erythrocyte deformability*

In large arteries, normal erythrocytes (Brooks et al. 1987) change their shape in response to external forces depending upon cytoskeleton, actin/myosin tensile status, and membranes viscoelasticity. Depending upon the values of the shear forces, erythrocytes can simply rotate as a rigid body, or also present a convective motion of the cellular envelope around the interior content. This is also important for its influence upon red cell aggregation.

*1.4.1.4. Interactions between red blood cells and with the endothelium*

Red blood cells can easily and spontaneously stack up forming aggregates, often with shapes of *rouleax*, variously interconnected; this erythrocyte aggregation is reversible under flow conditions, when cells are in blood layers that move at different velocities. The difference in the velocity of blood layers is called shear rate. If shear rate values overcome the reciprocal attraction of erythrocytes, they are disaggregated. Aggregation occurs when red cells are suspended in autologous plasma, or in a solution containing large polymers. Although the exact mechanism of this process has not been fully elucidated, the presence of fibrinogen in normal blood, as well as other high molecular-weight components, such as alpha-2 macroglobulin and immunoglobulin M, is essential (Neu et al. 2007). Furthermore, aged erythrocytes (i.e. close to their end of life) are more prone to aggregation (Nash et al. 1987). Obviously, the same molecules also influence plasma viscosity.

Unlike platelets and white cells that can carry out their functions (haemostasis and inflammation) via adhesion to the endothelium, red blood cells tend not to interact with the vessel wall (Brooks et al. 1987). In fact, in conditions of laminar (i.e. non-turbulent) flow, due to their propensity to the aggregation, red cells tend to transit into small arteries as long rouleax in the central area of the

vessel cross section; in large arteries also, they do not occupy regions close to the endothelium (Gaehtgens et al 1987).

*1.4.1.5. Blood viscosity*

All the above reported features of normal blood influence, in various ways, the viscosity of the whole blood (Matrai et al. 1987). The two main factors are haematocrit values, and red cell aggregation. The latter resulting in blood viscosity being inversely related to the shear rate; in other words, blood viscosities are low at high shear rates, and whole blood behaves as a non-Newtonian fluid (i.e. viscosity changes with shear rate) with a pseudoplastic behaviour (i.e. presenting an inverse and nonlinear relationship between these two values) (Figure 1.3).

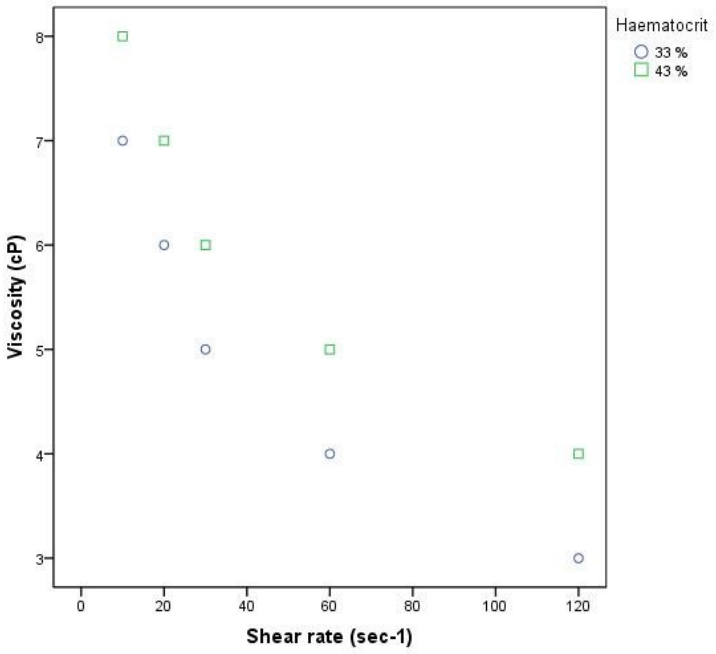



**Figure 1.3: Variations of whole blood viscosity with shear rate at different haematocrit values**

## *1.4.2. Vessel wall stresses*

*1.4.2.1. Surface haemorheology*

The dynamic behaviour of blood moving along a vessel following pressure gradients generates some stresses/strains on the vessel wall (Humphrey et al. 2009), schematically represented in Figure 1.4. First, the frictional force of the blood on the endothelial surface is named wall (flow) shear stress, and its mechanotransduction towards deeper large artery structures has significant biological effects, as described below. Therefore, wall shear stress is also the main topic of the present Thesis. Shear stress is directly proportional to volumetric blood flow and viscosity, and inversely to the cube of the vessel radius, following the Hagen-Poiseuille formula. Second, the normal (cross-sectional) component of the pressure on the vessel wall induces circumferential stress, causing a dilation mainly of large arteries containing less actomyosin fibers in their elastic media than muscular smaller arteries. Circumferential stress is computed as the product of blood pressure and vessel radius, which is often divided ("normalized") by the vessel wall thickness; the latter tends to increase in response to elevated pressure. Third, the axial (longitudinal) stress, driven by the axial load, tends to elongate the arteries. The role of axial stress and its biological effects are less studied, compared to the other two arterial stresses.

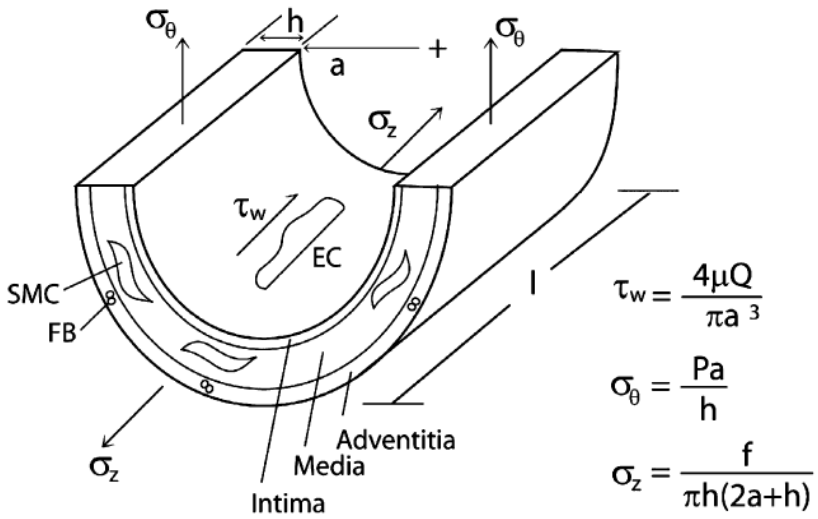



**Figure 1.4: Three primary geometric variables (radius a, thickness h, and length l) defining a straight segment of an artery and the associated three primary stresses: shear stress ($\tau_w$), circumferential stress ($\sigma_\theta$) and axial stress ($\sigma_z$) (SMC = smooth muscle cells; FB = fibroblasts; $\mu$ = blood viscosity; Q = blood flow volume; P = blood pressure; f = axial load). (Humphrey et al. 2009, with permission).**

In an ideal reconstruction of a long rigid straight artery (Figure 1.5), shear stress exerted by the blood flow is determined by the shear rate (i.e. velocity gradient) at the wall multiplied by blood viscosity (Wentzel et al. 2012). Therefore, assuming a fully developed blood flow velocity profile along the vessel as if blood was a Newtonian fluid in a laminar flow, the magnitude of shear stress is directly proportional to blood viscosity and mean velocity, and inversely to arterial diameter; this simplification is only valid for Poiseuille flow.

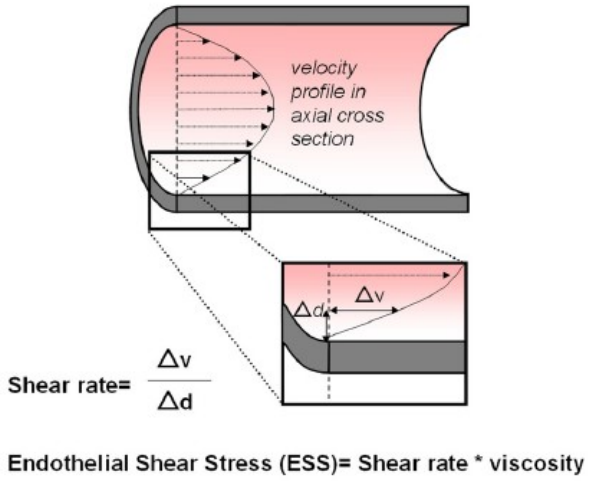



**Figure 1.5: Wall shear stress (Wentzel et al. 2012, with permission).**

However, in vivo blood flow in large arteries differs from Poiseuille flow in several aspects (Matrai et al. 1987). First, arteries are curved, branched, and/or too short (generally less than 10 times their diameters) for flow to become fully developed. Second, due to the shear-thinning behaviour of blood, blood flow velocity profile tends to be blunt at the centre compared to a fully-developed parabolic velocity profile for a Newtonian fluid. Third, because of non-uniform shear rate and the shear rate dependence of blood viscosity, blood viscosity is not constant in the vessel cross section or during a cardiac cycle. Fourth, even if blood flow is generally laminar in a straight large artery, acceleration or deceleration of flow during the cardiac cycle might trigger turbulence. A measure of

flow transition to turbulence is given by the Reynolds number, which is defined as the product of vessel diameter, blood velocity and density, and divided by blood viscosity; its value > 2000 (dimensionless) indicates transition to turbulence. Fifth, the endothelial surface is not smooth, due to nuclei and glycocalix of the cells encroaching into the lumen. Therefore, due to the differences between an ideal Poiseuille model and real flow, in vivo shear stress measurement is desirable, but is still technically very challenging. This will be further discussed later.

*1.4.2.2. Mechanotransduction of shear stress*

Endothelial cells sense shear forces via a complex network of mechano-transductors, which work together to exert vascular effects in response to changes in wall shear stress via biochemical signals (Collins et al. 2011). Starting from the endothelial surface, glycocalyx (Kang et al. 2011), calcium (Li et al. 2014) and potassium channels (Hoger et al. 2002) are some of the most important external mechanosensitive structures protruding into the vessel lumen and provided of transmembrane domain, able to respond to shear stress with conformational changes. Particularly, calcium channels are able to directly regulate intracellular calcium concentration, which is one of the major cytoplasmic second messengers of shear stress. Under the plasma membrane, cytoskeleton is composed of rigid structures surrounded by tensional elements, able to undergo both acute and stable conformational changes in response to cell stretch (Ueki et al 2010). Cytoskeletal reorganization is one of the major effectors of the alignment of endothelial cells along the blood flow (del Alamo at al. 2008). Deeper into the cell, cytoskeleton interacts with transmembrane adhesion proteins named integrins, that are also drag force sensors (Tzima et al. 2005); outside of the cell, integrins are physically and functionally linked to the intercellular matrix proteins of the subendothelial space, and to other integrins belonging to the adjacent endothelial cells (Melchior et al. 2010).

*1.4.2.3. Vascular effects of shear stress: molecular and cellular mechanisms*

Wall shear stress has been recognised to have a significant influence on vascular biology, and supporting evidence has been obtained from *in vitro* and *in vivo* experiments (Chatzizisis et al. 2007). Figure 1.6 shows the role of low shear stress in the development of atherosclerosis and the associated molecular and cellular mechanisms.

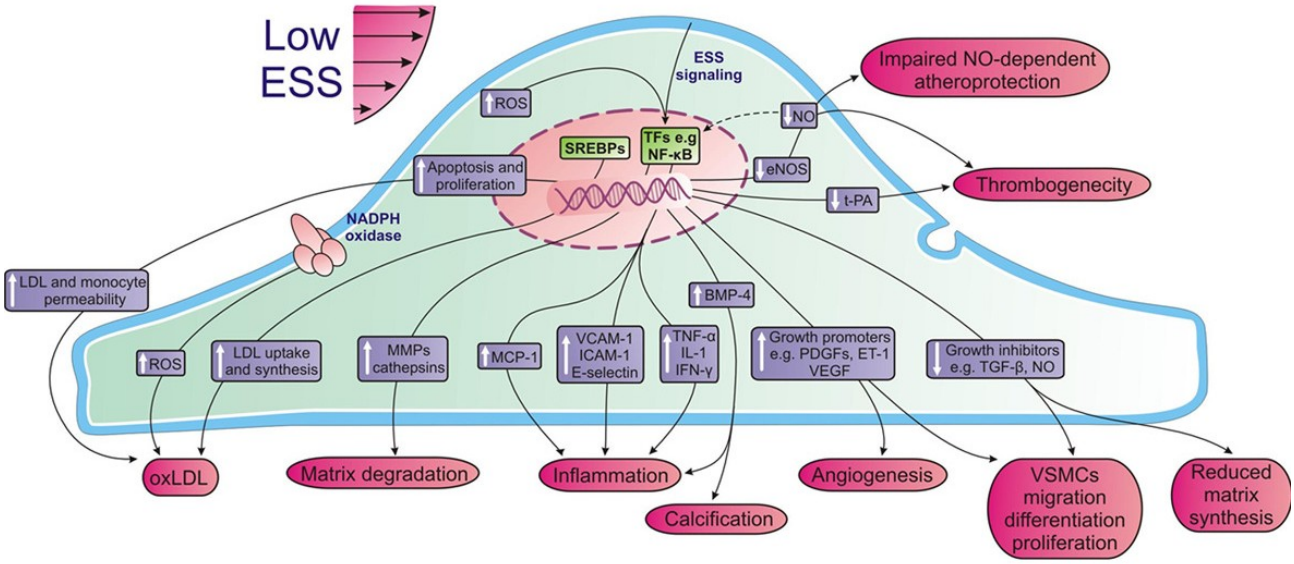



**Figure 1.6: Molecular and cellular mechanisms through which low shear stress promotes atherosclerosis. BMP=bone morphogenic protein; eNOS=endothelial nitric oxide synthase; ET=endothelin; ESS=endothelial shear stress; ICAM=intercellular adhesion molecule; IFN=interferon; IL=interleukin; LDL=low-density lipoprotein cholesterol; MCP=monocyte chemoattractant protein; MMP=matrix metalloproteinase; NO=nitric oxide; PDGF=platelet-derived growth factor; ROS=reactive oxygen species; SREBP=sterol regulatory elements binding protein; TF=transcription factor; TGF=transforming growth factor; TNF=tumour necrosis factor; t-PA=tissue plasminogen activator; VCAM=vascular cell adhesion molecule; VEGF=vascular endothelial growth factor; VSMC=vascular smooth muscle cell (Chatzizisis et al. 2007, with permission).**

Shear stress is crucial for fundamental physiological arterial functions. It has been shown that high shear stress promotes angiogenesis, flow augmentations and cell replication, whereas low wall shear stress may cause vessel obliteration, flow reduction and cell apoptosis (Chatzizisis et al. 2007). Endothelial mechanisms are by post-transcriptional protein modifications, or via the phosphorylation of several transcription factors, which involves binding of shear stress responsive

elements with promoters of mechanosensitive genes (Brooks et al. 2002). Studies of endothelial pathophysiology show that normal shear stress experienced in areas of laminar flow stimulates the expression of various atheroprotective genes, whereas in regions with low and/or oscillating shear stress pro-atherogenic genes are upregulated (Humphrey 2008). This is realized through several pathways which are described below.

First, physiologic shear stress is a potent stimulus for endothelial nitric oxide production through prostacyclin synthase, elicitating its vasodilating, antiplatelet and antiproliferative activities (Koller et al. 1995), while downregulating endothelin-1, a potent vasoconstrictive and mitogenic molecule (Malek et al. 1992). Second, via the endothelial activation of sterol regulatory elements binding proteins, low shear stress upregulates the expression of genes encoding LDL receptor, cholesterol synthase, and fatty acid synthase, favouring lipid accumulation (Liu et al. 2002). Also by these ways, shear stress influences plaque size and composition (Segers et al. 2008). Third, inflammation is another important pathway activated by low shear stress, via the expression of vascular and intercellular adhesion molecules, and cytokines as tumour necrosis factors (Wara et al. 2008). On the other hand, low shear stress might also be a consequence of chronic inflammatory diseases as in periodontitis (Carallo et al. 2010; Carallo et al. 2013), the treatment of which allowed a reversion of the haemodynamic impairment (Carallo et al. 2015). Fourth, bone morphogenic protein-4 is upregulated by oscillating shear stress; it participates in plaque calcification, suggesting a potential role of low shear stress in the formation of arterial spotty deposits of calcium (Sorescu et al. 2003). Fifth, oxidative stress is elevated in endothelial cells experiencing a disturbed haemodynamic profile, by an upregulation of NADPH oxidase (Hwang et al. 2003). Sixth, neovascularisation is involved in low shear-induced atherosclerosis, via an upregulation of the vascular endothelial growth factor and angiopoietin-2 (Dai et al. 2004). Finally, haemostatic processes are modulated by haemodynamic disturbances, both by a downregulation of anticoagulant plasma mediators as tissue-plasminogen activator (Papadaki et al. 1998), and via an enhancement of platelet aggregation and adhesion, both in vitro and in vivo (De Franceschi et al. 2015, Tanahashi et al. 1999).

## 1.5. Pathophysiology of vascular ageing in large arteries, and atherosclerosis development

### 1.5.1. Introduction

The incidence of major cardiovascular diseases, such as heart disease and stroke, increases exponentially with ageing, accounting for more than 40% of all deaths among people aged 65 – 74 years and almost 60% at the age of 85. Ageing results in phenotypic changes rendering the cardiovascular system prone to atherosclerosis, even more if in presence of its risk factors, such as hypertension, diabetes, and obesity (Ungvari et al 2010). In addition, clusters of interrelated atherosclerosis risk factors as in the metabolic syndrome, and its frequent epiphenomenon of hepatic steatosis, are also associated with carotid atherosclerosis (Carallo et al. 2009).

### 1.5.2. Molecular mechanisms: endothelial dysfunction

One of the main underlying mechanisms of arterial damage in the elderly involves endothelial dysfunction, particularly via a failure of vasodilator nitric oxide; this kind of damage also occurs in healthy older people, but earlier in males than in females (Celermajer et al. 1994). The presence of a cardiovascular risk factor such as arterial hypertension predisposes the development of endothelial dysfunction (Taddei et al. 1997), and this effect is additive in clusters of cardiovascular risk factors (Vlachopoulos et al. 2015). Nitric oxide depletion is partly a consequence of redox cellular balance (Adler et al. 2003), but because one of the most potent activators of nitric oxide production is fluid shear stress, this dysfunction partially results from shear stress reduction and endothelial insensitivity to shear stress (Collins et al. 2011). As a result of decreased nitric oxide availability, ageing vessels often exhibit endothelial cell senescence and increased oxidative stress, whereas physiological shear stress in vitro has also been shown to rejuvenate endothelial cells and improve

the ability of endothelial progenitor cells to repair mechanical endothelial damage (Fadini at al. 2012).

### *1.5.3. Molecular mechanisms: inflammation, endothelial microparticles*

Aged central arteries exhibit a chronic proinflammatory profile that relies on several biochemical mediators. On the cell surface, an alteration in signalling systems of angiotensin II and endothelin 1, together with the recruitment of inflammatory molecules by advanced glycation products, have been described (Wang et al. 2014). Elicited mechanisms include increases of transforming growth factor-beta1 and metalloproteinases (Wang et al. 2006). Furthermore, endothelial microparticles, recently recognized as both a marker and effector of vascular damage, have been found to be strongly associated with ageing. The mediators of these interaction might be a modification of estrogen levels and a pro-coagulative state (Markiewicz et al. 2013).

### *1.5.4. From cellular to clinical consequences of molecular endothelial alterations with ageing*

As a result of this phenotypic shift, aged dysfunctional endothelium undergoes proinflammatory proliferation, migration, secretion, senescence, and extracellular matrix deposition within the aged arterial wall. Microscopically, all these contribute to: progressive accumulation of advanced glycation end products, by which the glycated proteins alter their physical properties and cause fibre stiffness; calcium deposition; matrix protein degradation, elastin fracture, and fibrosis; increase in the number of vascular smooth muscle cells and of their expression of adhesion molecules; disruption of the endothelium, intima-media thickening, amyloidosis (Figure 1.7) (Lee et al. 2010, and Wang et al. 2014).

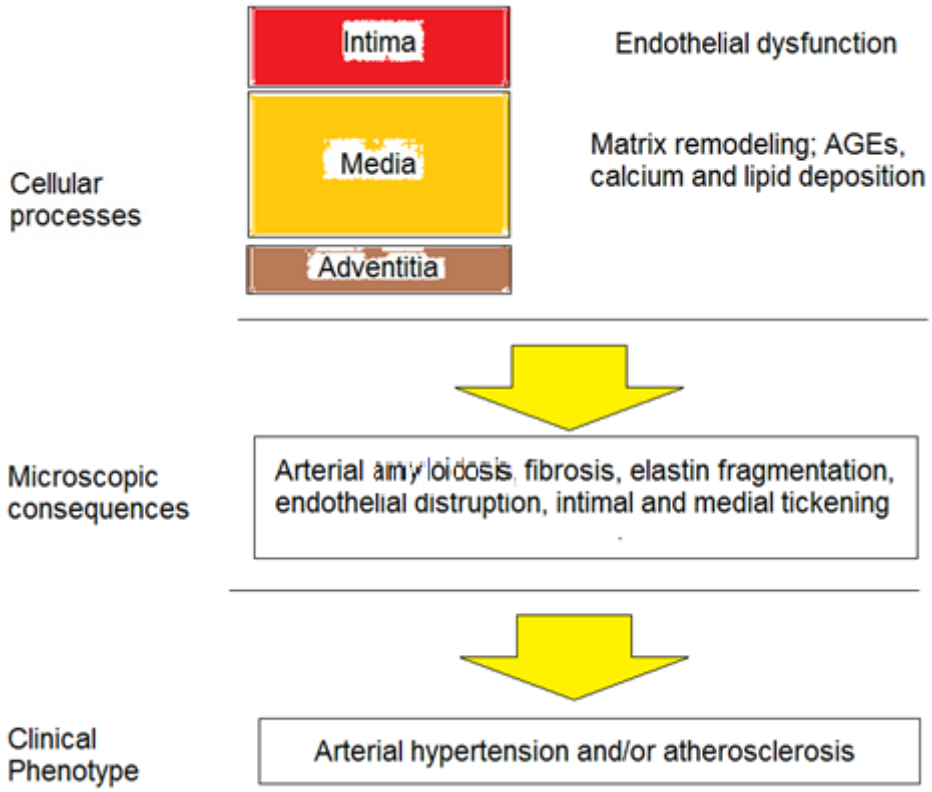



**Figure 1.7: Age-associated arterial changes.**

In order to counterbalance the loss of elastin power with ageing, collagen accumulation and cross-link formation of extracellular matrix proteins take place, which help to stiffen the wall of large arteries (Barodka et al. 2011). The degeneration of arterial tunica media is also favoured by fatigue fractures of the structural matrix proteins, collagen and elastin (O'Rourke 1990). Macroscopically, resulting wall changes are circumferential dilation, thickening, and stiffening (Lee et al. 2010). Clinically, these changes result in augmentation of central vs peripheral blood pressure and increased systolic and decreased diastolic pressure (causing an elevation of pulse pressure) (Nilsson 2014).

In fact, changes in arterial structure with age differ between central and peripheral arteries. With advancing age, there is marked aortic stiffening which increases aortic pulse wave velocity, by which reflected waves from the peripheral arteries arrive early in the systole thereby increasing

central systolic blood pressure. In contrast, the stiffness of large muscular arteries changes relatively little. Furthermore, aortic stiffening results in reduction of the protective compliance gradient normally present between the heart and peripheral arteries and enhances transfer of excessive, potentially harmful pulsatile energy into the periphery. In response, distal arteries increase peripheral resistance and limit vasodilatory reserve, potentially compromising resting and reserve flow organs, leading to end-organ damage (Mitchell et al. 2008).

Regardless of the mechanisms, clinical results of the atherosclerotic process are influenced by the known cardiovascular risk factors which must be controlled in clinical practice. Prevention programme includes physical exercise; smoking cessation; management of dyslipidemia, hypertension, diabetes, and obesity; and management of depression, social isolation, return to work, and other psychosocial issues (Williams et al. 2002).

## *1.6. Clinical studies in the field of ageing, haemodynamics and atherosclerosis*

### 1.6.1 Shear stress variations with ageing

Several cross sectional studies have reported that wall shear stress is lower in older people. The first observation was based on 21 healthy male subjects in an age range of 21-64, mean 35.9 years (Gnasso et al. 1996). The common carotid arteries were chosen, studied and pooled from the left and the right body side (42 samples). Maximum and mean wall shear rates during the cardiac cycle were calculated using the Poiseuille formula, based on maximum and mean blood flow velocities and maximum and minimum arterial diameters measured by Doppler and two-dimensional ultrasound images, respectively. Shear stress was calculated by multiplying shear rates by the whole blood viscosity measured at elevated shear rate. Both maximum and mean shear stress values

during the cardiac cycle were inversely associated with age (Figure 1.8). The peak shear stress was reduced by approximately 7 dynes/cm$^2$ for every 10 years increase in age, while the mean shear stress data followed the same trend.

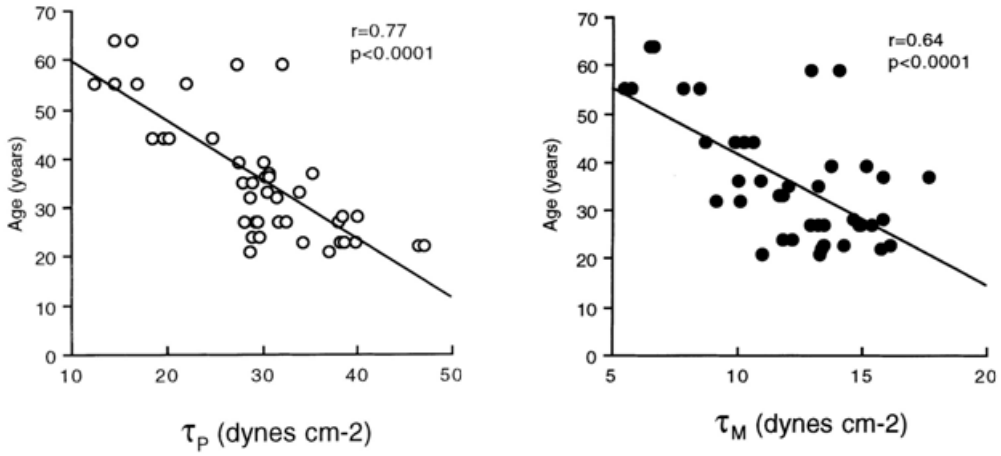
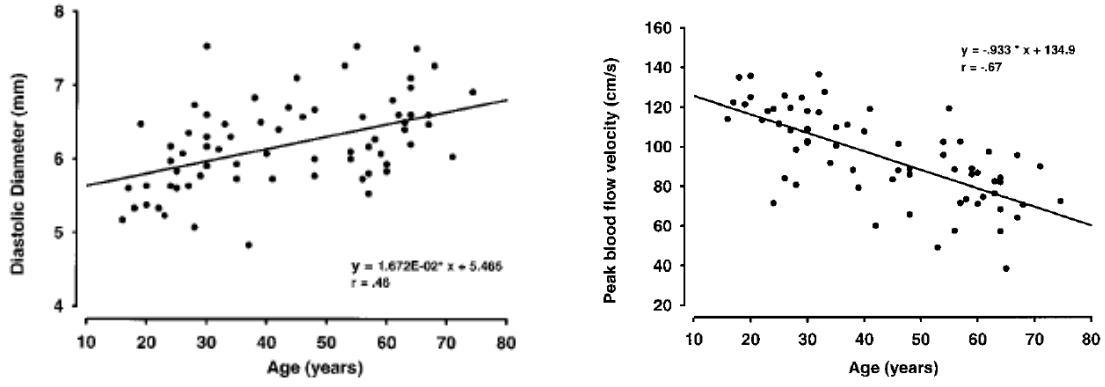



**Figure 1.8: Age vs peak (left) and mean (right) shear stress (Tau) (Gnasso et al. 1996, with permission).**

Similar finding has been reported by others (Schmidt-Trucksäss et al. 1999), with respect to vascular parameters that are determinants of shear stress. In 69 healthy males, the diastolic diameter of the right common carotid was measured from one-dimensional ultrasound images, and the systolic peak blood flow velocity from Doppler signals. As expected, diameters were larger and velocities lower in older vs younger subjects (Figure 1.9).




(A) (B)

**Figure 1.9: Modification with age of the common carotid artery in 69 healthy male subjects. A. Diastolic arterial dilation. B. Reduction of peak blood flow velocity (Schmidt-Trucksäss et al. 1999, with permission).**

In the same time period, another similar cross sectional study was conducted (Samijo et al. 1998). This study examined the right common carotid artery in a total of 111 healthy subjects, which also included females whose data were analyzed separately. Another difference from the previous studies was that blood flow velocity profiles along the cross sectional vessel axis were measured, instead of simply centreline velocities. Although velocity profiles provided more detailed information on blood flow, the intra-subject reproducibility was increased from 4 and 5 % for peak and mean wall shear stress respectively in the first report (Gnasso et al. 1996), to 15 and 12 % in this study. Furthermore, for shear stress calculation, blood viscosity was simply calculated from plasma viscosity and haematocrit, and not measured. Nevertheless, this study confirmed a significant inverse correlation between peak wall shear stress and age in males and reported for the first time in females. Furthermore, mean values of peak wall shear stress were lower in the female cohort than in males (Figure 1.10).

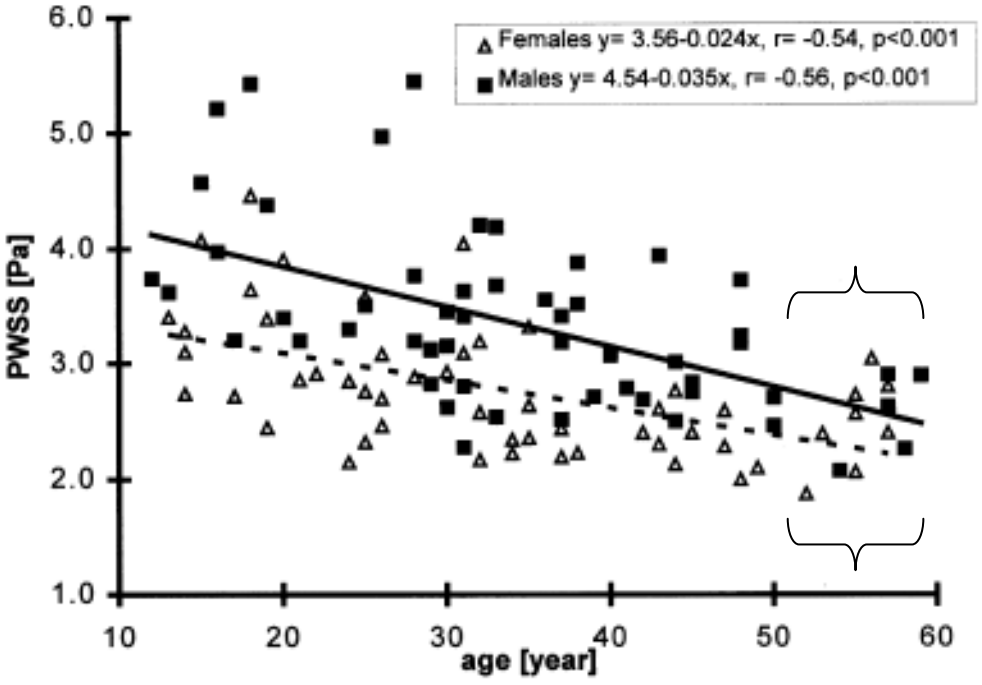



**Figure 1.10: Peak wall shear stress (PWSS) in the right common carotid artery as a function of age in the female and male groups (Samijo et al. 1998, with permission).**

Further examination of the data in Figure 1.10 suggested that if the analysis was restricted to the older subjects (highlighted in brackets), there would be no reduction in peak wall shear stress with age in females. Furthermore, at the age of 50-60 years (presumably after menopause) no difference between the two sexes seems evident.

Several years later, two large cross sectional studies were performed on men and women bearing various common risk factors for atherosclerosis, including diabetes, obesity, hypertension, smoking, and dyslipidemia. The first study was based on 1296 middle-aged volunteers participating in a cardiovascular prevention program which included magnetic resonance brain imaging; 21% of them were found to have small vessel diseases. Common carotid peak wall shear stress, computed from peak centerline blood velocity, systolic arterial diameter, and calculated blood viscosity, was inversely related to age in a simple regression analysis (Okada et al. 2014). The second study was on 950 middle aged patients who underwent coronary angiography; 30% of them were discovered to have coronary artery disease. Common carotid shear stress (as a mean of the two body sides) was computed from centerline blood velocity, arterial diameters, and calculated blood viscosity. Intra-subject variations were 6 and 8% for peak and mean shear stress, respectively. In this population, mean shear stress was also inversely related to age, independently of the presence of risk factors for atherosclerosis (Cho et al. 2016). Table 1.1 summarizes the above reported findings.

**Table 1.1: Overview of Literature concerning shear stress variations with ageing - cross sectional studies on common carotid artery.**

| *First Author and Year/ Shear stress method(s)* | *Number/ Gender/ Status* | *Age range or mean age ± SD* | *Shear stress values* |
|---|---|---|---|
| Gnasso 1996<br>Poiseuille+viscometry | 21/Males<br>Healthy | 21-64 | Lower at higher ages |
| Schmidt-Trucksäss 1999<br>Poiseuille | 69/Males<br>Healthy | 18-74 | Lower at higher ages |
| Samijo 1998<br>Cross-sectional profile | 111/Both<br>Healthy | 12-59 | Lower at higher ages |
| Okada 2014<br>Poiseuille | 1296/Both<br>I-II card. prevention | 65.4±9.1 | Lower at higher ages |
| Cho 2016<br>Poiseuille | 950/Both<br>I-II card. prevention | 59.7±13.1 | Lower at higher ages |

*Explanations about shear stress methods can be found in the text.*

Although the present study is focused on the common carotid arteries, it should be noted that shear stress reduction with ageing has been observed in other large arteries. For example, in a study based on 329 elderly males and females who were voluntarily recruited among participants to a United States Federal health program and their relatives, magnetic resonance imaging of the internal carotid artery was performed to obtain wall shear rate values. Despite a very narrow age range (70 to 82 years), peak wall shear stress was found to be inversely related to age in a simple regression analysis (Mutsaerts et al. 2011).

A separate study examined vessels in the limbs. The popliteal artery and the radial artery were examined using ultrasound and Doppler in 24 healthy subjects of both genders (Nishiyama et al. 2008). In the leg vessel, the older group had lower shear rates than the younger group (24±5 vs 30±3 $s^{-1}$), while the arm vessel - despite having a more muscular structure - showed similar differences (72±15 vs 94±16 $s^{-1}$). The same trend was found for the common femoral artery in

similar patient groups, with higher retrograde shear stress in the older group, resembling pro-atherogenic oscillatory shear stress conditions (Young et al. 2010). Furthermore, low basal shear stress values might also be a link by which ageing influences vascular function. In fact, resting brachial artery wall shear stress is directly related to flow-mediated vasodilation (Figure 1.11), as demonstrated by a study involving 27 healthy males (Gnasso et al. 2001). In this study, a multiple regression analysis was performed to test the independent contribution to flow-mediated dilation of wall shear stress, age, body fat, glucose, and lipids, and the result showed that wall shear stress displaced age in its known association with the endothelial function (standardized beta coefficient = 0.690, $p < 0.005$).

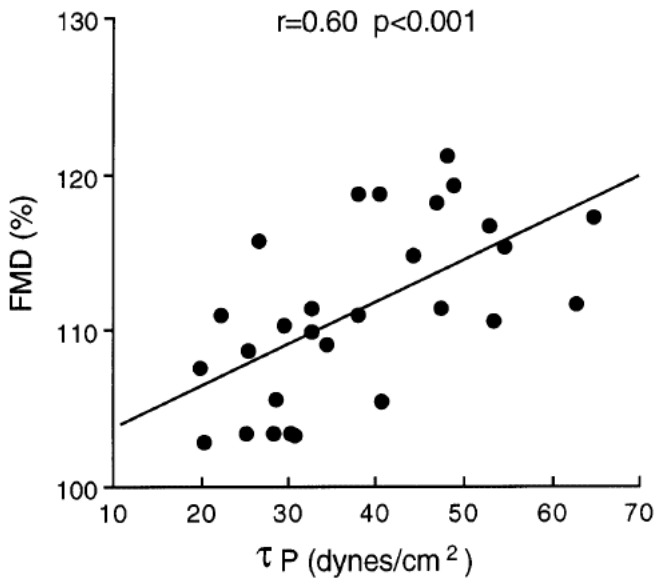



**Figure 1.11: Scatterplot showing flow-mediated vasodilation (FMD) by peak wall shear stress (Tau P) in the brachial artery of 26 healthy male subjects (Gnasso et al. 2001, with permission).**

## 1.6.2. Ageing, haemodynamics and atherosclerosis: cross-sectional studies

*1.6.2.1. Introduction*

In this field, most of the knowledge is derived from the extrapolation of data by laboratory models, patient-specific flow modelling data, or short-term human intervention studies. The latter have demonstrated that acute reductions of wall shear stress cause endothelial dysfunction, a precursor of atherosclerosis development. Particularly, a minimally invasive model was applied to the arm of 10 healthy men, with a brief occlusion of the forearm circulation causing flow disturbances (low mean and high oscillatory shear stress) in the proximal (brachial) artery. The local venous outflow, although not all derived from the large artery perfusion, demonstrated endothelial distress, by an elevated concentration of endothelial microparticles (Jenkins et al. 2013).

Among the data directly derived from the observation of the ageing process, they mainly come from cross-sectional human studies, whereas longitudinal studies investigating the chronic effect of decreasing shear stress on atherosclerosis development are lacking. This is probably due to the complexity of wall shear stress measurement, and to the relatively recent development of reliable techniques for its evaluation (Tortoli et al. 2006; Pedersen et al. 2014). Table 1.2 summarizes the reported large body of evidence regarding cross sectional associations of atherosclerosis prevalence with low carotid shear stress in elevated age.

**Table 1.2: Overview of Literature concerning atherosclerosis and carotid shear stress variations with ageing - cross sectional studies.**

| *First Author and Year/ Shear stress method(s)* | *Number/ Gender/ Status* | *Age range or mean±SD* | *Vascular feature related with lower shear stress in ageing* |
|---|---|---|---|
| Gnasso 1996<br>Poiseuille+viscometry | 21/Males<br>Healthy | 21-64 | CCA intima-media thickness |
| Schmidt-Trucksäss 1999<br>Poiseuille | 69/Males<br>Healthy | 18-74 | CCA intima-media thickness |
| Kornet 1998<br>Poiseuille | 53/Both<br>Healthy | 18-67 | CCA intima-media thickness |
| Augst 2007<br>3DUS, CFD | 14/Both<br>Healthy | 26-75 | CCA intima-media thickness |
| Gnasso 1997<br>Poiseuille+viscometry | 23/Both<br>I-II card. prevention | 59.5±7.8 | Carotid plaques |
| Jiang 1999<br>Poiseuille+viscometry | 78/Both<br>Healthy and HT | 58.0±11.0 | Carotid plaques |
| Carallo 2006<br>Poiseuille+viscometry | 25/Both<br>II card. prevention | 68.8±8.2 | Stroke |
| Mutsaerts 2011<br>Magnetic resonance | 329/Both<br>II card. prevention | 70-82 | Stroke |
| Okada 2014<br>Poiseuille | 1296/Both<br>I-II card. prevention | 65.4±9.1 | Stroke |
| Cho 2016<br>Poiseuille | 950/Both<br>I-II card. prevention | 59.7±13.1 | Coronary atherosclerosis |

*Explanations about shear stress methods can be found in the text. CCA: common carotid artery; 3DUS: three-dimensional ultrasound; CFD: computational fluid dynamics; HT: hypertensives.*

### *1.6.2.2. Studies concerning intima-media thickening as a precursor of atherosclerosis*

The wall intima-media thickness has been adopted frequently as an index of initial atherosclerosis and plaque precursor, although at least an amount of vessel wall thickness in healthy people is a physiological wall hypertrophy counterbalancing circumferential wall stress due to the blood pressure (Augst et al. 2007). The first cross-sectional human study investigating the relationship

between large artery wall shear stress and atherosclerosis with respect to ageing was reported in 1996 (Gnasso et al. 1996), and concerned with common carotids of healthy male subjects studied by ultrasound and viscometry. Results show that aged people who presented lower shear stress also had thicker arterial walls (Figure 1.12). In the same population, shear stress values explained all the age-related variability of wall thickness, independently of other interfering parameters such as plasma lipids and glucose, blood pressure, and body weight. In a subsequent study, both common carotid diameter and age independently predicted local wall thickening in a multiple regression analysis, whereas blood flow velocity did not. However, shear stress was not calculated in this study (Schmidt-Trucksäss et al. 1999).

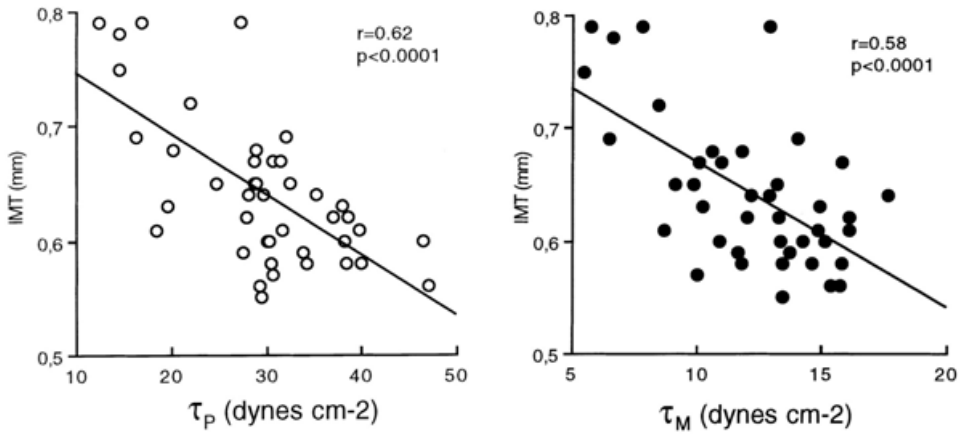



**Figure 1.12: Inverse relationship in common carotids between peak ($\tau_P$) or mean ($\tau_M$) wall shear stress, and intima-media thickness (IMT) (Gnasso et al. 1996, with permission).**

These observations were supported by other studies in the same artery with different techniques. In 53 healthy subjects of both genders and various ages, the right common carotid wall shear rate value was obtained by ultrasound images of arterial diameter and blood flow velocity profile along the cross sectional vessel axis (Kornet et al. 1998). Again, intima media thickness measured in the last part of common carotid, just proximal to the bulb as in the previous papers, was inversely related to shear stress and directly to age; no multiple regression analyses were performed. The

authors also noted that, repeating the measurements a few centimeters upstream, where the wall thickness was consistently smaller, the statistical significance was weakened; this could be explained by the concept described earlier that small intima-media thickening might simply be physiological hypertrophy. In 2007, the left common carotid wall shear stress of 14 healthy subjects of both genders was derived from anatomical data acquired by 3D ultrasound, and by computational fluid dynamic reconstruction of blood flow velocity patterns. Shear stress and wall thickness of the common carotid were again inversely related in simple regression analyses, although multiple regression analyses including age were not performed, due to the small sample size (Augst et al. 2007).

*1.6.2.3. Studies concerning carotid plaque as a marker of atherosclerosis*

Among participants to a cardiovascular prevention campaign, ageing influenced the presence and progression of carotid atherosclerotic plaques, whereas common carotid arteries shear stress influenced their localization in one of the two carotid trees (Gnasso et al. 1997); age was not taken into account by multiple regression analyses.

A similar study by another group confirmed elevated presence of carotid plaques in patients with low common carotid shear stress, but failed to investigate the role of age in this association (Jiang et al. 1999).

*1.6.2.4. Studies regarding major atherosclerotic cardiovascular diseases*

In a study involving 25 patients suffering from unilateral ischemic stroke derived from the carotid arteries, common carotid shear stress was found to be 28 % lower in the affected side (Carallo et al. 2006). This comparison among the two common carotids from the same patients corrected the influence of the presence of cardiovascular risk factors and age. A large study involved 329 elderly participants, almost all suffering from cerebrovascular diseases. After stratification for the aetiology of stroke, internal carotid wall shear stress at various times during the cardiac cycle was measured

by magnetic resonance imaging. Patients presenting a large artery stroke showed an elevated age and a reduced shear stress for almost the entire cardiac cycle; the association between shear stress and stroke was independent of age and common risk factors for atherosclerosis (Mutsaerts et al. 2011). More recently, two large studies were reported. In the first (Okada et al. 2014), common carotid peak wall shear stress was associated with silent lacunar cerebral infarctions, a subtype of small vessel diseases, independently of age and other risk factors of cardiovascular diseases. The second study (Cho et al. 2016) reported that both common carotid wall shear stress and age were associated with the presence of coronary atherosclerosis, independent of each other and of other cardiovascular risk factors, but among patients shear stress was only associated with the total coronary plaque area.

Looking at other arteries, a large cohort study yielded different results. This study involved 1583 participants of both genders, of whom 7 % was healthy, and the remaining suffered from various risk factors for atherosclerosis; one half had also coronary and/or lower limb cardiovascular disease. In order to obtain mean wall shear stress of the brachial artery, diameter and blood flow velocity were investigated with ultrasound, and viscosity was assumed constant. Brachial shear stress was found to be inversely associated with age, but not with clinical atherosclerosis (Chung et al. 2009). However, plaque presence in the brachial artery was an exclusion criteria in this study; additionally, the muscular brachial artery wall structure, different from the elastic carotid, might have played a role.

*1.6.2.5. Studies including other haemodynamic factors*

Rheological factors, such as whole blood viscosity, were found to be associated with the presence of carotid plaques (Carallo et al. 1998). This was a case-controlled study of 28 middle aged male participants per group; measured blood viscosity was elevated among patients presenting carotid atherosclerosis, whereas age did not differ between groups. Likewise, a larger study with similar features, but including clinical events such as stroke, showed that blood viscosity was also increased

in cerebrovascular diseases; differently to the previous study, age here was more elevated in the case group, but a multiple regression analysis was not performed (Szapary et al. 2004).

Circumferential wall tension was less studied compared to shear stress in the interplay between haemodynamics/biomechanics and carotid atherosclerosis with ageing. The first study involved 116 common carotid arteries from 58 healthy males of age 21-74 years, with ultrasound and blood viscosity measurements in order to compute their haemodynamic conditions. Multiple regression analyses demonstrated that shear stress and blood cholesterol only were independent predictors of intima-media thickening, whereas age and wall tension were not (Carallo et al. 1999). From the same group, a subsequent study on patients suffering from unilateral ischemic stroke demonstrated that, like shear stress, common carotid wall tension was also different between the two body sides, whereas the experimental model chosen displaced the influence of age (Carallo et al. 2006). Another study employed computational fluid dynamic reconstruction of common carotid blood velocities for 14 healthy subjects, with the carotid systolic blood pressure being measured with an applanation tonometry and calibrated by the brachial pressure. Although wall tension was not calculated, the obtained blood pressure values were associated with wall thickness not only in the common carotid, like shear stress, but also in the carotid bulb, where local shear stress values were not (Augst et al. 2007). In the above reported study of 1296 middle-aged participants in a cardiovascular prevention program where one out of five suffered from cerebrovascular diseases, central systolic blood pressure was obtained by the analysis of radial pressure waveform, by which peak common carotid wall tension of both body sides was calculated using local maximum arterial diameter acquired by ultrasound. Wall tension was associated with the two subtypes of small vessel cerebral diseases, both silent lacunar infarction and deep subcortical white matter hyperintensity, whereas shear stress was related to silent lacunar infarction only; all these associations were independent of age and other cardiovascular risk factors in multiple regression analyses (Okada et al. 2014). However, it should be noted that cerebral ischemia resulting from diseases of small

vessels are conventionally recognized as derived not from atherosclerosis, and possibly from blood pressure elevations (Adams et al. 1993).

### 1.6.3. Ageing, haemodynamics and atherosclerosis: longitudinal studies

Reported longitudinal studies on the relationships between haemodynamic forces and atherosclerosis are based on a very short follow-up period. Nevertheless, data obtained within months of follow-up have already demonstrated the progression of atherosclerotic lesions in native segments of coronary arteries presenting low wall shear stress (Stone et al. 2003; Samady et al. 2011; Stone et al. 2012). Specifically, this was a series of studies by two workgroups, sharing the arterial region examined (coronary district) and the technique used (intravascular ultrasound and computational fluid dynamics simulations, with the addition of coronary angiography in the studies by Stone et al. In their most recent report (Stone et al. 2012) involving 329 patients with acute coronary syndromes, the decrease of lumen area (secondary end point) after a follow-up of 6-10 months was independently associated with low baseline shear stress, whereas the primary end point of change in plaque area was not. Other results reported in this paper appear less robust: a post-hoc end point such as coronary plaque burden increase (an index of vascular thickening) was independently associated with low baseline shear stress; additionally, probably also due to the low number of percutaneous coronary interventions (n=31), the predictive values for the combination of low baseline shear stress plus large plaque burden versus the incidence of the procedure were 92 % as negative predictive value but 41% only as positive predictive value.

The only longer-term observation comes from a prospective study, demonstrating the reduction of internal carotid wall shear stress after 2.7 years of follow-up, but atherosclerosis development/progression was not evaluated (Box et al. 2007). In this investigation, 178 elderly patients suffering from vascular disease or carrying its risk factors were studied by magnetic

resonance imaging. Blood viscosity was assumed identical among participants. Measured reduction in mean wall shear stress was slightly less than 3% for each year of follow-up.

Regarding the viscosity of the whole blood and of the plasma /serum, a large prospective analysis with a prolonged follow-up, taking into account almost all known inflammatory, haemostatic and rheological factors, indicated only plasma and not blood viscosity as an independent risk factor for myocardial infarction and stroke (Tzoulaki et al. 2007). Furthermore, its clinical relevance remained doubtful. Fibrinogen, instead of blood viscosity, was found to be associated with atherosclerotic disease development (Tzoulaki et al. 2007). The same patient setting showed that both plasma viscosity and blood viscosity predicted peripheral arterial disease development, but "their clinical utility is likely to be limited" (Tzoulaki et al. 2007). However, a similarly large prospective study on the importance of plasma viscosity in the prediction of coronary disease (Yarnell et al. 2004) confirmed the role of this rheological variable. By comparing two multivariate models - a lipid model (total and HDL-cholesterol, triglycerides) and a haemostatic/inflammatory model (fibrinogen, plasma viscosity and white cell count), this study also showed a "non inferiority" for rheology in the prediction of coronary disease. These somewhat conflicting clinical evidences probably support the hypothesis that plasma viscosity or whole blood viscosity might influence the development and progression of atherosclerosis, though the clinical relevance is unknown and the precise pathways still need to be identified.

## 1.7 Summary and objectives of the study

In summary, there is evidence suggesting that both low arterial wall shear stress and ageing are involved in the atherosclerotic process. Furthermore, low shear stress and ageing were often found together in cross-sectional studies. Therefore, low shear stress might also serve as a mediator of atherosclerosis in the pathophysiology of ageing process.

The objectives of the present research are: firstly to carry out a longitudinal study to investigate non-invasively "*in vivo*" in both genders the age-dependent changes in human carotid artery morphology, haemodynamics and wall shear stress in particular; secondly, to compare these changes to the incidence of atherosclerosis.

# Chapter 2. Procedures on patients and haemodynamic measurements

*The present Chapter describes, in its first "medical procedures" section, the physical and instrumental examinations applied throughout the Thesis. The second, extended, section is then dedicated to the rheological measurement of blood and plasma viscosity, and to haemodynamic techniques for measuring common carotid wall shear stress, circumferential wall tension and stiffness.*

## 2.1. Medical procedures

### *2.1.1. Anthropometric measurements*

The acquisition of anthropometric data, an essential aspect of physical examination, was performed by Hospital Nurses trained in this procedure. Standing height without shoes was measured to the nearest 0.5 cm. Weight was measured to the nearest 0.1 kg in ordinary street clothes. Body mass index was computed as weight (in kg) divided by height (in m) squared. Systolic and diastolic blood pressures were measured, on the right arm, after the participant had been resting for at least 5 min, with a standardized sphygmomanometer. Supine or sitting positions were allowed. Three measurements were made every three minutes; the average of the second and third of the readings was computed. Mean blood pressure was computed as diastolic blood pressure plus one third of the differential pressure (i.e. systolic minus diastolic blood pressures).

### *2.1.2. ECG and clinical ultrasound approaches*

The acquisition of ECG was performed by Hospital Nurses trained in this procedure; interpretation of the results was by Hospital Doctors qualified in Internal Medicine. A standard 12 lead ECG was used with a connection to a PC for storage.

In order to obtain clinical prevalence data about the atherosclerosis of the carotid tree, an echo-Doppler examination was performed by a trained Physician in a quiet room at 22 °C with an ECG-triggered high-resolution instrument which was equipped with a multifrequency linear probe (details of the instrumentations are reported in the Section 2.2.3.7.). The extracranial sections of the carotid tree were divided into four different segments: common carotid artery, carotid bulb, external carotid artery, and internal carotid artery. Ultrasound was performed by longitudinal and transverse arterial planes, with anterior, lateral and posterior approaches. These arteries were examined to evaluate the presence of plaques. Atherosclerotic plaque was defined as localized intima-media lesion of upper and/or bottom walls on B-mode images with a thickness of at least 1.3 mm, no spectral broadening or only in the deceleration phase of systole, and systolic peak velocity of less than 120 cm/s. Normal segments were scored 0, and those with plaque were scored 1. A global carotid plaque score was computed by adding the scores of all segments (Irace et al. 2009).

## *2.1.3. Biochemical measurements*

Venous blood was collected after overnight fasting for routine analyses by Hospital Nurses. Attention was paid to avoid venous stasis and the haemostatic loop, when used, was immediately removed after cannulation of the vein. Blood glucose and lipids were measured by routine methods.

## *2.1.4. Physical examination*

After collecting the medical history and performing a complete physical examination, diagnostic procedures were performed by Hospital Doctors qualified in Internal Medicine. Diabetes was defined as fasting blood glucose ≥ 126 mg/dl and/or use of antidiabetic agents. Hyperlipidaemia

was defined as total cholesterol and/or triglycerides exceeding 200 mg/dl and/or use of lipid lowering drugs. Hypertension was defined as systolic blood pressure / diastolic blood pressure ≥ 140/90 mmHg and/or use of antihypertensive agents. Obesity was defined as body mass index exceeding 29.9 $Kg/m^2$.

## *2*.2. Haemodynamic techniques

### *2.2.1. Overview of haemodynamic measurements in large arteries*

In blood flow measurement, key variables are blood velocity, vessel diameter, and blood viscosity, along with their variations during a cardiac cycle. In the present study, we will focus on the determination of wall shear stress, which can be derived from the three haemodynamic parameters listed above.

Several methods are available to measure all these parameters; they differ by handling, operator dependence, and mainly cost and time efforts.

Blood viscosity is generally measured in vitro, with rotational viscometers, reproducing the shear rate conditions found in vivo by measuring diameter and velocity. The most commonly used viscometers are cone-plate rotational viscometers (Figure 2.1), where the configuration ensures that the entire blood sample is exposed to the same shear rate. Among them, the choice depends upon shear rate ranges desired (Hardeman et al. 2007).

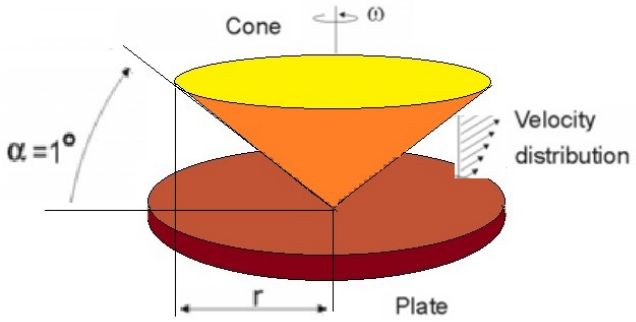



**Figure 2.1: Cone-plate rotational viscometer.**

If the viscosity of the fluid is known, the apparatus can also be used to impose a controlled shear stress to a monolayer of endothelial cells grown on the plate. Viscometry is an operator-dependent technique; for this reason, successful application of this technique is discussed in the appropriate section below.

Blood velocities are usually acquired by Doppler ultrasound, velocity-encoded magnetic resonance imaging, or angiography (Shaaban et al. 2000). In order to determine wall shear stress, it is essential to have information on velocity profile or near wall velocities. Almost all measurement techniques assume that blood velocity at the vessel wall is zero. Depending upon the technique used, the velocity gradient near the vessel wall may be interpolated or extrapolated to obtain the wall shear rate (Figure 2.2).

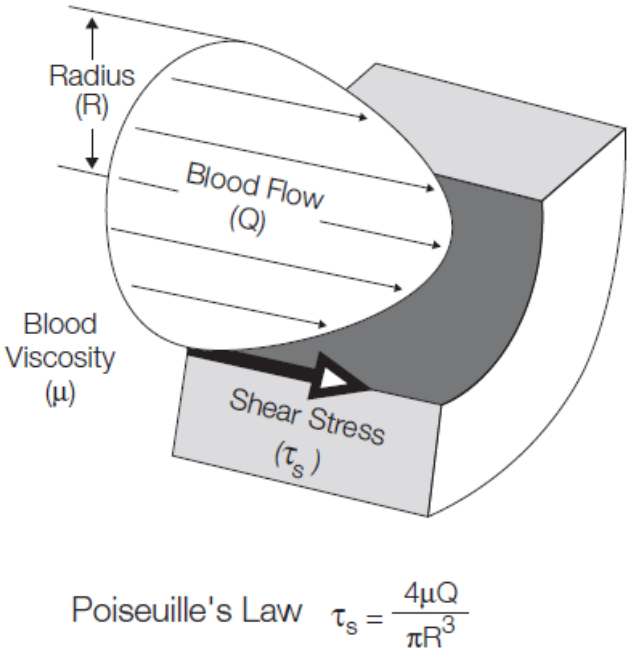



**Figure 2.2: Cross-sectional schematic diagram of a blood vessel illustrating haemodynamic shear stress, $\tau_s$, the frictional force per unit area acting on the inner vessel wall and on the luminal surface of the endothelium as a result of the flow of viscous blood. (From Malek et al. 1999, with permission).**

Pulsed Doppler ultrasound is a non-invasive technique commonly used in medical examinations. It measures the velocity component along the direction of insonation and in a sample volume only. A correction is then made to determine the velocity component along the axis of the vessel. With these limitations, velocity profiles determined from Doppler measurements are generally not accurate enough particularly near the wall, where the velocity gradient is often high, or in regions of disordered flow where velocity profiles are not parabolic. A simpler method is the assumption of laminar flow with a parabolic velocity profile of a Newtonian fluid in a long straight rigid tube with a uniform circular cross-section, as in Figure 2.2. These Poiseuille flow assumptions showed moderate confidence in respect to the common carotid artery (Augst et al. 2007).

Velocity-encoded phase-contrast magnetic resonance imaging relies on the phase shift of spins moving along the direction of a bipolar velocity-encoding gradient; although its measurements typically provide less temporal resolution than pulsed Doppler ultrasound and it is more time

consuming and costly, magnetic resonance imaging can be used to examine almost any large vessel and flow velocity profile in the body.

Diameter measurement can be made with ultrasound, or magnetic resonance imaging. The pros and cons of these techniques are similar to those for blood flow velocity measurements: ultrasound gives accurate vessel dimensions along the cardiac cycle, also in 3D mode if image reconstruction software is used, but it fails to approach deep vessel structures.

The two techniques have previously been compared for haemodynamic computations of the carotid tree (Glor et al. 2004) and, apart from a slight superiority of magnetic resonance in common carotid diameter measurements, their overall agreement for wall shear stress was satisfactory.

In the last two decades, two new techniques have become available for a more reliable determination of arterial wall shear stress, despite their increasing complexity. One of these techniques integrates computational fluid dynamics with ECG-gated intravascular ultrasound and biplane coronary angiography (Figure 2.3): here the progression of radio-opaque material gives blood flow velocity, internal lumen is depicted by angiograms and adventitia boundaries are derived from ultrasound. Then wall shear stress is calculated by flow velocity computations (Stone et al. 2003). In detail, ultrasound is performed with controlled pullback at 0.5 mm/s. Coronary blood flow is calculated directly from the time required for the volume of blood contained within the section to be displaced by radio-opaque material during a contrast injection. The arterial wall is considered in the diastole and assumed to be stiff; blood is assumed as homogeneous, Newtonian, and with a steady flow. The shear stress at the luminal surface of the artery is calculated as the product of viscosity and the gradient of blood velocity at the wall; its reproducibility has an r value of 0.91 ($P<0.0001$).

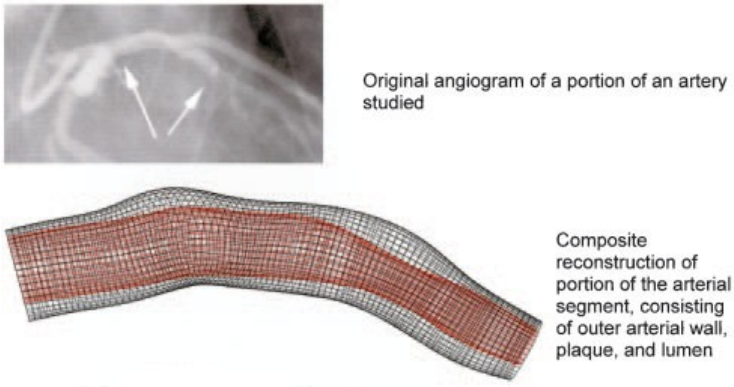



**Figure 2.3: Example of coronary angiogram and a portion of the 3D reconstructed artery (Stone et al. 2003, with permission).**

Another approach involves the combination of computational fluid dynamics with magnetic resonance imaging or 3D ultrasound. In this case, the echo probe should be equipped with a probe guide, or with an electromagnetic position and orientation measurement device, in order to locate the acquired ultrasound image slices. In this method also, three-dimensional flow patterns are reconstructed by numerical methods. Although these numerical methods governing three-dimensional blood movements, such as the Navier Stokes equations, involve time-consuming computations, this method is applicable non-invasively to complex arterial geometries and flow patterns; this is because shear stress values also depend upon arterial shape and vary extensively both in different arterial sections and at the same point during a cardiac cycle (Augst et al. 2007). Very often, those haemodynamic reconstructions give complex pictures, mainly at branching points (Figure 2.4).

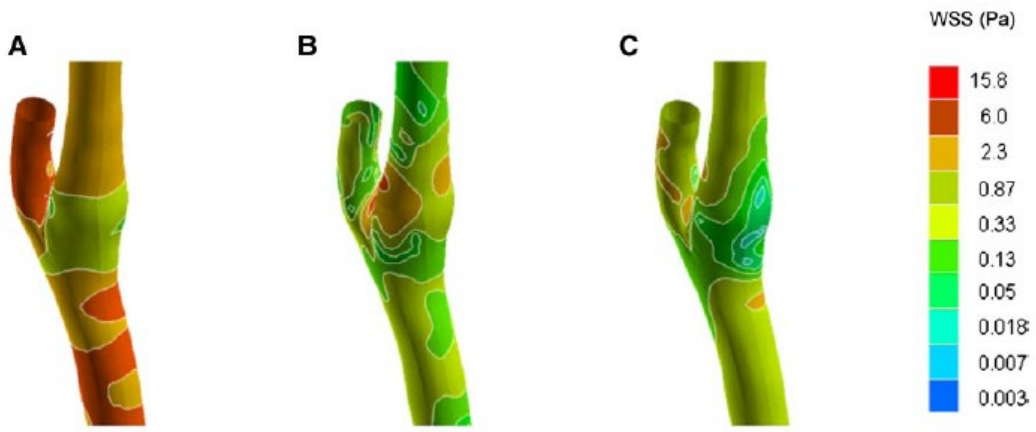



**Figure 2.4: Instantaneous wall shear stress (WSS) patterns in a representative subject during systolic acceleration (A), systolic deceleration (B), and diastole (C) (Modified by Augst et al. 2007, with permission).**

## *2.2.2. Blood and plasma viscosity measurements*

*2.2.2.1. Procedures conducted on participants*

During these studies, participants undergoing rheological measurements had been fasting for at least 12 hours. Smoking was not allowed in the morning of the blood examination. The subject was in a sitting or supine position for at least five minutes. A haemostatic loop on the arm was removed just after a forearm vein was cannulated and before filling the blood tube, in order to avoid any possible influence on the haematocrit value. Blood and plasma viscosity were measured within 2 hours of blood withdrawal, and heparin (35 I.U./mL) was added to the blood specimen.

*2.2.2.2. Instrumentation used and its maintenance*

Viscosity measurements were performed at 37 °C with a cone-plate viscometer (Wells-Brookfield DV-III, U.S.A.) equipped with a cp-40 spindle; this instrumentation was the same during both examinations and is suitable for blood viscosity measurement. The viscometer was regularly checked during the intervening years by sending it to the manufacturer for control and maintenance every year; it was also overhauled before the follow-up study and some spare parts were installed

by the manufacturer in order to ensure normal functioning of the instrument. Typically, the jewelled bearing of the main shaft was replaced every 2 or 3 years. Every day of rheological measurement, after zeroing of the instrumentation, a standard fluid with a known viscosity (Standard Fluid 5 CPS, Wells-Brookfield, USA) was used for checking the instrument. Even if the measurement took place in an air-conditioned room, a screw allows the user to counterbalance any modifications in the materials constituting the instrumentation (e.g., thermal dilations of metals in the summer), aligning the output of the rheometer to the known value of the standard fluid.

*2.2.2.3. Best practice for accurate blood viscosity measurement*

Viscosity measurements need a number of important precautions. First, all the metal parts of the viscometer in contact with the sample, and the blood sample itself, need to be at the desired temperature (37 °C here, reproducing physiologic conditions) just before starting the evaluation. Second, careful attention should be paid to the mixing of the blood sample, due to the tendency of the red blood cells to prompt sedimentation; otherwise, the viscosity of the blood sample placed into the measuring plate might not correspond to the circulating blood. Third, due to the speed with which thin blood films dry on the plate, the examiner needs to be fast in spreading the sample. Fourth, when the spreading the blood, the examiner has to overcome both the yield stress (the stress that must be applied in order to produce a structured fluid flow) and the surface tension of the blood with only the help of the tip of the pipette, and while avoiding haemolysis; for these reasons, a circular movement of the plate, as well as tilting it slightly might help. Fifth, air bubbles need to be avoided in the sample since they may influence the measurement. Last, due to the small sample volume (1 mL) and to the thin gap between cone and plate, dirt or impurities (e.g. left over cleaning papers, talcum from gloves, etc.) even if small, must be kept away.

*2.2.2.4. The choice of the appropriate shear rate for the measurement*

Blood viscosity was recorded at shear rates ranging between 450 – 22.5 $s^{-1}$. Blood changes its rheological features depending upon flow conditions (Matrai et al. 1987). Under high flow velocity, simulated in vitro by shear rates higher than 90 $s^{-1}$, blood behaves like a Newtonian fluid and shows near constant viscosity: in these conditions blood viscosity is only dependent on haematocrit. Below that threshold, erythrocyte aggregation and rigidity cause a progressive increase in viscosity. Measurement at shear rate of 450 $s^{-1}$ was not feasible in all subjects, because in hyperviscosity syndromes (e.g. polycythaemia in heavy smokers) the higher blood viscosities are out of range of the measurement window of the instrumentation; therefore, blood viscosity at a shear rate of 225 $s^{-1}$ was considered, which is indicative of the high flow velocity typical of large arteries such as the common carotid.

*2.2.2.5. Reproducibility of the measurement*

The laboratory operator at follow-up was the same as at baseline, but was blinded with regard to the results of the first examination.

Overall, the coefficient of variation for blood viscosity was below 3%. In detail, before the first examination, the intra-patient inter-day coefficient of variation was computed, performing the measurement for five consecutive days in five participants. Their mean coefficient of variations, computed as standard deviation divided for mean value, were: 0.4 at a shear rate of 225 $s^{-1}$, 0.7 at a shear rate of 90 $s^{-1}$, 1.4 at a shear rate of 45 $s^{-1}$, 2.3 at a shear rate of 22.5 $s^{-1}$. These measurements were repeated before the follow up, and the reported values were similar.

*2.2.2.6. Cells/whole blood percent ratio measurement*

Haematocrit is a standard laboratory procedure; it was measured in a glass microtube placed into a microcentrifuge, without correction for plasma trapping, using the same instrument in both examinations.

## *2.2.3. Common carotid wall shear stress, circumferential wall tension and stiffness*

*2.2.3.1. Ultrasound data acquisition for the computation of haemodynamic parameters*

For arterial diameter and blood flow velocity measurements, the examination was performed as previously described (Gnasso et al. 1996). Echo-Doppler parameters were acquired in the common carotid arteries, 1 cm proximal to the line dividing the common carotid and the carotid bulb. The portion of the common carotid within 10 cm upstream of the measurement site was considered; in this area, vessels not straight (in the comparison between an electronic line and the lumen-intima interface of the near and the far wall in the anterior, lateral and posterior longitudinal approaches), or presenting carotid plaques, were excluded because of their impact on haemodynamics (Markl et al. 2010), as also demonstrated in other arterial districts (Wood et al. 2006). The exact distance from the carotid bulb was recorded. Internal diameter was defined as the distance between the leading edge of the echo produced by the intima-lumen interface of the near wall and the leading edge of the echo produced by the lumen-intima interface of the far wall. Internal diameter was measured at the R and T waves of the ECG, representing the minimum and maximum carotid diameter, respectively (Gnasso et al. 1996). Blood flow velocity was recorded, with the sample volume being reduced to the smallest possible size (1 mm) and placed in the centre of the vessel. The angle between the ultrasound beam and the longitudinal vessel axis (θ) was kept between 44° and 56°, and was recorded. The maximum Doppler frequency shift, i.e. systolic peak velocity, and mean velocity were automatically recorded with auto-tracking as the mean of three cardiac cycles. The peak velocity was used to calculate peak wall shear stress and the time-averaged peak velocity to calculate mean wall shear stress. It has been suggested (Gnasso et al. 1996) that the mean velocity of flow can be estimated more accurately by time-averaged peak over an integral number of cardiac cycles. In this study time-averaged peak was evaluated over three cardiac cycles.

*2.2.3.2. Ways to improve the quality of haemodynamic evaluation*

First, because blood viscosity is in the formula used for shear stress computation, ultrasound was performed on the same morning as the viscosity evaluation and blood pressure measurement, but after blood withdrawal, in order to reduce the possible influence of anxiety in respect to the vein puncture on the carotid haemodynamic (peripheral resistances, heart rate, etc.). Second, the patient was resting in a supine position for at least ten minutes before starting the evaluation for haemodynamic stabilizations, and no conversation was carried out before and during the examination to avoid possible anxiety. Third, head position was varied before the measurement until the last ten centimetres of the common carotid was straight (see above), and for this reason a pillow was not allowed. Fourth, sleeping was not permitted during the ultrasound procedures in order to avoid its sympathetic effects. Fifth, if coughing, swallowing or extrasystolic beats occurred during the recordings, data were discarded and acquired again. Last, blood pressure measurement was made just after the ultrasound.

*2.2.3.3. Calculations*

Peak ($\tau$P) and mean ($\tau$M) wall shear stresses were calculated according to the following formulas:

$$\tau P \text{ (dynes/cm}^2) = 4 \cdot \eta \cdot V_{SP}/ID_T$$

$$\tau M \text{ (dynes/cm}^2) = 4 \cdot \eta \cdot V_M/ID_R$$

where blood viscosity ($\eta$) is expressed in poise; systolic peak ($V_{SP}$) and mean ($V_M$) velocities in cm/s; maximum ($ID_T$) and minimum ($ID_R$) carotid diameters in cm.

*2.2.3.4. Reproducibility of shear stress measurements*

To test the reproducibility of the wall shear stress calculation, five healthy adult male volunteers were studied three to five times over two months (Gnasso et al. 1996). For each subject, the average of these examinations was calculated, and the ratio between each individual value and the average

value was determined: this value never exceeded 9%. The mean coefficient of variation represents the average of the coefficients of variation calculated in each subject: values were 4.0 ± 2.2 and 5.0 ± 2.4 for peak and mean values respectively. Pearson correlation coefficients were 0.98 and 0.97 for peak values, and 0.93 and 0.95 for mean values (all $P < 0.05$), for the first versus the second and the first versus the third measurements, respectively. The Kendall's W coefficients of concordance were highly significant: $W_{0.05,5,3}=11.4$ ($P<.001$) and $W_{0.05,5,3}=8.8$ ($P<.01$), for peak and mean values respectively.

*2.2.3.5. Method validation for shear stress evaluation*

During the period when the method for this study was developed, another group published shear rates data for the common carotid calculated by ultrasound (Hoeks et al. 1995), but without blood viscosity measurement. Looking at their data of young male volunteers, and multiplying their shear rates for the mean viscosity measured in the present study, their shear stress values were 27.5 and 9.5 dynes/cm$^2$ for peak and mean values, respectively. In the present Thesis, for a similar population, values were similar (31.7 and 12.7 dynes/cm$^2$ for peak and mean values respectively). Slight differences might be attributed to the fact that, in contrast to the present Thesis, Hoeks et al. used a *multigate* Doppler system in order to attempt to design the shape of the cross-sectional blood flow velocity distribution.

*2.2.3.6. Other haemodynamic components*

Blood flow (BF) was computed as the product of mean cross-sectional velocity ($V_M/2$) expressed in cm/s, and area ($ID_R$ is the minimum carotid diameter, in cm), according to the following formula:

$$BF\ (mL/s) = V_M/2 \cdot \pi \cdot (ID_R/2)^2$$

Peak ($T_P$) and Mean ($T_M$) circumferential wall tensions were:

$$T_P \text{ (dynes/cm)} = SBP \cdot (ID_T / 2)$$

$$T_M \text{ (dynes/cm)} = MBP \cdot (ID_R / 2)$$

where systolic (SBP) and mean (MBP) blood pressures are expressed in dynes/cm$^2$, maximum ($ID_T$) and minimum ($ID_R$) carotid diameters in cm.

Peterson's elastic modulus (P) was used as an index of arterial stiffness according to the following formula:

$$P \text{ (dynes/cm}^2) = (SBP - DBP) \cdot ID_R/(ID_T - ID_R)$$

The brachial blood pressure was used instead of the central (carotid) pressure; limitations deriving from this approximation (Manisty et al. 2009) are discussed elsewhere (Sections 5.4 and 6.2.3).

Intima-media thickness of the common carotid artery was measured off-line, as previously described (Gnasso et al. 1996). Images were selected from video recordings, displayed on a computer screen and analysed manually. For each participant, three measurements pertaining to the anterior, lateral and posterior projections of the far wall were performed on each side. The average of the six measurements was used to calculate intima-media thickness.

*2.2.3.7. Ways of improving the similarity between the experimental conditions at follow-up versus baseline examinations*

The operator for ultrasound at follow-up was the same as at baseline, and was blinded with regard to the results of the first examination.

For each patient the same machine settings were used; that is, gain, focal depth, transducer aperture size, beam steering, depth and size of sample volume, beam-vessel angle, and the exact distance from the carotid bulb where the diameters were taken, in order to reproduce the same method and obtain data that should be comparable.

The instrument used at baseline was an ATL UltraMark 9 HDI (Philips), equipped with a linear phased array multifrequency probe operating at 5-10 MHz. In the follow-up study an ATL HDI 3000 (Philips) was used, equipped with a similar linear phased array multifrequency probe operating at 5-10 MHz. Obviously, even if the same brand and type of ultrasound and its equipment were chosen for the follow-up examination, the technical improvement in a decade might have influenced the results. Unfortunately, data from a phantom was not taken at the baseline examination. The Author of the present Thesis therefore decided to use, as a reference, ultrasound data derived from various populations with identical anthropometric and clinical features to the participants at baseline, as is discussed more fully in Section 5.2.1.1.

# Chapter 3. Cross-sectional description of the baseline cohort, and the follow-up at recall

*The present Chapter describes the cross sectional data from the baseline examination; discusses procedures for recall and drop-outs, and compares baseline versus follow-up clinical and biochemical data.*

## 3.1. Patient cohort and methods

### *3.1.1. Patient selection*

A cohort of free-living participants, voluntarily recruited into a cardiovascular disease prevention programme in the city of Catanzaro (Italy) and its suburbs, were, from 1996, also used for the evaluation of haemodynamic forces within the common carotid arteries. The prevention programme was started and performed by the Metabolic Unit of the local University Hospital, by a direct contact with patients and their relatives at the outpatient clinic. All participants were informed about the aim of the campaign and signed an informed consent. The protocol was approved by the local Ethics Committee.

Exclusion criteria were: ECG not in sinus rhythm, females not in menopause, use of diuretic and anticoagulant drugs, severe anaemia (haemoglobin < 10g/dL), polycythaemia (red blood cells > 6 x $10^6$ cells per microL), clinically significant renal, hepatic or pulmonary disease, severe hyperglycaemia (fasting plasma glucose >250 mg/dL), heart failure. Furthermore, as discussed in

more detail in Paragraph 2.2.3, participants presenting at ultrasound without straight common carotid arteries, or plaques at the arterial site of haemodynamic measurements, haemodynamically significant stenoses of the carotid arteries downstream of the common carotid, where excluded from the study. Finally, baseline data was taken from 147 selected subjects.

### *3.1.2. Methods*

Participants included in the study underwent a complete clinical examination and blood withdrawal, for glucose, lipid profile, blood and plasma viscosity sampling, and carotid haemodynamic evaluation; all these procedures are described in Chapter 2.

For statistical analysis, Pearson's correlation coefficient was used to test the correlation between continuous variables. Linear multiple regression analyses were applied in order to test the independent association between intima-media thickness and its determinants, such as atherosclerosis risk factors and haemodynamic features. The Student t test for paired data and the chi-square test were used to compare baseline versus follow-up data for continuous and categorical variables, respectively. Triglycerides, not normally distributed, were log transformed. All statistical analyses were performed by SPSS 17.0 for Windows.

A description of the chosen statistical approaches is reported in Appendix 2.

## 3.2. Data analysis of baseline examination

Some parts of this baseline cross-sectional analysis were previously reported (Carallo et al. 1999). Clinical, laboratory, and haemodynamic values of the participants (n=147) at baseline are reported in Table 3.1.

**Table 3.1: Baseline characteristics of the participants (n=147).**

| | |
|---|---|
| Age (yrs) | 54.4±12.9 |
| Body mass index (kg/m$^2$) | 27.15±3.84 |
| Systolic blood pressure (mmHg) | 127±18 |
| Diastolic blood pressure (mmHg) | 79±9 |
| Total cholesterol (mg/dl) | 221±44 |
| HDL-cholesterol (mg/dl) | 54±12 |
| Triglycerides (mg/dl) | 148±76 |
| Glucose (mg/dl) | 118±54 |
| Obesity (%) | 34 |
| Hypertension (%) | 24 |
| Hyperlipidaemia (%) | 31 |
| Diabetes (%) | 22 |
| Cigarette smoking (%) | 8 |
| Blood viscosity 225 (cP) | 4.51±0.50 |
| Peak shear stress (dynes/cm$^2$) | 19.9±5.2 |
| Mean shear stress (dynes/cm$^2$) | 10.4±2.0 |
| Intima-media thickness (μm) | 689±127 |
| Peak circumferential wall tension (x10$^4$ dynes/cm) | 5.0±0.7 |
| Mean circumferential wall tension (x10$^4$ dynes/cm) | 3.6±0.6 |
| Peterson's modulus (x10$^5$ dynes/cm) | 9.3±7.1 |

*Values are mean ± SD (unless otherwise indicated)*

Intima-media thickness was directly associated with age (r=0.33), total cholesterol (r=0.25), glucose (r=0.30) systolic blood pressure (r=0.35), body mass index (r=0.38), peak (=0.32) and mean (r=0.34) circumferential wall tension, and inversely with peak (r=0.357) and mean (r=0.340) shear

stress (all p < or = 0.01). Blood viscosity and Peterson's modulus were not associated with intima-media thickness.

A multiple regression analysis including age, total cholesterol, glucose, systolic blood pressure, body mass index, mean circumferential wall tension, and mean shear stress as independent variables, and intima-media thickness as the dependent variable, showed that only mean shear stress and total cholesterol were independently associated with intima-media thickness (Table 3.2). The results were unchanged if peak instead of mean values were used for the haemodynamic variables.

**Table 3.2: Stepwise regression analysis at baseline (outcome: intima media thickness).**

| Step | Variable | Multiple $R^2$ | Significance |
|---|---|---|---|
| 1 | Mean shear stress | 0.30 | 0.001 |
| 2 | Total cholesterol | 0.32 | 0.05 |

# 3.3. The follow-up analysis

## *3.3.1. Patients selection*

A part of the follow-up analysis, after 11.6 years, has also been reported already (Irace et al. 2012).

Starting from 2008, the subjects (n=147) were recalled by phone and mail: 8 subjects had died, 70 were unavailable or had moved away, and 19 refused to participate. Fifty subjects agreed to participate: after clinical examination, one subject was excluded because of severe anaemia and one

because of a malignancy; 48 (15 women and 33 men) signed informed consent, underwent the haemodynamic examination, and were included in the follow-up (Figure 3.1).

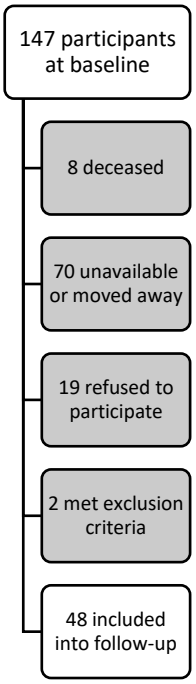



**Figure 3.1: Flow chart of inclusion of the participants at follow-up.**

## *3.3.2. Analysis of drop-outs*

Only about one third of the baseline cohort participated in the follow-up examinations. This was because of the large proportion of participants (n=70) that were unavailable or moved away. Apart from people who had relocated for life events, the most common difficulty was that of contacting the baseline population after almost 12 years. In fact from the middle/late 1990s, mobile phones began to take the place of home phones. In most cases, therefore, the recruitment for follow-up was by regular mail, a procedure carrying a low recruitment rate.

Even if, as might be expected, more diseased people with respect to healthy people were lost to follow-up, a statistical comparison of the baseline clinical features of the former population (n=147, Table 3.1), with respect to the baseline clinical features of the sample participating in the follow-up (n=48, first column of Table 3.3), showed no significant differences. Nonetheless, all the analyses of follow-up versus baseline were performed within the population of 48 participants (n= 96 carotid arteries), thus avoiding bias due to the elevated drop-out size.

## *3.3.3. Clinical and biochemical results at follow-up*

*3.3.3.1. Analysis of the whole population*

Table 3.3 shows clinical and biochemical characteristics of participants (n=48, male 69%). The mean (±SD) follow-up was 11.63±0.85 years. At baseline 16 subjects had no coronary heart disease risk factors, 19 had one and 13 had more than one coronary heart disease risk factor. At follow-up, 9 subjects remained free of coronary heart disease risk factors while the number of obese subjects doubled and that of subjects with hypertension was almost threefold. The number of participants with hyperlipidaemia or diabetes slightly increased during the study. The very small group (n=4) of cigarette smokers at the first visit was halved at the follow-up. All together 25 subjects worsened their coronary heart disease risk profile at follow-up and 23 remained stable.

**Table 3.3: Clinical and biochemical characteristics of the participants at baseline and follow-up (n=48).**

| | Baseline | Follow up | p= |
|---|---|---|---|
| Age (yrs) | 53.6±9.8 | 65.3±9.5 | |
| Body mass index (kg/m$^2$) | 26.85±2.98 | 28.65±3.72 | .0001 |
| Systolic blood pressure (mmHg) | 123±16 | 139±21 | .0001 |
| Diastolic blood pressure (mmHg) | 79±8 | 80±10 | NS |
| Total cholesterol (mg/dl) | 211±38 | 202±39 | NS |
| HDL-cholesterol (mg/dl) | 50±11 | 45±10 | .02 |
| Triglycerides (mg/dl) | 132±66 | 116±41 | NS |
| Glucose (mg/dl) | 108±41 | 106±41 | NS |
| Obesity, n (%) | 16 (33) | 31 (65) | 0.02 |
| Hypertension, n (%) | 11 (23) | 28 (58) | 0.0001 |
| Hyperlipidaemia, n (%) | 14 (29) | 16 (33) | NS |
| Diabetes, n (%) | 9 (19) | 10 (21) | NS |
| Cigarette smoking, n (%) | 4 (8) | 2 (4) | NS |

*Values are mean ± SD (unless otherwise indicated)*

Table 3.4 shows participants on therapy during the study. None of the participants with hypertension were taking diuretics, while the majority was taking renin angiotensin system-inhibitors. Total cholesterol rose from 195±30 to 208±39 mg/dl in subjects classified as non hyperlipidaemic, and decreased from 250±24 to 188±35 mg/dl in hyperlipidaemics; this was for the increase of treatment in hyperlipidaemics at follow-up. HDL cholesterol was unchanged in the former group (50±10 vs. 47±9 mg/dl, p=NS) and markedly decreased in the latter (50±12 vs. 40±11

mg/dl, p<0.05). Among subjects with diabetes, they were all taking metformin and/or sulphonylureas. None was under insulin or glitazone treatment.

**Table 3.4: Participants on pharmacological therapy during the study.**

| *Patients on therapy for:* | Baseline | Follow up |
|---|---|---|
| Arterial hypertension, n (%) | 11 (100) | 26 (93) |
| Hyperlipidaemia, n (%) | 0 (0) | 12 (75) |
| Diabetes mellitus, n (%) | 6 (67) | 8 (80) |

Body mass index at follow-up was correlated with its value at baseline (r=0.66, p<0.0001), and the same was observed with systolic blood pressure (r=0.54, p<0.0001) and diastolic blood pressure (r=0.42, p<0.0001). No correlation was found for blood lipids and glucose between baseline and follow-up measurements.

*3.3.3.2. Gender differences*

Clinical and biochemical characteristics of subjects, at baseline and during the follow-up visit, divided according to gender (69 % male), are reported in Table 3.5. Females were older than males, though not significantly. They had significantly higher systolic pressures, but comparable levels of blood lipids and glucose. The mean number of cardiovascular risk factors was higher in females (1.47±1.18 vs. 0.76±0.83, females and males, respectively). Systolic blood pressures and body mass index were significantly higher in both genders at the follow-up visit compared to baseline.

15 women and 33 men

**Table 3.5: Clinical and biochemical profile of participants (n=48) investigated at the start and the end of follow-up**

| | Females (n=15) | | Males (n=33) | |
|---|---|---|---|---|
| | Baseline | Follow up | Baseline | Follow up |
| Age (yrs) | 55.9±7.7 | 67.7±7.8 | 51.6±11.5 | 63.1±11.1 |
| Systolic blood pressure (mmHg) | 132±19 | 148±23§ | 118±14* | 133±17§ |
| Diastolic blood pressure (mmHg) | 80±11 | 81±11 | 77±7 | 79±8 |
| Body mass index (kg/m$^2$) | 27.8±3.5 | 29.5±3.1§ | 26.6±3.0 | 28.5±4.0§ |
| Total Cholesterol (mg/dl) | 214±37 | 197±34 | 208±40 | 203±38 |
| HDL- Cholesterol (mg/dl) | 52±12 | 49±11 | 50±11 | 44±13 |
| Triglycerides (mg/dl) | 111±50 | 130±42 | 145±68 | 112±38 |
| Glucose (mg/dl) | 109±47 | 109±42 | 110±40 | 104±40 |

*Values are mean ± SD;*=vs. Females at baseline; §=vs. baseline (all p at least <0.01)*

The analysis of follow-up data regarding rheological and haemodynamic variables is reported in the following Chapters 4 and 5.

# Chapter 4. Blood viscosity in an ageing population

*The present Chapter analyses the variation of blood viscosity and its components with ageing, and in relation to other risk factors for cardiovascular diseases.*

## 4.1. Introduction

As reported in Section 1.6.3., whole blood viscosity might influence the development and progression of atherosclerosis. The association between elevated blood viscosity and age has previously been reported by other researchers in healthy humans in cross-sectional settings (Seki et al. 2006, Ajmani et al. 1998, De Simone et al. 1990). Looking at the diseased population, however, a large study found this association to be weak; i.e. limited to a statistic significance, but probably with a limited clinical importance (Feher et al. 2006). Observations in this field, however, originated from cross-sectional studies; furthermore, studies performed on a diseased population also have several confounding factors in respect to the therapies used, influencing blood rheology.

The aim of the study presented in this section was to analyse the change in blood viscosity with ageing applying a longitudinal approach after a 12 year life span.

The cohort was described in the previous chapter, but three participants (two women and one man) lacked a plasma viscosity measurement. In the end, therefore, 45 of them (13 women and 32 men) were included in this section of the study (Carallo et al. 2011).

Methods are presented in Chapter 2. Here, apart from clinical and biochemical measurements, blood and plasma viscosity evaluations have been applied.

For the statistical analysis, matching continuous variables measured at baseline and follow up, Student *t* test for paired data was used to compare means, and Pearson's correlation coefficient was used to test the correlations. Triglycerides, not normally distributed, were log transformed. All statistical analyses were performed by SPSS 17.0 for Windows.

Appendix 2 provides a description of the statistical approaches used.

## 4.2. Results

Looking at rheological changes with ageing, Table 4.1 shows the haemorheological profile of the participants (n=45, male 71%). Haematocrit and plasma viscosity did not change during the study, whereas blood viscosities, both at high and low shear rates, significantly increased, slightly less than 1% for each year of follow-up. Adjustment for haematocrit did not alter these findings (shear rate 225/sec: 4.46±0.29 vs. 4.76±0.42; shear rate 45/sec: 6.20±0.46 vs. 6.62±0.59, basal and follow-up, respectively, both $p<0.0001$).

**Table 4.1: Haemorheological parameters of the participants (n=45).**

| | Baseline | Follow up | p= |
|---|---|---|---|
| Haematocrit (%) | 45.46±4.15 | 44.92±3.88 | NS |
| Blood viscosity 225 (cP) | 4.46±0.49 | 4.81±0.54 | 0.0001 |
| Blood viscosity 45 (cP) | 6.19±0.67 | 6.65±0.79 | 0.0001 |
| Plasma viscosity (cP) | 1.42±0.10 | 1.46±0.11 | NS |

*Values are mean ± SD (unless otherwise indicated)*

Haematocrit at follow-up was correlated with its value at baseline ($r=0.58$, $p<0.0001$). Blood viscosity values at shear rates of 250/sec were also correlated ($r=0.40$, $p=0.007$), while plasma viscosity and blood viscosity at shear rate of 45/sec were not correlated ($r=0.17$, 0.14, respectively).

The percent variation in blood viscosity was not associated with the percent variation in body mass index, blood pressure, blood lipids (even after exclusion of subjects taking lipid lowering drugs) or glucose. Furthermore, the increase in blood viscosity was similar in subjects with coronary heart disease risk profile worsening or not (in subjects with stable coronary heart disease risk profile: shear rate 225/sec: 4.49±0.53 vs. 4.98±0.60; shear rate 45/sec: 6.20±0.67 vs. 6.80±0.84; in subjects with coronary heart disease risk profile worsening: shear rate 225/sec: 4.46±0.45 vs. 4.71±0.44; shear rate 45/sec: 6.16±0.65 vs. 6.47±0.68; all $p<0.05$).

## 4.3. Discussion

### *4.3.1. Evaluation of the results*

The results of the present study demonstrate that blood viscosity increases with age, thus probably contributing to the elevated coronary heart disease risk associated with ageing.

Blood viscosity increased even in participants not experiencing a worsening of their coronary heart disease risk factors; furthermore, it is not related to haematocrit and plasma viscosity. All this suggests a possible direct effect of ageing on red blood cells.

In previous studies, plasma volume reductions in the elderly (mean age 66 years) with respect to young people (25 years) has been found to have a role in the haematocrit elevation, mainly due to alterations of thirst mechanisms (Davy et al. 1985). These diverse results can probably be explained by differences in the ages of the recruited populations, which were more varied in the cited study.

In contrast to previous cross-sectional reports, the present research is a longitudinal study to evaluate the influence of ageing on haemorheological parameters, with an important follow-up of almost 12 years.

No correlation was found between the variation in blood viscosity and the modification of classical coronary heart disease risk factors, i.e. blood pressure, lipids and glucose. Even in subjects who remained free from coronary heart disease risk factors during the 11.6 years observation period blood viscosity and red cell rigidity significantly increased. Therefore, it is possible that factors not analysed in the present study may be responsible for the viscosity increase. In fact, erythrocyte membrane fluidity is markedly decreased in elderly subjects, probably as a consequence of high oxidative stress and decreased antioxidant defences (Goi et al. 2005). In this cross-sectional study, a comparison between elderly people and young subjects reveals that older people are subject to higher oxidative stress. As known, activity differences were also observed when erythrocytes were further distinguished according to their biological age. This structural perturbation of the erythrocyte membrane, apart from affecting the behaviour of some membrane enzymes and receptors, also influences the physical properties of blood, i.e. increases the viscosity and red cell rigidity.

The elevation of blood viscosity with ageing found in the present study might have clinical consequences in some clinical contexts; in fact, blood viscosity was found to be increased in cerebrovascular diseases (Banerjee et al. 2000, Velcheva et al. 2008)

## *4.3.2. Sources of experimental errors*

It is very unlikely that blood viscosity has been overestimated at follow-up. The whole procedure was carefully checked during the study, the instrument was calibrated with a standard solution before each use and plasma viscosity was not affected. It is also unlikely that the results are influenced by statin therapy, for at least two main reasons. Firstly, exclusion of subjects taking a

statin does not alter at all the results (data not shown). Secondly, available data suggest no influence or at most a decrease in blood viscosity following statin therapy (Banyai et al. 2001). However, the increase in blood viscosity observed in subjects taking a statin (though the number is quite small and any conclusion must be drawn with caution) is similar to that of subjects not taking the drug, despite a marked reduction of blood cholesterol. This suggests that blood cholesterol might have negligible influence on blood viscosity.

The present study has some limitations. The first limitation is the relative heterogeneity of enrolled subjects. The reason for this lies with inclusion criteria. Subjects were free-living participants in a cardiovascular disease prevention program: some were healthy; some were sick and/or under medication. In order to avoid the effect of diseases and/or drug therapy on blood viscosity, very strict exclusion criteria should have been adopted. This would exclude or at least reduce the possible confounding influence of external factors. Moreover, some important factors, like fibrinogen, have not been evaluated, thus reducing the possibility of an exhaustive interpretation of the findings.

Furthermore, the study protocol was very strict. Blood withdrawal was performed without venous stasis (see section 2.2.2.), viscosity measurement was done at different shear rates and within two hours, the viscometer used was the same and, most importantly, the whole procedure was precisely the same at both examinations.

## 4.4. Summary

In conclusion, ageing per se and independently of coronary heart disease risk factors causes an increase in blood viscosity, and this might contribute to the increased risk of developing atherosclerosis frequently observed in advanced age.

# Chapter 5. Carotid haemodynamics in an ageing population, and the development of atherosclerosis

*The present Chapter reports the changes in the haemodynamics of the common carotid artery with ageing, and its associations with atherosclerosis development. This is achieved both by multivariate analyses and by comparing the two pairs of arteries in the same person over time, if they have different haemodynamic variations.*

## 5.1. Introduction

### *5.1.1. Rationale, participants, and procedures*

As discussed in Chapter 1, atherosclerosis is a systemic disease, with focal manifestations strongly dependent on local haemodynamic factors. Wall shear stress, the friction exerted by blood moving on the endothelium, is an important factor involved in arterial structure and function; it was found to be lower in elevated ages, favouring arterial damages.

Apart from a few short studies, there is a lack of longitudinal data regarding the effects of ageing on shear stress and atherosclerosis progression. The aims of the research presented in this Chapter, therefore, were to evaluate the influence on atherosclerotic plaque development of baseline and ageing-related changes in common carotid wall shear stress and other arterial physical properties.

As described in detail in Chapter 3, 48 of the participants to a cardiovascular programme conducted starting from the late 1990s were recalled after 12 years and included in this study. They underwent all the procedures described in Chapter 2: a complete clinical examination, ECG, lab reports, viscometry and ultrasound of common carotids. When data included arterial parameters, analyses were performed on carotid arteries on both sides of the body (n = 96 samples). Wall shear stress, circumferential wall tension, and Peterson's elastic modulus were computed based on echo-Doppler and blood viscosity data; carotid plaques and intima-media thickness were also analysed (Carallo et al. 2016).

### *5.1.2. Statistical analysis of haemodynamic variations and arterial wall thickening*

Student t tests for paired and unpaired data were used to compare continuous variables as appropriate. Pearson's correlation coefficient was computed to evaluate the association between age and diameter, velocity and shear stress. The multiple linear regression analysis was used to assess the association between the percentage change in intima-media thickness and the percentage change of the haemodynamic forces and traditional cardiovascular risk factors. Percentage change was calculated as the follow-up value divided by the baseline value and multiplied by 100. Triglycerides, not normally distributed, were log transformed. All statistical analyses were performed by SPSS 17.0 for Windows.

Appendix 2 provides a description of the statistical approaches used.

### *5.1.3. Statistical analysis of haemodynamic variations and the development of atherosclerotic plaques*

All continuous variables, except carotid plaque score, followed a normal distribution. Male sex, cardiovascular risk factors (smoking, obesity, hypertension, diabetes, dyslipidaemia) and carotid atherosclerosis development at follow-up were used as categorical variables (yes/no). Carotid atherosclerosis development was defined as any increase in plaque score at follow-up.

Student t-tests for paired data were used to compare baseline and follow-up data as appropriate. Pearson's correlation coefficient was applied to evaluate the association between baseline and follow-up wall shear stress, for both peak and mean values.

Binary logistic regression analysis was used to verify if the appearance of cardiovascular risk factors and haemodynamic variations during follow-up predicted carotid atherosclerosis development. This model was structured in two blocks as follows: in order to correct for the presence of risk factors at baseline, the first block (mode: enter) included baseline values of age, male gender, smoking, obesity, hypertension, diabetes, dyslipidaemia, plus each individual haemodynamic measurement; the second block (mode: stepwise forward) included the development at the follow-up visit of smoking, obesity, hypertension, diabetes, dyslipidaemia, plus percentage variation of the individual haemodynamic measurement included into the first block.

In order to exclude a cluster effect derived by the analysis of both carotids together, the infraclass correlation coefficient was calculated to estimate the variance explained by variability within the patient, on haemodynamic variables resulted associated to carotid atherosclerosis.

Then, analyses were restricted to participants presenting, at the follow-up, a decrease both in peak and in mean wall shear stress on one common carotid only. This was to verify if deterioration in haemodynamic values alone influences the development of atherosclerosis, with all other risk

factors being equal. The cut-off value chosen was a reduction by 5%, corresponding to the mean coefficient of variation of the present method for shear stress measurement (Gnasso et al. 1996). In this group, basal and follow up values of carotid plaque score, haemodynamic variables and their determinants were compared within each body side (decreased or unchanged in haemodynamics). Percent variations of carotid diameters and velocities at the follow-up were also compared between the two body sides. Student t-test for paired data and Wilcoxon test were used as appropriate. All these statistical analyses were performed by PASW Statistics 18.0.0 (IBM Corporation, Armonk, NY).

Appendix 2 provides a description of the statistical approaches used.

# 5.2. Results

## *5.2.1. Variations of haemodynamic factors*

*5.2.1.1. Comparison between subjects studied in the former analysis and matched controls studied in the pre-study analysis*

In order to test if the instrumentation used in the follow-up examination gave similar results as at baseline, before starting the follow-up two populations were recruited, that were similar to groups of participants at baseline. For this pre-study analysis, data were initially collected from a first "standard" subject group, consisting of 15 healthy subjects carefully matched for age, gender, body mass index and blood pressure with the 15 available healthy subjects studied in the initial examination between 1996 and 1998. The results are reported in Table 5.1.

**Table 5.1: Clinical, biochemical and haemodynamic characteristics of 15 healthy subjects studied now, against matched controls from those investigated 12 years ago.**

| | Former analysis | Last analysis |
|---|---|---|
| Systolic blood pressure (mmHg) | 118±6.6 | 118±2.5 |
| Diastolic blood pressure (mmHg) | 76±5.0 | 71±3.9 |
| Body mass index (kg/m$^2$) | 23±1.5 | 21.5±3.9 |
| Minimum carotid diameter (mm) | 5.3±0.4 | 5.4±0.5 |
| Maximum carotid diameter (mm) | 6.0±0.4 | 6.0±0.4 |
| Systolic peak velocity (cm/s) | 118.5±19.0 | 95.4±15.8 |
| Mean velocity (cm/s) | 43.8±4.9 | 41.7±6.0 |
| Blood viscosity 225 (cP) | 4.78±0.34 | 5.31±0.66 |
| Peak shear stress (dynes/cm$^2$) | 36.8±6.1 | 33.8±8.9 |
| Mean shear stress (dynes/cm$^2$) | 15.6±1.8 | 16.3±3.3 |

*Values are mean ± SD*

Then, a similar analysis was repeated in a second group (Table 5.2), but considering gender, age and patients suffering from various diseases. For this reason, the data of the participants examined in 2008 were compared to those of controls precisely matched for age, sex, presence or absence of hypertension, diabetes mellitus, obesity and dyslipidaemia, evaluated in 1996. For 36 of the 48 subjects examined in 2008 it was possible to find a suitable control among data recorded at baseline, from 1996. All measured and calculated variables were similar in both groups, with the exception of diastolic blood pressure, slightly but significantly higher in controls examined from 1996.

**Table 5.2. Clinical, biochemical, and haemodynamic characteristics of a sample of 36 subjects studied now, compared with matched cases investigated 12 years ago.**

| | Study Group | Control Group |
|---|---|---|
| Age (years) | 61.5±10.3 | 60.50±10.2 |
| SBP (mmHg) | 133±18 | 138±22 |
| DBP (mmHg) | 78.1±7.5 | 83.9±11.0* |
| Body mass index (kg/m$^2$) | 28.9±3.5 | 27.4±3.9 |
| Glucose (mg/dl) | 106±34 | 112±43 |
| Triglycerides (mg/dl) | 134±60 | 133±54 |
| Total Cholesterol (mg/dl) | 207±41 | 218±44 |
| HDL-Cholesterol (mg/dl) | 48±11 | 53±15 |
| Minimum carotid diameter (mm) | 5.9±0.7 | 5.9±0.8 |
| Maximum carotid diameter (mm) | 6.3±0.7 | 6.4±0.8 |
| Systolic peak velocity (cm/s) | 60.5±15.7 | 65.6±16.3 |
| Mean velocity (cm/s) | 28.7±8.2 | 30.9±7.5 |
| Blood viscosity 225 (cP) | 4.90±0.58 | 4.64±0.33 |
| Peak shear stress (dynes/cm$^2$) | 19.0±6.3 | 18.9±6.6 |
| Mean shear stress (dynes/cm$^2$) | 9.6±3.3 | 9.7±3.3 |

*Values are mean ± SD, unless otherwise indicated; *p<0.01*

In both the comparisons between these groups, therefore, all measured (diameter, velocity and viscosity) and calculated (shear stress) variables were similar and no statistically significant difference was detected. These results led us to pursue the study, in the belief that the measurement of haemodynamic forces in the carotid district, even at this distance of time, would be reliable. In

fact, measurements made in this study were not influenced by the type of equipment used and/or the skill of the operator, clearly different after 12 years.

*5.2.1.2. Common carotid diameter, flow velocity and volume*

In order to compare the arterial parameters at baseline and follow-up, the right and left common carotid artery were analysed separately. Therefore, the results presented in the following sections refer to a sample size twice (n=96) the number of participants (n=48). However, if two separate analyses, one for each body side, were performed, the results were similar.

Common carotid artery diameter was similar in males and females at each observation time and significantly increased in the follow-up throughout the cardiac cycle, by 5.5 (3.3%) in females and by 7.4 (6.3%) in males, at R and T wave of ECG, respectively. Systolic peak velocity and mean velocity were significantly lower in females at baseline compared to males, however in both sexes velocities significantly decreased during the observation period: by 10.7 (8.9%) in females, and by 14.2 (18.1%) in males, respectively. Common carotid blood flow was higher in males than in females in both sets of observations. At the follow-up visit, however, it decreased by 12.7% ($p<0.002$) in females, while in males the reduction was less marked (-6.8%; $p=0.07$) (Table 5.3).

**Table 5.3: Haemodynamic profile of 96 arteries from 48 subjects investigated at the start and the end of follow-up.**

| | Females (n=33) | | Males (n=15) | |
|---|---|---|---|---|
| | Baseline | Follow up | Baseline | Follow up |
| Minimum carotid diameter (mm) | 5.5±0.5 | 5.9±0.7§ | 5.7±0.6 | 6.1± .7§ |
| Maximum carotid diameter (mm) | 6.0±0.5 | 6.3±0.7§ | 6.2±0.6 | 6.5±0.8§ |
| Systolic peak velocity (cm/s) | 60.3±11.0 | 53.3±12.0§ | 75.4±19.8* | 63.7±15.9§ |
| Mean velocity (cm/s) | 31.9±6.9 | 25.3±8.0§ | 35.4±7.8* | 28.9±8.2§ |
| Blood flow (mL/s) | 3.86±0.68 | 3.37±0.85§ | 4.43±1.08* | 4.13±1.24 |
| Blood viscosity 225 (cP) | 4.19±0.48 | 4.63±0.54§ | 4.59±0.43* | 4.89±0.53§ |
| Peak shear stress (dynes/cm$^2$) | 16.7±3.3 | 15.6±4.8 | 22.6±6.5* | 19.7±6.3§ |
| Mean shear stress (dynes/cm$^2$) | 9.7±2.5 | 8.4±3.3§ | 11.6±3.0* | 9.6±3.2§ |
| Peak wall tension (x10$^4$ dynes/cm) | 5.3±0.8 | 6.2±1.0§ | 4.9±0.6* | 5.8±0.7#§ |
| Mean wall tension (x10$^4$ dynes/cm) | 3.6±0.5 | 4.1±0.6§ | 3.4±0.4* | 3.9±0.4#§ |
| Peterson's module. (x10$^5$ dynes/cm$^2$) | 97.4±79.5 | 170.1±113.3§ | 85.6±68.6 | 109.6±58.3#§ |
| Intima-media thickness (mm) | 715±119 | 924±222§ | 676±128 | 811±183#§ |

*Values are mean ± SD; *=vs. Females at baseline; §=vs. baseline; #=vs. Females at follow-up (all p at least < 0.01)*

### *5.2.1.3. Wall shear stress*

Looking at baseline versus follow-up haemodynamic data, males had higher baseline levels of peak and mean wall shear stress compared to females. At follow-up, in the male group, peak and mean shear stresses significantly decreased by 13.0% and 17.5%. In the female group, peak shear stress did not change at follow-up , while mean shear stress significantly decreased by 11.8% (Table 5.3). In the pooled group, baseline and follow-up wall shear stress were highly correlated (Figure 5.1), with a Pearson's correlation of: $r^2 = 0.57$, $p < 0.001$.

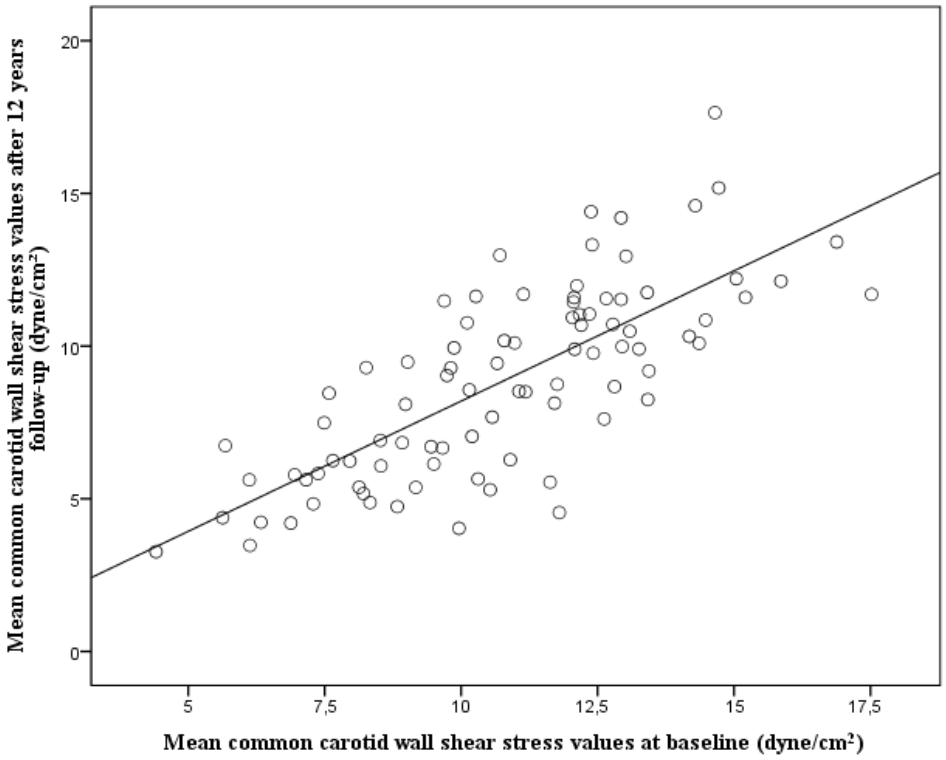



**Figure 5.1: Baseline and follow-up wall shear stress correlation.**

*5.2.1.4. Arterial stiffness and wall tension*

Arterial stiffness (Peterson's elastic modulus) and circumferential wall tension were significantly different between sexes (Table 5.3), and significantly increased at follow-up.

Peterson's modulus, at baseline and follow-up, was weakly but significantly inversely related to the respective shear stress ($r=0.39$, $p<0.01$ between baseline stiffness and baseline mean shear stress, $r=0.24$, $p=0.02$ between baseline stiffness and follow-up mean shear stress; other analyses gave similar results).

## *5.2.2. Haemodynamic variations and arterial wall thickening*

*5.2.2.1. Intima-media thickness*

In the present analysis, attention was paid to wall thickness variations, and to determinants of these changes. At follow-up, intima-media thickness significantly increased by 29% in females and 20% in males (Table 5.3). Stepwise linear regression analyses, including the percentage change of all clinical and biochemical and haemodynamic measured variables (blood pressure, body mass index, blood lipids, glucose, years of follow-up, sex, Peterson's elastic modulus, shear stress, and circumferential wall tension, the last two being either peak or mean values) as independent variables, showed that only the percentage change in mean shear stress was a significant predictor of the percentage change in intima-media thickness (R=.251, p=0.041).

*5.2.2.2. Entity of the time-dependent haemodynamic variations*

This section contains a comparison between the variations of key haemodynamic parameters with time predicted by the cross-sectional baseline analysis, with respect to those observed. The correlation between age and vessel diameter, flow velocity and shear stress was therefore evaluated across the entire population studied between 1996 and 1998 (n=147). Based on these results the variation of each of these parameters was estimated for the 48 subjects re-evaluated in 2008, and the results are shown in Table 5.4 along with the change actually measured.

**Table 5.4: Pearson correlation coefficients between age and vessel diameter, flow velocity and shear stress in the entire population examined between 1996 and 1998 (n=147), and estimated and measured variations in these parameters in the population examined in 2008 (n=48).**

| | | Females | | Males | |
|---|---|---|---|---|---|
| Minimum | *Correlation* | r=0.346 | p<0.001 | r=0.477 | p<0.0001 |
| carotid | *Estimated Δ* mm / yr | + 0.020 | | + 0.026 | |
| diameter | *Measured Δ* mm / yr | + 0.033 | | + 0.034 | |
| Maximum | *Correlation* | r=0.355 | p<0.0001 | r=0.368 | p<0.0001 |
| carotid | *Estimated Δ* mm /yr | + 0.028 | | + 0.021 | |
| diameter | *Measured Δ* mm / yr | + 0.025 | | + 0.026 | |
| Systolic | *Correlation* | r=0.069 | p=NS | r=0.757 | p<0.0001 |
| peak | *Estimated Δ* cm /s / yr | - 0.10 | | - 1.21 | |
| velocity | *Measured Δ* cm / s / yr | - 0.59 | | - 1.02 | |
| Mean | *Correlation* | r=0.340 | p<0.001 | r=0.618 | p<0.0001 |
| velocity | *Estimated Δ* cm / s / yr | - 0.28 | | - 0.37 | |
| | *Measured Δ* cm /s / yr | - 0.56 | | - 0.56 | |
| Peak | *Correlation* | r=0-194 | p=0.061 | r=0.765 | p<0.0001 |
| shear | *Estimated Δ* dynes / $cm^2$ / yr | - 0.10 | | - 0.42 | |
| stress | *Measured Δ* dynes / $cm^2$ / yr | - 0.09 | | - 0.24 | |
| Mean | *Correlation* r=0.386 | p<0.0001 | | r=0.670 | p<0.0001 |
| shear | *Estimated Δ* dynes / $cm^2$ / yr | - 0.12 | | - 0.17 | |
| stress | *Measured Δ* dynes / $cm^2$ / yr | - 0.11 | | - 0.17 | |
| Intima- | *Correlation* r=0.255 | p=0.013 | | r=0.487 | p<0.0001 |
| media | *Estimated Δ* μm / yr | + 4.61 | | + 5.26 | |
| thickness | *Measured Δ* μm / yr | + 17.7 | | + 11.6 | |

## *5.2.3. Haemodynamic variations and the development of atherosclerotic plaques*

*5.2.3.1. Logistic regression analysis*

In Table 5.5, data pooled by gender of shear stress, circumferential wall tension, and carotid plaque score (as defined in the Section 2.1.2.) are presented. All haemodynamic parameters of the 96 common carotids worsened with ageing. Peak and mean wall shear stress decreased by 12% and 18%, respectively; peak and mean circumferential wall tension significantly increased, as well as arterial stiffness by Peterson's modulus. Carotid plaque score almost doubled after 12 years.

**Table 5.5: Haemodynamic features of all 96 common carotids at baseline and follow-up.**

| | Baseline | Follow up |
|---|---|---|
| Peak shear stress (dynes/cm$^2$) | 20.6±5.6 | 18.0±5.5$^*$ |
| Mean shear stress (dynes/cm$^2$) | 10.8±2.7 | 8.9±3.1$^*$ |
| Peak circumferential wall tension (x10$^4$ dynes/cm) | 4.9±0.8 | 5.9±1.2$^*$ |
| Mean circumferential wall tension (x10$^4$ dynes/cm) | 3.5±0.5 | 4.0±0.7$^*$ |
| Peterson's modulus (x10$^5$ dynes/cm) | 9.1±7.2 | 12.4±8.0$^*$ |
| Carotid Plaque Score | 0.39±0.72 | 0.67±0.86$^*$ |

*Baseline vs Follow-up Student paired t-test statistical significances (Wilcoxon test for Echo Doppler Score):* $^*p \leq 0.01$

Determinants of carotid plaque development were then analysed with time. Looking at the incidence of cardiovascular risk factors and the change of vessel parameters during follow-up, only the percent variation of wall shear stress after 12 years was significantly and independently related to plaque progression or development: subjects with more marked wall shear stress reduction also developed higher carotid plaque score (Table 5.6 for peak shear stress; mean shear stress yielded

similar results, although those are not shown here). Percent variation of circumferential wall tension and Peterson's modulus did not enter into the model.

**Table 5.6: Binary logistic regression analysis on 96 common carotids (final model, variables entered in second block). Dependent variable: atherosclerotic plaque development at follow up visit (presence/absence).**

| *Variable* | *B* | *Odds ratio* | *95% confidence interv.* | *p* |
|---|---|---|---|---|
| Common carotid peak shear stress variations (%) | -0.063 | 0.94 | 0.89 – 0.99 | 0.01 |

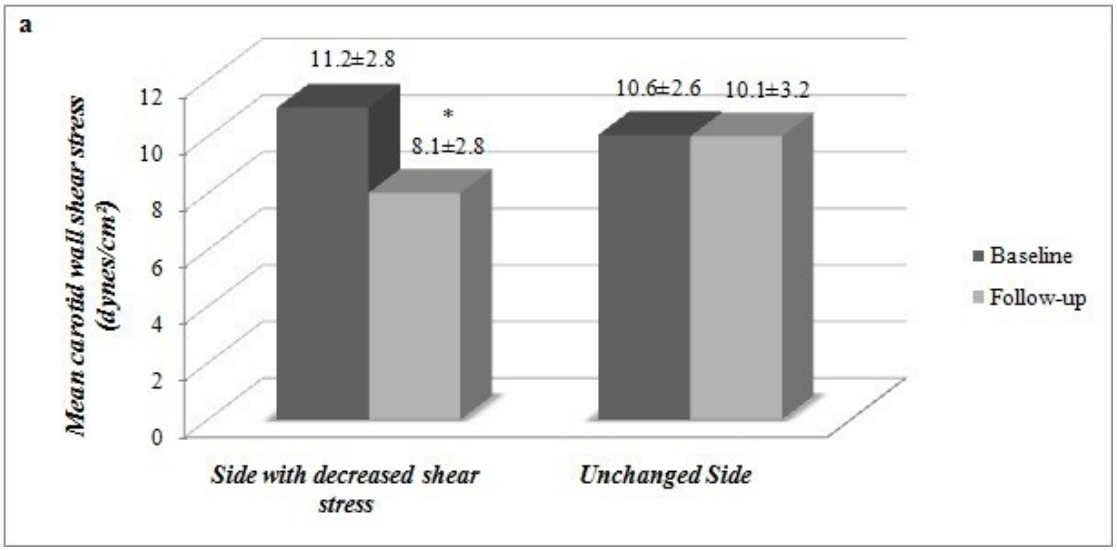
*Independent variables were entered in two blocks: first block (method: enter) included baseline values of age, male gender, smoking, obesity, hypertension, diabetes, dyslipidaemia, common carotid peak shear stress; second block (method: stepwise forward) included the appearance at the follow-up visit of smoking, obesity, hypertension, diabetes, dyslipidaemia, plus common carotid peak shear stress percentage variations.*

Considering the left and right carotids of the same patient as a possible cluster, the infraclass correlation coefficient was calculated for all shear stress values. The estimated correlations between measurements ranged from 0 to 0.28, indicating that the variability of the within-patient measurements was similar to the between-patients variability.

*5.2.3.2. Comparison between the left and right carotids*

The investigation also focused on possible different haemodynamic behaviour (asymmetry) in the two sides of the participants' bodies, irrespective of which side (left or right) was considered. This approach highlights if 1) age and chronic diseases have the same impact on haemodynamics in the two common carotids of the same body, and 2) two pairs arteries, eventually exposed to different haemodynamic conditions but identical risk factors, develop atherosclerotic lesions differently with ageing. It was found that age-related wall shear stress reduction was asymmetric in 21 (44%) subjects who showed peak and mean shear stress reduction ≥5% at follow-up on one side only of the body (57% of them on the right common carotid). Variations in peak and mean values of

haemodynamic variables showed similar results; therefore, to avoid redundancy, only mean values are presented (Figure 5.2). On the side with shear stress reduction (-21% for peak and -28% for mean values) plaque score increased from 0.52±0.98 to 0.90±0.94 ($p \leq 0.05$). In the opposite carotid artery, with stable shear stress, no atherosclerosis developed despite worsening in circumferential wall tension.



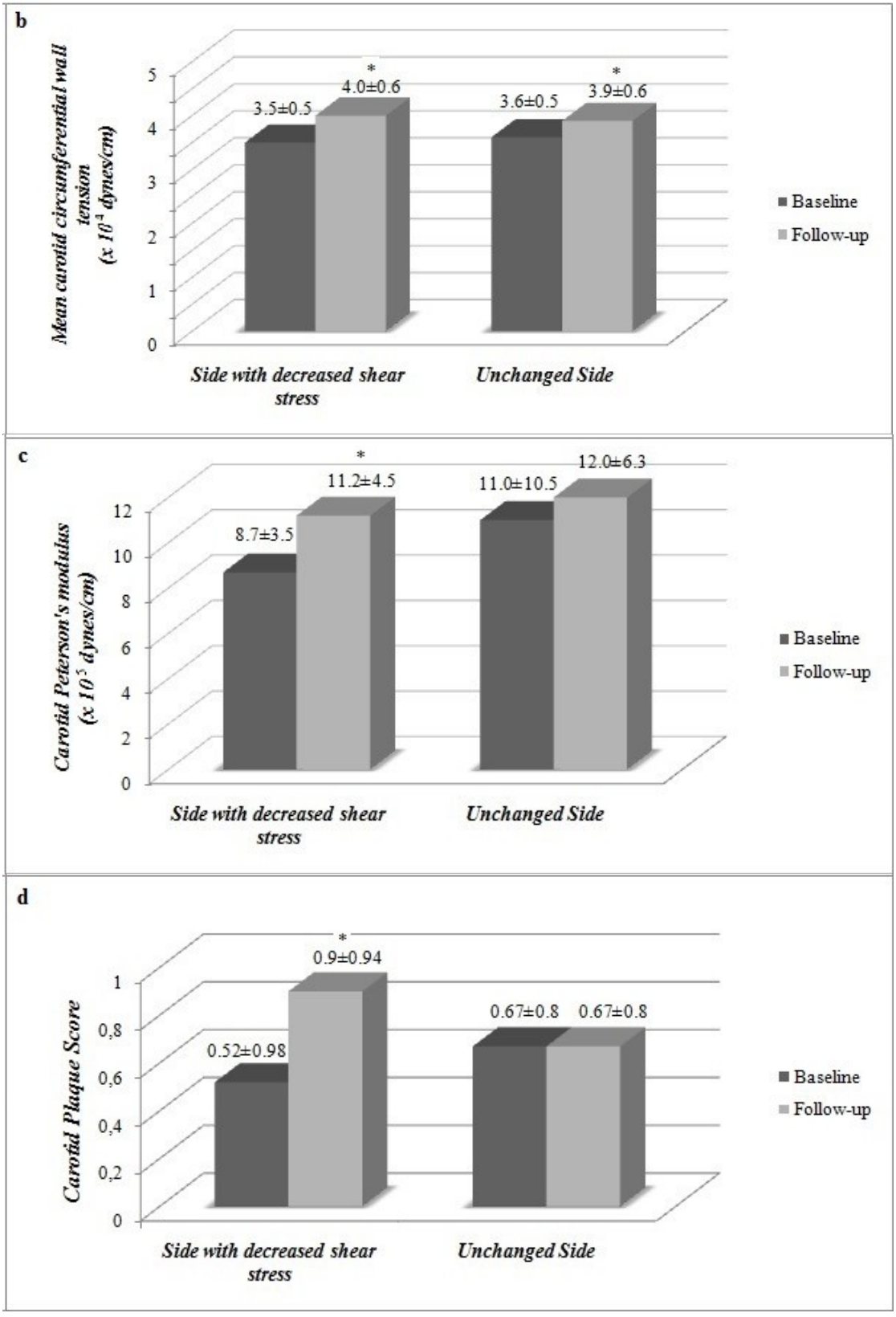



**Figure 5.2: Participants with common carotid peak and mean wall shear stress decrease ≥ 5% at follow up on one common carotid only (n=21). Student t-test statistical significances (Wilcoxon test for Carotid Plaque Score): * p<0.05 vs baseline.**

Looking at the determinants of these haemodynamic variations, they were a consequence of enlarged carotid diameters and reduced blood flow velocities, differently in the two body sides. Minimum diameter increased by 10.2 ± 1.3% in the carotid with shear stress reduction vs 3.9 ± 0.7% in the opposite side ($p=0.02$). Peak systolic velocity decreased by 27.6 ± 12.7% vs 11.6 ± 15.0% respectively ($p < 0.001$).

Asymmetrical changes in circumferential wall tension and arterial stiffness were found in two and one subjects, respectively.

## 5.3. Discussion

The results shown in this chapter represent the first longitudinal study of age-dependent changes in wall shear stress of the common carotid arteries. Our data prospectively demonstrate that ageing, in a middle-aged free-living population, induces changes in common carotid wall shear stress that can promote localised intima-media thickening. Furthermore, age-related wall shear stress reduction is an independent predictor of carotid plaque development and/or progression. Other parameters commonly used to evaluate the arterial wall, such as circumferential wall tension and wall stiffness, seem to play a minor role.

In detail, it was found that peak wall shear stress decreased with ageing in males whereas mean wall shear stress decreased in both males and females. These changes were caused by an increase in arterial diameter and a reduction in blood velocity. Furthermore, common carotid blood flow slightly decreased with ageing in our study population.

Furthermore, comparing the haemodynamic alteration with ageing among the two common carotids of the same individual, the analysis also revealed asymmetric shear stress reduction in almost half of the participants. Looking at this subgroup as a model, excluding the influence of many possible confounding factors such as chronic diseases and their therapies, it was clearly demonstrated that the development and/or the progression of plaques occurred exclusively in the side where shear stress decreased. In previous cross-sectional studies by the Author of the present Thesis and his Colleagues, where the left and right common carotids of the same individuals were compared, we found a strong association between wall shear stress and plaques in subjects with asymmetrical carotid atherosclerosis, fully in line with the present results (Gnasso et al. 1997), and also that wall shear stress is lower in the carotid artery responsible for a unilateral ischemic stroke (Carallo et al.

2006). The present findings confirm previous cross-sectional reports, and emphasize the importance of the role of haemodynamic forces also in the development of atherosclerosis.

In males, peak wall shear stress decreased as a consequence of diameter enlargement (+6.3%) and a fall in peak blood flow velocity (-14.2%), while there was also an increase in blood viscosity (+7.2%). In females, the enlargement of internal diameter and the fall in blood velocity were less marked (+3.3% and -10.7%, respectively) causing no change in peak wall shear stress. Women have stiffer arteries at follow-up and it is known that menopause per se may increase arterial stiffness (Staessen et al. 2001). Increased arterial stiffness is associated with an increase in the propagation speed of waves, with augmentation of the interaction of the incident and reflected waves (O'Rourke et al. 2007; Nichols et al. 2005; Segers et al. 2008). This might at least in part explain the less marked decrease in peak blood flow velocity in women. The marked increase in blood viscosity (+13.2%), furthermore, compensated for diameter and flow velocity variations causing peak wall shear stress to be almost unchanged in women.

The finding that peak wall shear stress in females remained unchanged is apparently in contrast with previous cross-sectional studies, demonstrating an inverse correlation between wall shear stress and age. In the study by Samijo et al. (1998), for example, a significant inverse correlation has been reported both in males and females. However, by limiting the analysis in Samijo's study to older subjects with comparable age to the present population, we could reach the same finding, i.e. peak wall shear stress in females did not decrease. Furthermore, in the study by Samijo, where viscosities were only derived from haematocrit and not measured in a laboratory, an increase in blood viscosity was steeper in females than males, and by the age of 55-65 years no difference between the two sexes was found, similarly to what we measured in the present population. Considering absolute values, both velocity and diameter measurements, and consequently blood flow calculations, are similar to those reported by Samijo et al (1998), with the exception of a 4%

smaller diameter in our population. Obviously, quantitative comparisons can only be made if similar echo-Doppler equipment were used and study populations were age and sex matched.

The reasons and the implications for a different trend in peak wall shear stress due to gender are as yet unknown and need further investigation. Mean wall shear stress decreases in both sexes due to a similar level of reduction in mean blood flow velocity (~18%) in both sexes. Mean blood flow velocity is less influenced by arterial stiffness.

The observed decrease in mean wall shear stress is at variance with the hypothesis that it should be maintained at the same level, through arterial wall remodelling. However, all published studies have shown that shear stress decreases with age. Probably, where there are temporary changes of blood flow supply needs, mean wall shear stress tends to remain constant in conductance arteries and also acts as a regulator of blood flow in arterioles by its influences on vasomotion (Koller et al. 1995). With ageing, however, shear stress is reduced, which in turn causes impaired functioning of the vessel predisposing to atherosclerosis. Atherosclerosis may in turn cause deterioration in haemodynamic conditions, creating a vicious cycle. In addition, the data of the present study demonstrate for the first time that changes in mean shear stress are associated with variations of intima-media thickness, after adjustment for changes in traditional cardiovascular risk factors. This could have important implications, allowing monitoring of the effect of therapeutic interventions in a clinically meaningful time frame.

The development of plaque in arterial segments with reduced shear stress has already been demonstrated in the coronaries after a few months of follow-up (Stone et al. 2003, Samady et al. 2011, Stone et al. 2012). In contrast to the previously reported data, the present results focused on ageing-related wall shear stress reduction, with a follow-up of ~12 years; furthermore, the present results looked at the carotid bifurcation, thereby also making the haemodynamic evaluation more comprehensive.

Arterial diameter, blood flow velocity and blood viscosity are also strongly interrelated; therefore, it is important to analyse the reciprocal influence of their variations in our population.

Concerning the interaction between viscosity and blood flow velocity, elevated blood viscosity could increase peripheral vascular resistance (Martini et al. 2005), which, in turn, might contribute to reduce blood flow and blood flow velocity. About the relationship between viscosity and arterial diameter, it has been reported that, in acute haemoconcentration experiments, the endothelial relaxation factor (nitric oxide) is stimulated causing an increase in arterial diameter in order to keep constant wall shear stress (Martini et al. 2005; Giannattasio et al. 2002). Despite this, we could notice that viscosity elevation should not be the only cause of the increase of arterial diameter with ageing in our population, since we have recorded a shear stress *decrease* with ageing.

As regards the relationship between velocity and diameter, it should be noted that an increase in arterial diameter is accompanied by a reduction in blood velocity if the total flow rate remains the same. In fact, looking at the inverse relationship among velocity and diameter, 71% (male) and 64% (female) of the mean blood velocity reduction with ageing, is predicted by the arterial enlargement with ageing in our population. The rest of the reduction of the blood flow velocity in the carotid artery with age might be attributed to impaired heart function (Homma et al. 2009) and/or to an increase in cerebrovascular resistance in the elderly (Robertson et al. 2010). In fact, a small reduction of carotid mean blood flow rate with ageing was also found in our population.

In short, the main ageing phenomenon in the carotid arteries, in our population, is arterial diameter enlargement; the present data also extend this conclusion in a context of the different behaviour exhibited between the two carotid arteries of the same patient. It can be hypothesized that this, in turn, may contribute to the development of an haemodynamic profile prone to atherosclerosis.

Increase in large artery diameter throughout life has been widely reported by many researchers, though the underlying mechanisms have not been fully clarified. According to the “stress fatigue” theory, the cyclic stress acting repetitively over many years on the arterial wall causes a subversion of the elastic component of the artery, leading to a loss of the retentive capacity of the elastic laminae (O’Rourke 1990; Wolinsky 1972). It has been postulated that an initial increase in blood flow velocity may lead to vessel dilation, in order to restore wall shear stress, which is followed by overcompensation (Glagov et al. 1987; Steinke et al. 1994; Jiménez-Quevedo et al. 2009). Arterial enlargement has also been attributed to the effort of the arterial system to maintain storage capacity (Reneman et al. 1986; Samijo et al. 1998). However, contrary to these hypotheses, in the present study we have observed a small reduction in carotid blood flow with ageing. The differences between the two sides are less easy to explain. Considering that head positions/rotations have a great effect on common carotid haemodynamics (Glor et al. 2004 2th), it is possible that head movements and consequent shear stress variations influence common carotid geometry differently during one’s lifespan.

Moreover, in the present study, common carotid stiffness showed conflicting results regarding its association with carotid plaque development. Here, the association has been partially verified in side to side model (right vs. left carotid comparison), but in the multiple regression analysis including the whole population it did not reach statistical significance. Reasonably, in the present study the limited sample size accounts for this discrepancy. Even if arterial stiffness is usually associated with incidence of cardiovascular events for the wide pathophysiological implications of its alterations (Zieman et al. 2005, Vlachopoulos et al. 2010), measurement methods for arterial distensibility and plaque presence and anatomic sites chosen for these measurements can strongly influence the results (Blaha et al. 2009). However, in a recent study with similar methods and length of follow-up, carotid arterial stiffness was found to be associated with incidence of ischemic stroke (Yang et al. 2012), disproving a finding presented in a previous study (Mattace-Raso et al. 2006).

## 5.4. Sources and analysis of experimental errors

It should be noted that extreme care was taken to ensure the reproducibility of the data (Chapter 2). Before starting the follow-up, new subjects and patients closely matching a sample of those previously examined, were recruited. In these new patients the same protocol and methods were adopted in all measurements (diameter, velocity and viscosity) and calculations (haemodynamic parameters) . The resulting data show no differences between the two sets of measurements.

Shear stress was calculated according to Poiseuille's law and equation. Poiseuille's law applies only to steady state laminar flow of a Newtonian fluid in a straight rigid tube of a uniform circular cross-section, conditions quite different from those observed in vivo. It has been demonstrated, however, that this model provides a useful estimation of wall shear (Stehbens 1979), and a fully developed parabolic flow profile is still at the basis of most of the recent literature (Moyle et al. 2006). This is despite the fact that velocity profiles are not parabolic in the common carotid, particularly in the deceleration phase, when shear stress evaluations are usually not performed (Ford et al. 2008). The choice of a parabolic flow model is largely driven by the need for simplification in clinical studies (Moyle et al. 2006). On the other hand, common echo-Doppler instrumentations, used in large clinical studies, are not able to discriminate flow velocity profiles; in the present study, therefore, it was necessary to keep the same settings as were used in the mid-1990s.

The use of Poiseuille’s law has been shown to be acceptable, however, at least for the mean value of arterial wall shear stress during the cardiac cycle (Schwarz et al. 2015). Furthermore, as far as flow in the common carotid artery is concerned, Poiseuille’s assumptions showed moderate agreement with computational fluid dynamics data, explaining 42% of its variability, even if with an

overestimation of absolute values; this agreement would not be the case at sites with complex arterial geometries, e.g. the carotid bulb (Augst et al. 2007).

The brachial blood pressure used for carotid circumferential wall tension calculations is only an index of central arterial pressure. Brachial blood pressure and central blood pressure may change differently with ageing, or upon different hypertensive therapies (Manisty et al. 2009). Although no data are available, it seems reasonable to assume that comparisons between the left and right carotid arteries of the same subject should have overcome this problem.

With regard to the present study design, it is clear that whenever an experimental approach bias has taken place, it should be replicated also in the follow-up. There are at least two exceptions to this, however. First, the shape of velocity profile may change with time in the same patient, particularly as arterial compliance decreases. This would cause elevation in peak centreline blood flow velocity and consequently shear stress in the second examination. Second, it is possible that ageing or change in tortuosity of the vessel during the follow-up period may influence the profiles of flow velocity in the second examination, particularly for peak shear stress measurements. These considerations will be accounted for in the interpretation of the results, even if, as previously explained, curved common carotids were excluded.

## 5.5. Summary

In conclusion, the results of this study show that, in a middle-aged population observed after almost 12 years, mean wall shear stress decreases significantly in both sexes, while reduction in peak wall shear stress is only significant in males. The reduction in mean shear stress is associated with increases in intima-media thickness. Arterial stiffness and circumferential wall tension, on the other hand, increase with ageing. Furthermore, wall shear stress and ageing-associated wall shear stress

reduction predict the development of new atherosclerotic plaque in the carotid arteries, independently of known cardiovascular risk factors.

Considering the clinical implications, this study strongly supports the importance of local haemodynamic factors in the development of atherosclerosis. The evaluation and control of these "physical" factors, namely low wall shear stress (Richter et al. 2006), may deserve clinical attention now focused almost exclusively on classical "chemical" cardiovascular risk factors, apart for hypertension that has an impact on haemodynamics as well (Levenson et al. 1998).

# Chapter 6. Conclusions and recommendations for future work

*After the general conclusions derived from the Thesis, the present Chapter presents some areas of future improvements in haemodynamic evaluation. All the basic rheological parameters, such as blood viscosity, arterial diameter, blood pressure, blood flow velocity, are addressed in an effort to present new techniques.*

## 6.1. Conclusions of the Thesis

The ageing process of the large arteries constitute a complex derangement from physiology. It relies upon multiple physical and biochemical alterations, often interrelated. The final effect, the atherosclerotic process, is a mix of two main phenomena, appearing differently in different patients/arteries: the partial or complete loss of vessel wall compliance, and of blood flow patency.

Among the physical causes of atherosclerosis, particularly wall shear stress, that is the friction exerted by the blood moving on the endothelium, is an important haemodynamic factor involved in arterial pathophysiology: low wall shear stress values activate a complex pro-atherogenic biochemical pattern of the vessel wall, and they have been found to be associated with the development of atherosclerosis.

The decline of wall shear stress values with ageing has been reported by several cross-sectional studies. The present approach is based upon a prospective approach, with two examinations after twelve years: it uses the common carotid as a model for conductance arteries, and ultrasound as a tool for repeated non-invasive haemodynamic measurements. The present data confirm that with seniority shear stress declines, giving also the entity of these variations in the two genders. Together with this wall shear stress reduction, the present results show an increase of blood viscosity and a

local atherosclerosis development; the relationship between shear values and atherosclerotic process with ageing results independent from any known cardiovascular risk factor.

Furthermore, despite of all "systemic" risk factors for cardiovascular diseases, here it has also been noted that frequently this haemodynamic impairment with ageing is not symmetric among pair arteries as the distal area of common carotids; in those cases, atherosclerotic process develops mainly in the side of the body where wall shear stress declines, irrespectively of the position (left or right) of this side.

Finally, looking at future research, multiple areas of improvements of haemodynamic measurement methods might be addressed. All the followings will be taken into consideration into shear stress calculations: a shear rate specific blood viscosity; several points of measurements of carotid haemodynamics during a cardiac cycle; the real shape of the carotid blood flow velocity profile. Taking them as a whole, all this will allow to reduce theoretical assumptions and finally to improve accuracy and precision of the haemodynamic measurements.

Perspectively therefore, also with these methodological improvements, it seems important to identify the mechanisms underlying local atherosclerotic derangement with ageing, and to verify eventual intervention procedures that could have a positive impact on large artery haemodynamics.

## 6.2. Overcoming some limitations in haemodynamic determinations

### *6.2.1. Shear rate-specific blood viscosity*

This section discusses some of the problems arising from considering blood viscosity as constant throughout the cardiac cycle in the common carotid.

It is known that under high shear rates, generally above 90 $s^{-1}$, blood behaves as a Newtonian fluid and shows near constant viscosity (Matrai et al. 1987). At lower shear rates, however, blood viscosity increases exponentially, as reported in Section 1.4.1.6 (pseudoplastic behaviour). Before choosing the shear rate for measuring blood viscosity *in vitro* for application to haemodynamic calculations, therefore, it is important to know the shear rates to which blood is exposed *in vivo* in the arterial section of interest.

In the present study the viscometer used was able to measure blood viscosities in the shear rate range from 450 to 45 $s^{-1}$. The calculated time-averaged mean shear rate in the entire sample at baseline and follow-up was above 200 $s^{-1}$. Consequently, for each patient a blood viscosity value measured at a shear rate of 225 $s^{-1}$ was chosen.

Obviously, an investigator might be interested to consider minimum shear stress during the cardiac cycle, and consequently pulse shear stress, since both are important haemodynamic determinants of vascular pathophysiology (Tedgui et al. 2001). If this is the case, in the late diastole in the common carotid, particularly in aged and/or diseased people, low shear rates might be found, at a level at which erythrocyte aggregation occurs, elevating blood viscosity and consequently wall shear stress.

In order to verify this hypothesis, further analyses were performed on five adults (three males) randomly extracted from the database of the study. First, end diastolic (minimum) blood flow velocities during the cardiac cycle were used to calculate minimum shear rates and stresses, based on the equation described in Section 2.2.3.3. Then, shear rate-specific blood viscosities for each carotid for each patient were used to calculate shear rate-specific minimum shear stress (for example, if a patient after his ultrasound examination had a calculated wall shear rate of 85 $s^{-1}$ on his left common carotid, then the calculation of his left minimum common carotid wall shear stress took into account the value that his blood presented in vitro at 85 $s^{-1}$).

Results are summarised in Table 6.1, which shows that the mean value in the population of minimum shear rate during the cardiac cycle is much lower at follow-up compared to baseline. Furthermore, shear rate-specific blood viscosities and the corresponding minimum shear stresses were 11% and 24% higher at baseline and follow-up, respectively, compared to the values without using shear-specific blood viscosities.

**Table 6.1: Mean values of age and minimum haemodynamic values during the cardiac cycle of ten common carotids (five patients) at baseline and follow-up.**

| | Baseline | Follow up |
|---|---|---|
| Mean age (years) | 52.8 | 64.4 |
| Minimum shear rate ($s^{-1}$) | 143.4 | 84.6 |
| Blood viscosity at $225s^{-1}$ (cP) | 4.6 | 4.5 |
| Shear rate-specific blood viscosity (cP) | 5.1 | 5.6 |
| Minimum shear stress (dynes/$cm^2$) | 6.6 | 3.8 |
| Shear rate-specific minimum shear stress (dynes/$cm^2$) | 7.3 | 4.7 |

Nevertheless, these calculations, even if one takes into account the effect of shear rate on blood viscosity, are for demonstration purposes only because they address only one aspect of the problem; i.e. the *in vivo* effects of changes of blood viscosity on shear rates (for example, a lowering of blood flow velocity in the areas where shear rates are low and blood viscosity increases) are not addressed by a Poiseuillean method for shear stress computation. A computational fluid dynamics model should be developed to accurately assess these effects.

## *6.2.2. Non-Newtonian models for shear rate-dependent blood viscosity*

### *6.2.2.1. Introduction*

As previously reported, the choice of flow model is crucial in haemodynamic analysis. According to the literature, at high shear rate conditions blood can be regarded as a Newtonian fluid; however blood exhibits non-Newtonian behaviour at low shear rates, particularly in some diseased conditions and in pulsatile flow, in which blood is subjected to cyclic low shear rates for a major part of the cycle (Matrai et al. 1987).

Some studies have reported a pronounced effect of shear-thinning on blood flow patterns (Gijsen et al. 1999; Chen et al. 2006; Siauw et al. 2000; Tu et al. 1996), while others have shown only a modest effect (Perktold at al. 1991; Cho et al. 1991; Valencia et al. 2006). The effect of non-Newtonian rheology depends explicitly on geometry and flow rate characteristics, and thus should be considered individually in each case (O'Callaghan et al. 2006).

The aim of this sub-study was to compare different non-Newtonian models when applied to the common carotid artery by using the viscosity and haemodynamic data described in the preceding section. Three well-known non-Newtonian models, namely the Carreau, Power-Law and Quemada models (Jung et al. 2004, Quemada et al. 1978) were tested. The flow rate at the inlet of the vessel was assumed to be the same for all models so that differences between the experimental value and simulated value of peak and mean shear stress by each model can be solely attributed to the model's rheological behaviour.

The density of blood is dependent on haematocrit and temperature. For the present study, the value of blood density is taken to be 1056kg/m$^3$ (Chmiel et al. 1980). Blood is a non-Newtonian fluid and therefore its viscosity is dependent on shear rate. Its shear-thinning behaviour implies that the viscosity is very high at low shear rates. It decreases gradually and maintains an almost constant value at high shear rates (asymptotic viscosity) where the behaviour of blood is Newtonian (see previous Section). The value of the asymptotic viscosity is very useful in calculating certain parameters introduced in the constitutive equation of blood and it is therefore of special interest.

*6.2.2.2.. Blood constitutive equations*

There are various models to describe the non-Newtonian behaviour of blood. In this work, three of the most widely known homogenous models are used and discussed below.

Quemada model

Quemada (Quemada et al. 1978) proposed the application of a rheological model for "concentrated disperse systems" as a constitutive equation for human blood. The Quemada's model, which is based on a minimum energy dissipation analysis, calculates the apparent viscosity as:

$$\mu_r = \mu_F(1 - \frac{1}{2}\tilde{k}\,\varphi)^{-2} \quad (1)$$

Where

$$\tilde{k} = \frac{k_0 + k_\infty \gamma_r^p}{1 + \gamma_r^p} \quad (2)$$

And

$$\gamma_r^p = \frac{\gamma}{\gamma_c}$$

Here $\mu_F$ and $\varphi$ are the viscosity of the suspending fluid (i.e. the viscosity of the human plasma) and the volume fraction of the dispersed phase respectively, therefore they should be patient specific. Under normal conditions, values of these parameters are: $\mu_F$ 0.0013 Pa.s; $\varphi$: 0.5073. For a shear-thinning fluid, $k_0 > k_\infty$ . For blood, the values of the constants were obtained by curve fitting to the experimental data of shear rate and viscosity (Figure 33), resulting in the following parameters:

$$k_\infty = 1.565,\ k_0 = 3.271 \text{ and } \gamma_c = 20.785\ [s^{-1}]$$

Carreau model

The complex rheological behaviour of blood can be approximated using the Carreau model (Jung et al. 2004), where the apparent viscosity is expressed as a function of shear rate:

$$\mu = \mu_\infty + [(\mu_0 - \mu_\infty)][1 + (\lambda * \dot{\gamma})^2]^{\frac{q-1}{2}} \quad (3)$$

and $\dot{\gamma}$, a scalar measure of the rate of deformation tensor, is given by:

$$\dot{\gamma} = \sqrt{\frac{1}{2}\sum_i \sum_j \dot{\gamma}_{ij}\,\dot{\gamma}_{ji}} \quad (4)$$

Here, $\mu_\infty$ is the viscosity of blood at infinite shear rate, $\mu_0$ is the viscosity of blood at zero shear rate, $q$ is the power law index and its value is obtained from the power law model curve fitting (see below).

The Carreau model was fitted to the experimental data (as shown in Figure 6.1) resulting in the following set of parameters:

$$\mu_\infty = 0.00345 \text{ Pa·s}, \mu_0 = 0.015 \text{ Pa·s}, \lambda = 2.329 \text{ s}, q = 0.7385.$$

Power Law model

Another commonly used blood rheological model is the power law written as:

$$\tau = k\,\dot{\gamma}^{q} \quad (5)$$

Or $$\tau = \mu\,\dot{\gamma}$$

Where, $$\mu = k\,\dot{\gamma}^{q-1}$$

Here $\mu$ is the apparent viscosity, $k$ is the flow consistency index (SI units Pa.$s^q$) and $q$ is the behaviour index (dimensionless). Blood is a pseudoplastic power law fluid which has progressively decreasing apparent viscosity with strain rate ($q$<1). At very high shear rates, the apparent viscosity becomes constant and equal to $\mu_\infty$, and the shear rate relationship becomes linear. Values of the parameters are:

$$k = 0.0198 \text{ Pa}.s^q \text{ , } q = 0.738$$

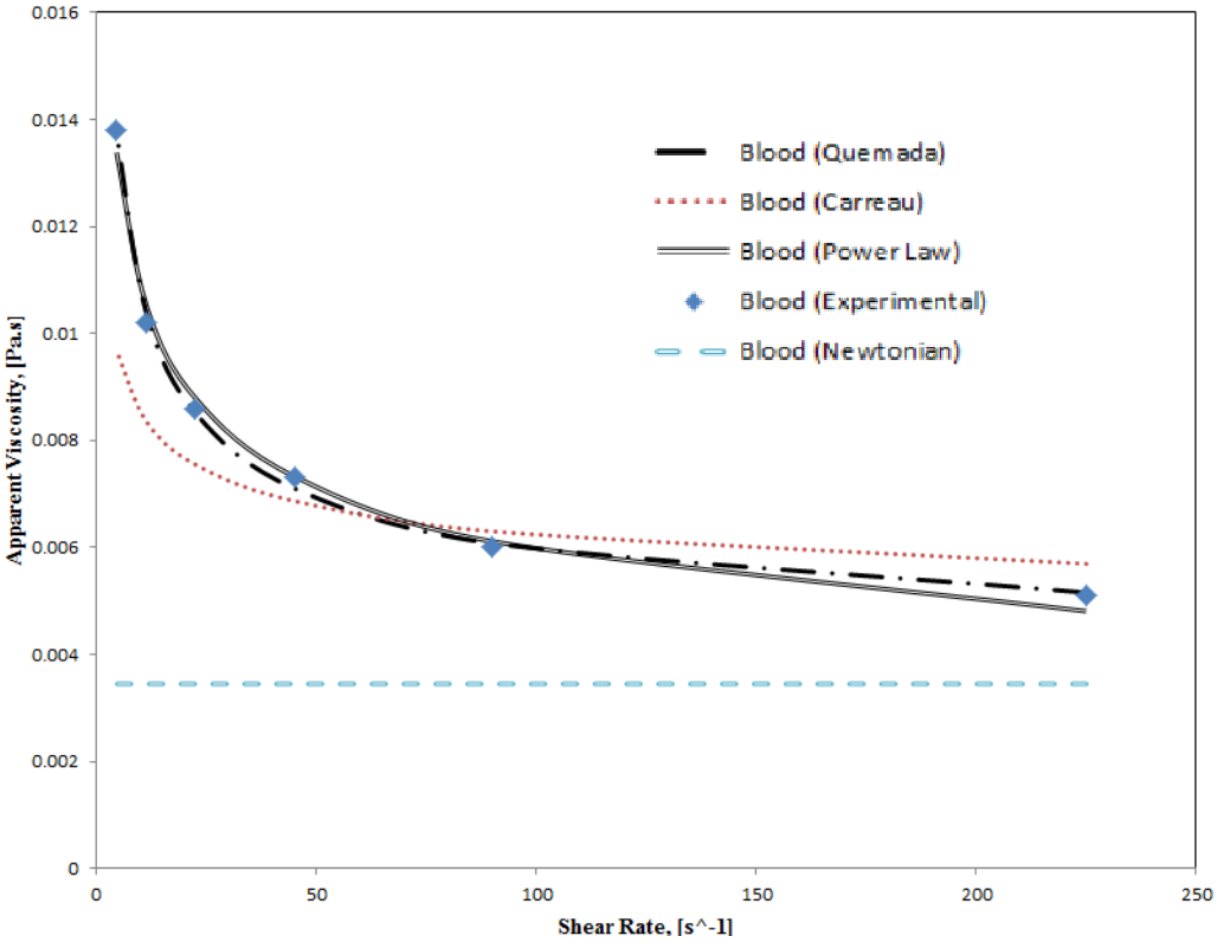



**Figure 6.1: Blood viscosity (averaged values of 5 subjects) versus shear rate for different non Newtonian models.**

*6.2.2.3. Computational models*

Simplified computational fluid dynamic models were built to calculate velocity profiles and wall shear stresses using each of the selected non-Newtonian models. Details of the computational fluid dynamic models are described below.

Geometry

The carotid geometry is assumed to be a cylindrical tube with a plane of symmetry along the length of the tube. The length of the tube was chosen so that fully-developed velocity profile could be established at the outlet. Hydrodynamic boundary layer equation for laminar flow

was used to estimate the length required ($x_{fd,h}$) as a function of tube diameter (D) and flow Reynolds number, $Re_D$.

$$\left(\frac{x_{fd,h}}{D}\right)_{\text{laminar}} \simeq 0.05\, Re_D \qquad (6)$$

Based on Reynolds numbers estimated from measured blood velocities and carotid artery diameters, the maximum value of length to diameter ratio is around 8. Therefore, a length equal to 10 times the tube diameter was used in the initial model to ensure fully developed flow at the outlet. After trial simulations it was found that the flow became fully developed fairly quickly, over a much shorter length of the tube, hence a length equal to 5 times the tube diameter was used in further simulations in order to save computational time.

Governing equations

It is generally accepted that blood flow in the common carotid artery is laminar under normal physiological conditions. The governing equations for blood flow are the conservation of mass and momentum equations for an incompressible and non-Newtonian fluid. These are three dimensional, time-dependent Navier-Stokes equations given below:

$$\frac{\partial u_i}{\partial x_i} = 0 \qquad (7)$$

$$\rho\left(\frac{\partial u_i}{\partial t} + u_j \frac{\partial u_i}{\partial x_i}\right) = -\frac{\partial p}{\partial x_i} + \frac{\partial \tau_{ij}}{\partial x_j} \qquad (8)$$

The shear stress tensor is defined by the constitutive equation (9):

$$\tau_{ij} = \mu \frac{\partial u_i}{\partial x_j} + \mu \frac{\partial u_j}{\partial x_i} \qquad (9)$$

For $i, j = 1, 2, 3$.

Numerical scheme

Parameters in the constitutive equations of various non-Newtonian models were obtained by fitting experimental viscosity data using Generalized Reduced Gradient-GRG2 nonlinear optimization code in Microsoft Excel Solver tool. The non-Newtonian models were

implemented in a general purpose CFD code, ANSYS-CFX, which employs a finite volume scheme based on the pressure-correction method and the SIMPLE (Semi-Implicit Method for Pressure-Linked Equations) algorithm. The boundary conditions of the problem are as follows: flat velocity profile at the inlet, fully developed flow with a zero relative pressure at the outlet, and no-slip condition on the wall which was assumed to be rigid. Standard tolerance value of $10^{-6}$ was specified in order to control numerical convergence. Simulations were run for two cycles to damp initial transients and results obtained from the third cycle were used for analysis. Solutions were obtained iteratively by following the basic steps described below.

1. A first approximation for the velocity (u, v and w) and pressure (p) distribution is assumed.

2. Pressure equation is solved.

3. Equations are solved and u, v and w are corrected.

4. Steps 2−3 are repeated until the convergence criterion is satisfied.

5. The above calculations are repeated at each time step for several cycles and the results obtained for each cycle are compared to those from the previous cycle.

A periodic solution is obtained when the variables calculated at different points and at different times over two successive cycles become almost identical.

*6.2.2.4. Discussion*

A quantitative comparison of the different non-Newtonian models is summarized in Table 6.2. The Carreau, Power Law, and Quemada models gave different shear stress results. Left and right common carotids were pooled for the analysis.

**Table 6.2: Mean shear stress values of 10 common carotids at the baseline examination calculated by three different models.**

| | Carreau | Power Law | Quemada |
|---|---|---|---|
| Peak shear stress (dynes/cm$^2$) | 20.4 | 23.5 | 18.6 |
| Mean shear stress (dynes/cm$^2$) | 13.6 | 8.54 | 14.8 |

Three constitutive laws (Quemada model, Carreau Model and Power Law) have been employed to describe the non-Newtonian shear-thinning blood viscosity. The results show that all three constitutive models are suitable for describing the non-Newtonian blood viscosity, but the Quemada model offers a better fit to the viscosity model and takes into account various important parameters, making it the most suitable model for common carotid blood flow analysis.

Regarding limitations of this method, the geometry adopted in the computational fluid dynamic model was highly idealised whereas in reality the common carotid artery may be curved with non-circular cross sections. Nevertheless, the idealised computational fluid dynamic model adopted here served the purpose of comparing the three most commonly used non-Newtonian models, while examining the potential discrepancy associated with the simple estimation of wall shear stress based on Poiseuille's law.

## *6.2.3. Central blood pressure measurement*

### *6.2.3.1. Introduction*

In order to evaluate circumferential wall tension more accurately, the measurement of carotid rather than brachial blood pressure would be desirable. In fact, central and peripheral arterial pressures are different, and this difference increases with ageing and the associated arterial stiffening, as better reported in Section 1.5.5 (Mitchell et al. 2008). A way to measure central arterial pressure non-invasively is by an *in situ* tonometric evaluation (Manisty et al. 2009), and this procedure is described in the following Section.

### *6.2.3.2. Method*

A tonometric examination of the right common carotid was performed on a participant at the follow-up visit, together with simultaneous diameter measurement. Several samples of the two variables were taken, as a mean of 3 or 19 cardiac cycles for diameter and pressure respectively. Measurements were taken from a patient who had been fasted and had been in the supine position for ten minutes. The patient abstained from nicotine and caffeine for at least twelve hours before the examination. A Miller tonometer (SPT-301) was used to acquire pressure in the common carotid artery (CCA), while the CCA diameter was measured with a Philips L12-3 ultrasound scanner.

Adobe Photoshop CS6 was used to overlay graphics of pressure, diameter, and electrocardiographic traces, and Autodesk Design Review 2013 was used to measure diameters on ultrasound images.
Data were acquired over 19 cardiac cycles and every cycle contained 21 frames with intervals of 46 milliseconds. Measurements were performed starting from the peak of the R wave as a fiducial point (Figure 6.2)

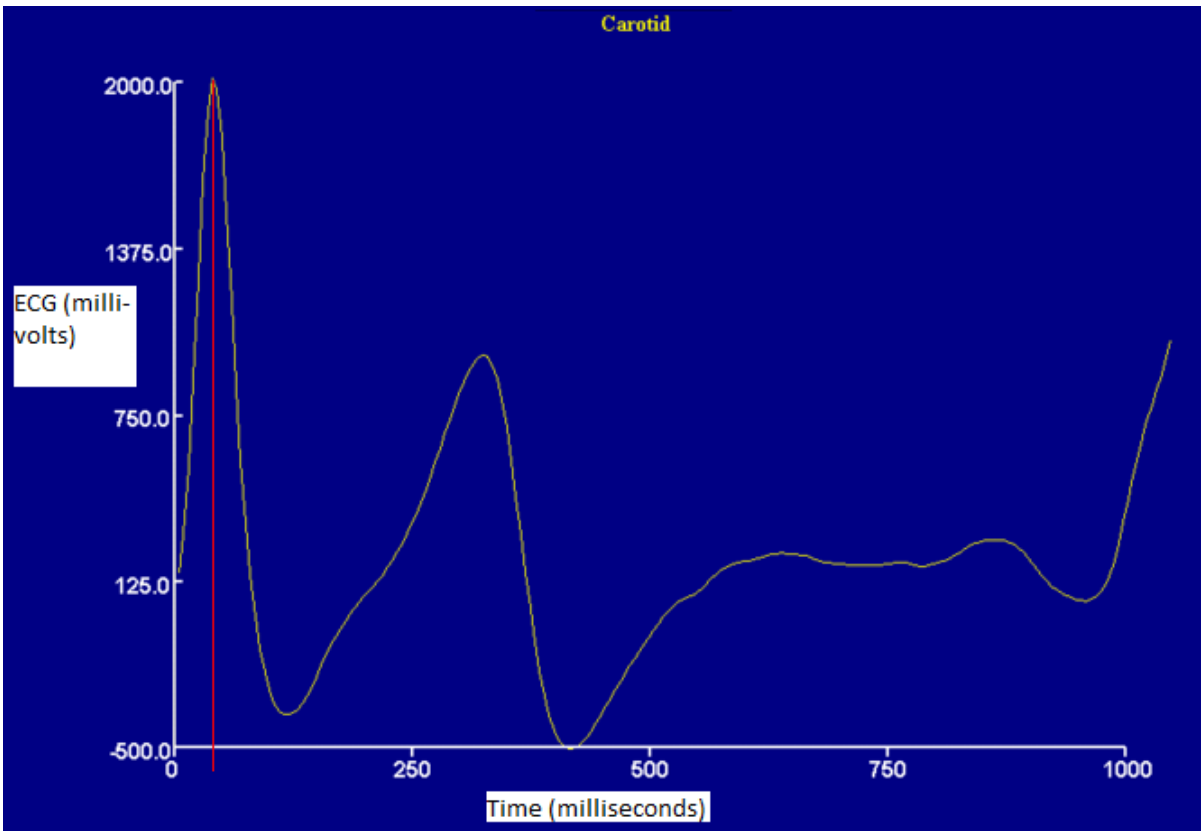



**Figure 6.2: Cardiac electric activity recordings.**

Graphics representing the average pressure and the ECG trace over 19 cardiac cycles have been drawn. Pressure curve quality in this sample was low, being only for demonstrative purposes. These two curves are overlaid as shown in Figure 6.3.

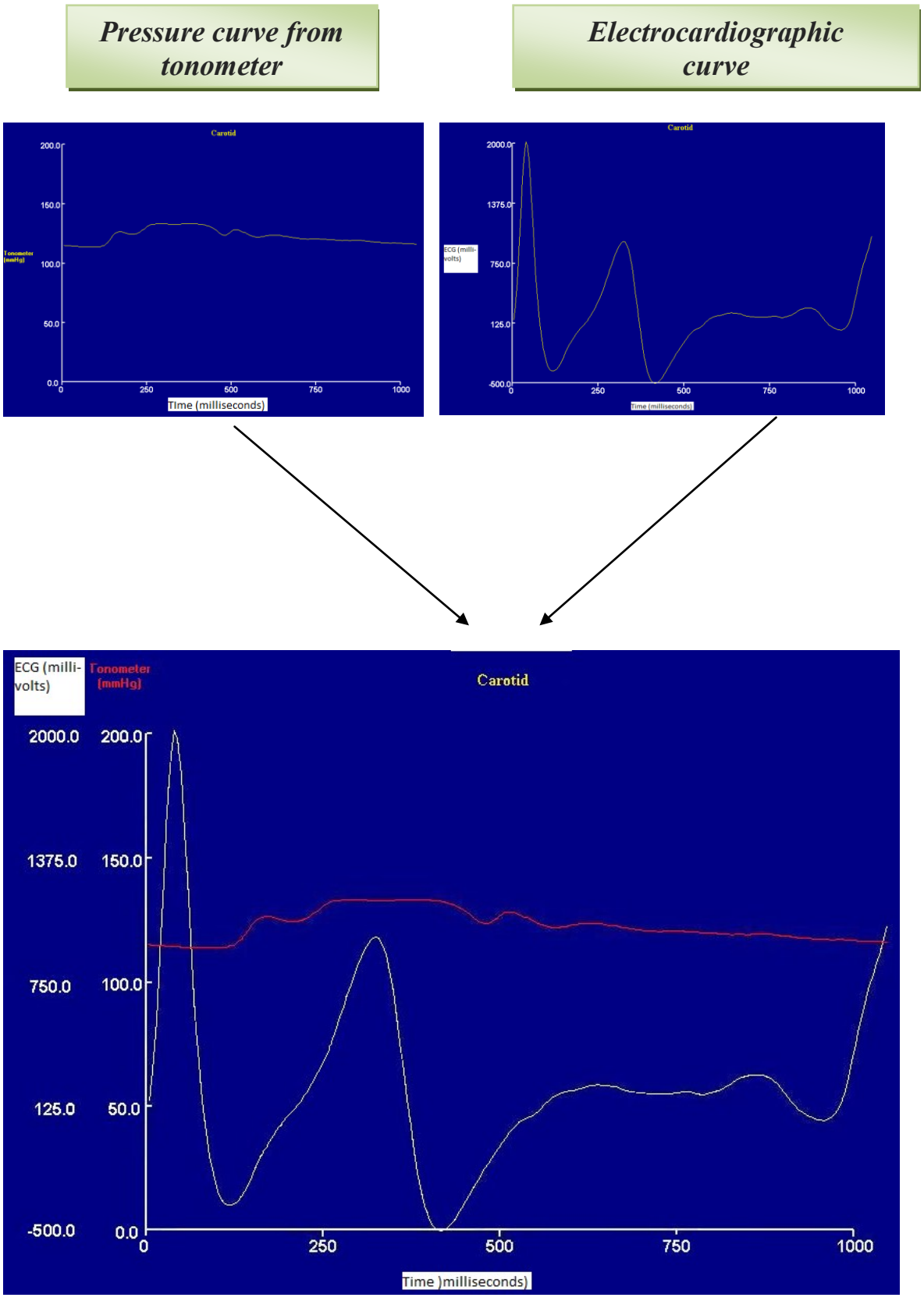



**Figure 6.3: Tonometer recordings of common carotid pressures along a cardiac cycle.**

CCA diameter was measured at 10 mm proximally to the carotid bulb (Figure 6.4).

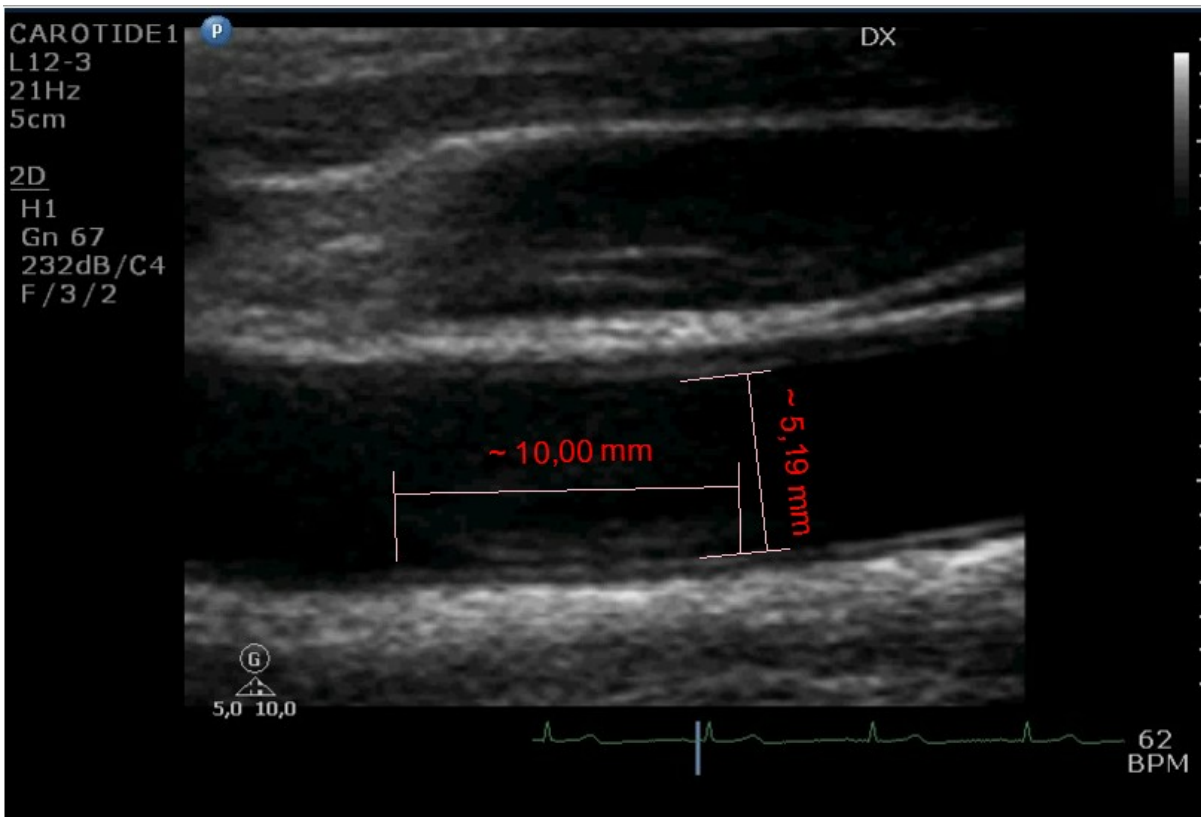



**Figure 6.4: Ultrasound image showing the measurement of common carotid diameter.**

Figure 6.5 shows measured CCA diameters at 21 time points (the same as those of pressure measurements) during a cardiac cycle, with peak R wave on ECG being used as a reference. Measurement at each time point was the average value over three cardiac cycles. It shows that CCA diameter varied between 5.07 mm and 5.56 mm during a cycle.

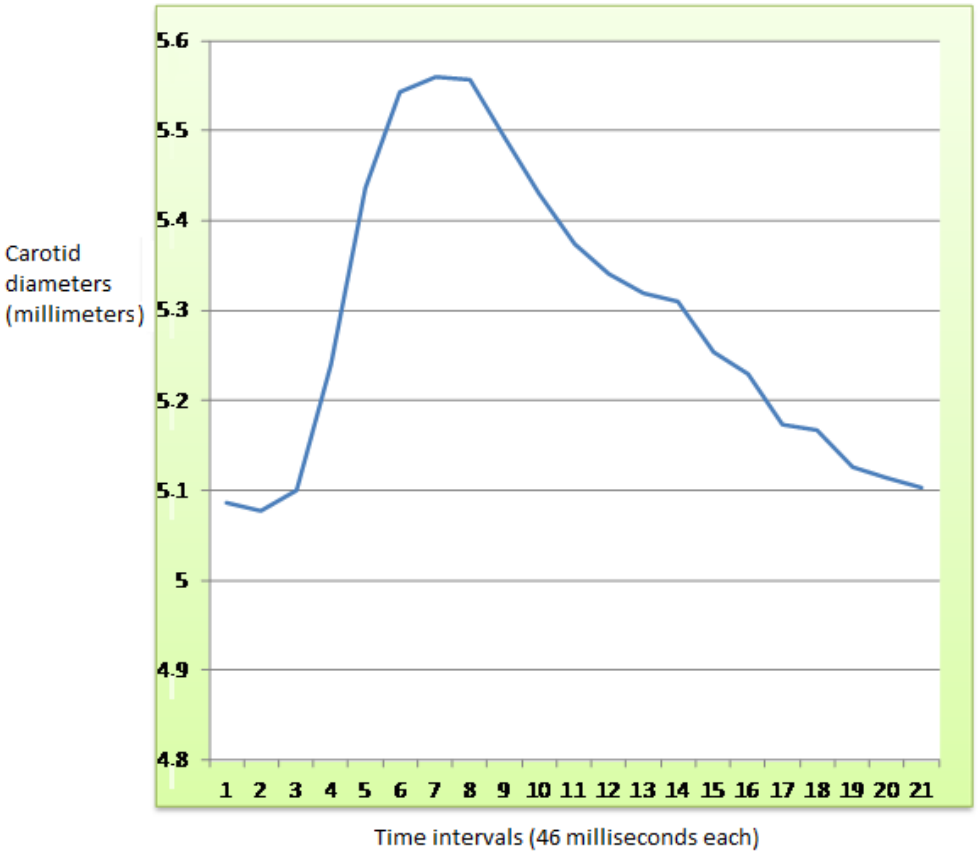



**Figure 6.5: Measured CCA diameters during a cardiac cycle.**

Similarly, CCA blood pressure measurements were obtained and the results are shown in Figure 6.6 without smoothing.

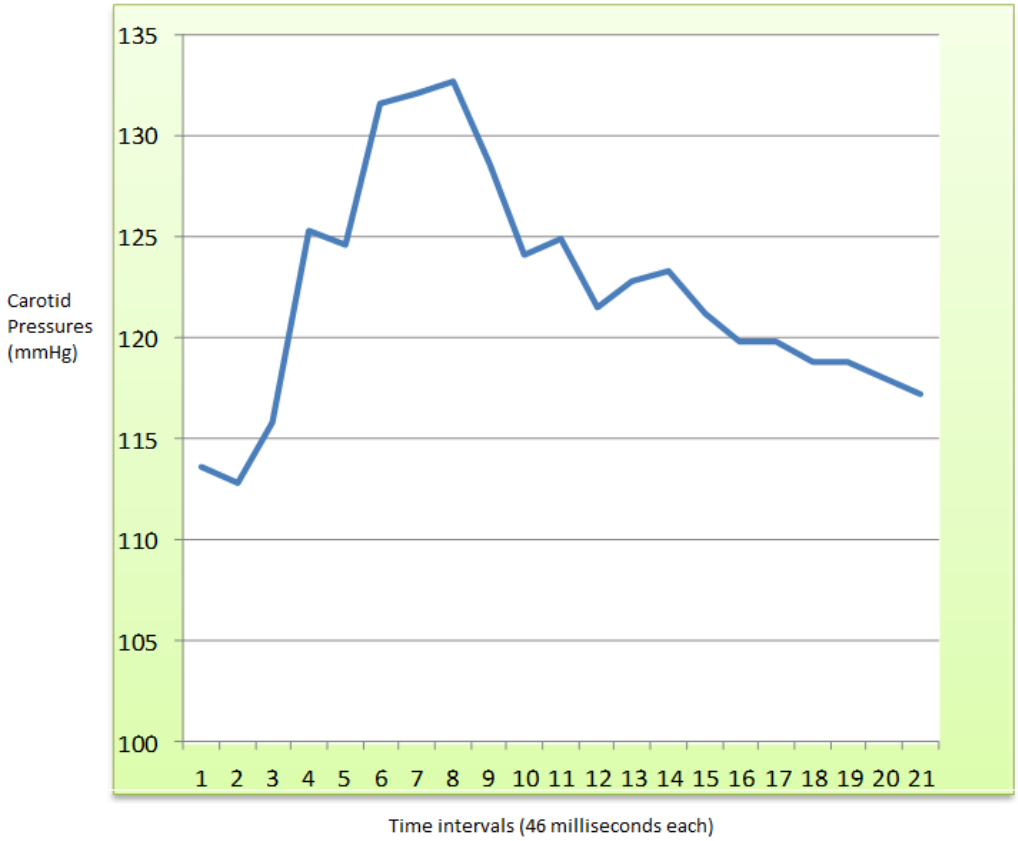



**Figure 6.6: Measured CCA pressures during a cardiac cycle.**

## *6.2.4. Multigate Doppler blood flow velocity acquisition*

*6.2.4.1. Introduction*

As discussed in Section 2.2.1, an important issue in computing wall shear stress is the determination of the wall shear rate. Indirect wall shear rate estimates based on the measurement of peak centreline velocity, or mean velocity through the entire vessel, are based on the assumption that the flow profile always has a parabolic shape. Such an assumption is not robust, however, particularly in vessels that are not straight or that present irregularities such as atherosclerotic plaques.

In order to overcome this limitation, some complex methods have been applied, based upon magnetic resonance and/or computational fluid dynamics. A direct method, based on high-resolution measurements of the velocity profile along a section of the vessel, might have the potential to overcome these difficulties. The following Section describes the use of a multigate Doppler providing direct measurements of blood velocity profiles in the carotid tree.

*6.2.4.2. Method*

An ULtrasound Advanced Open Platform (sold as ULA-OP), developed and kindly provided by the Department of Electronics and Telecommunications, Florence University, Italy (Tortoli et al. 2009), was used. All electronics necessary to control simultaneously up to 64 elements of a 192-element array probe was integrated in two boards, connected to a PC, as shown in Figure 6.7. The operational interface is shown in Figure 6.8.

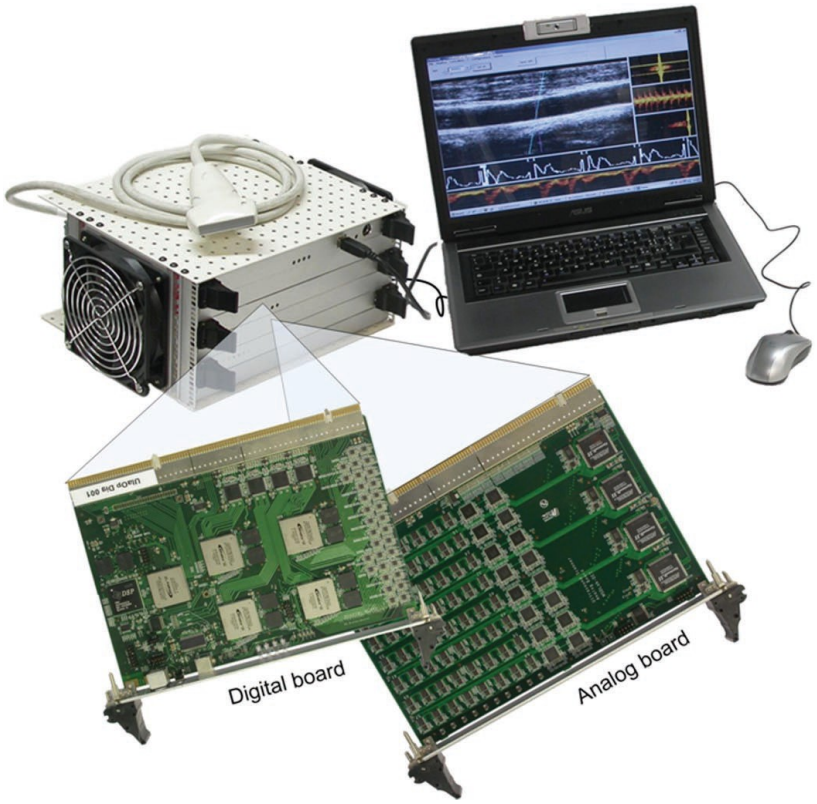



**Figure 6.7: Ultrasound Advanced Open Platform overview (Courtesy of Electronics and Telecommunications Dept., Florence University, Italy).**

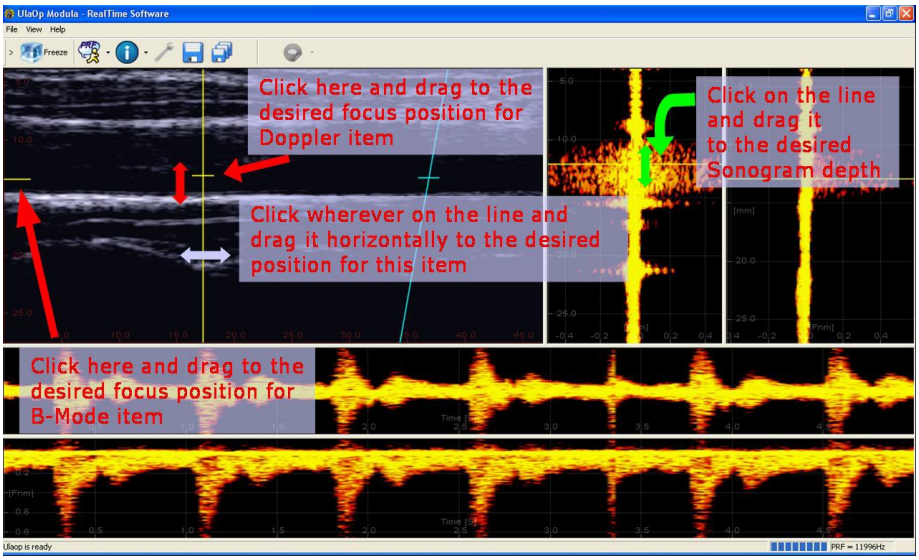



**Figure 6.8: PC-based user interface for Ultrasound Advanced Open Platform instrumentation (Courtesy of Electronics and Telecommunications Dept., Florence University, Italy).**

Sample blood flow velocity profiles along the carotid bifurcation in an healthy male subject are shown in Figure 6.9.

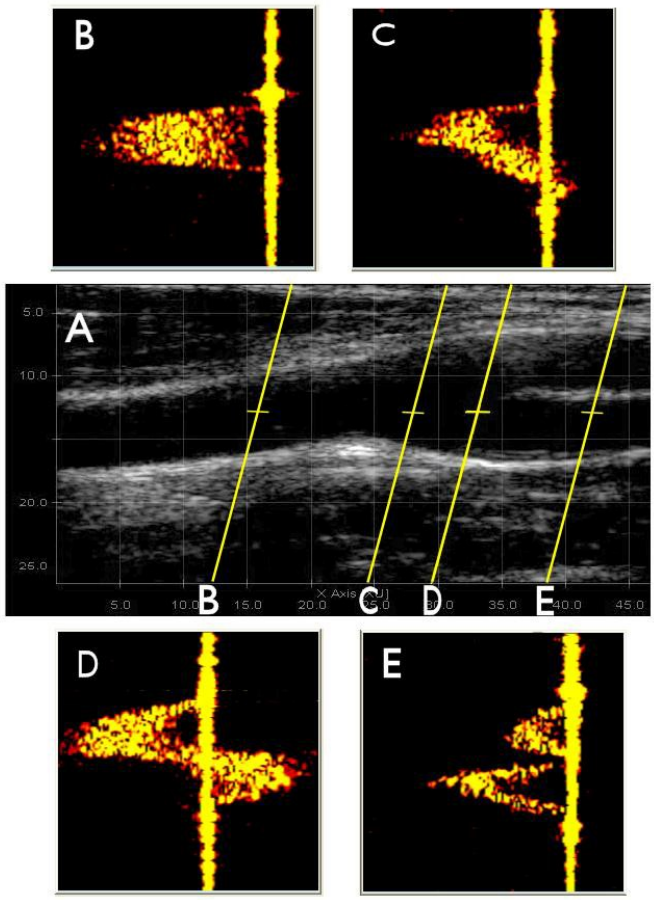



**Figure 6.9: B-MSD Mode flow velocity analysis of a carotid bifurcation. The spectral profiles (panels B,C,D,E) have been frozen during the systolic phase, in correspondence to the M-lines superimposed to the B-mode image (A). Flow is from left to right in panel A. (Courtesy of Electronics and Telecommunications Dept., Florence University, Italy).**

For each depth, the Doppler data collected over time can also be processed through a standard 128-point fast Fourier transform algorithm (Tortoli et al. 2011). This allows the interpolation of spectral profiles as displayed in Figure 6.10, reproducing a matrix of power spectral density points calculated from the echo data backscattered from 128 different sample volumes, according to a multigate spectral Doppler mode.

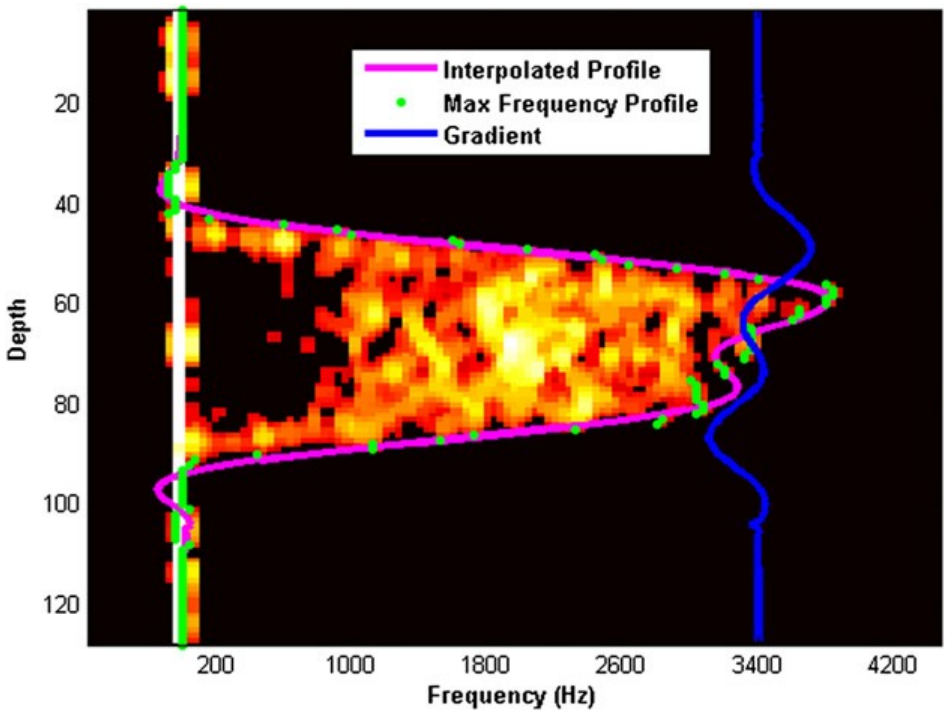



**Figure 6.10: A spectral blood velocity profile (Courtesy of Electronics and Telecommunications Dept., Florence University, Italy).**

The availability of a complete spectrum for each investigated depth allows the corresponding local mean velocity to be extracted from the spectral mean frequency, or the maximum velocity from the spectral peak frequency.

ULA-OP method for measurement of flow velocities has already been validated using a tube phantom and on common carotids of healthy volunteers (Ricci S et al. 2009). In the present setting, these sample carotid tree data were acquired from a middle-aged female patient suffering from type 2 diabetes mellitus and carotid atherosclerosis. Figure 6.11 shows the acquired blood flow profile in the common carotid artery of the patient described above. A non-flow-limiting plaque on the far wall can also be observed from the anterior longitudinal ultrasound image (a linear ultrasound probe was placed along the sagittal plane to the neck at the site of the carotid bifurcation). The shape of blood velocity profiles at this location does not appear to be influenced by this thin plaque, as expected. However, velocity profiles acquired downstream, at the entrance to the carotid bulb, are

distorted. It is clear from Figure 6.12 that there is a plaque encroaching the lumen at this site. The combination of bulb dilation and protruding plaque caused the velocity profile to deviate significantly from the assumed parabolic shape.

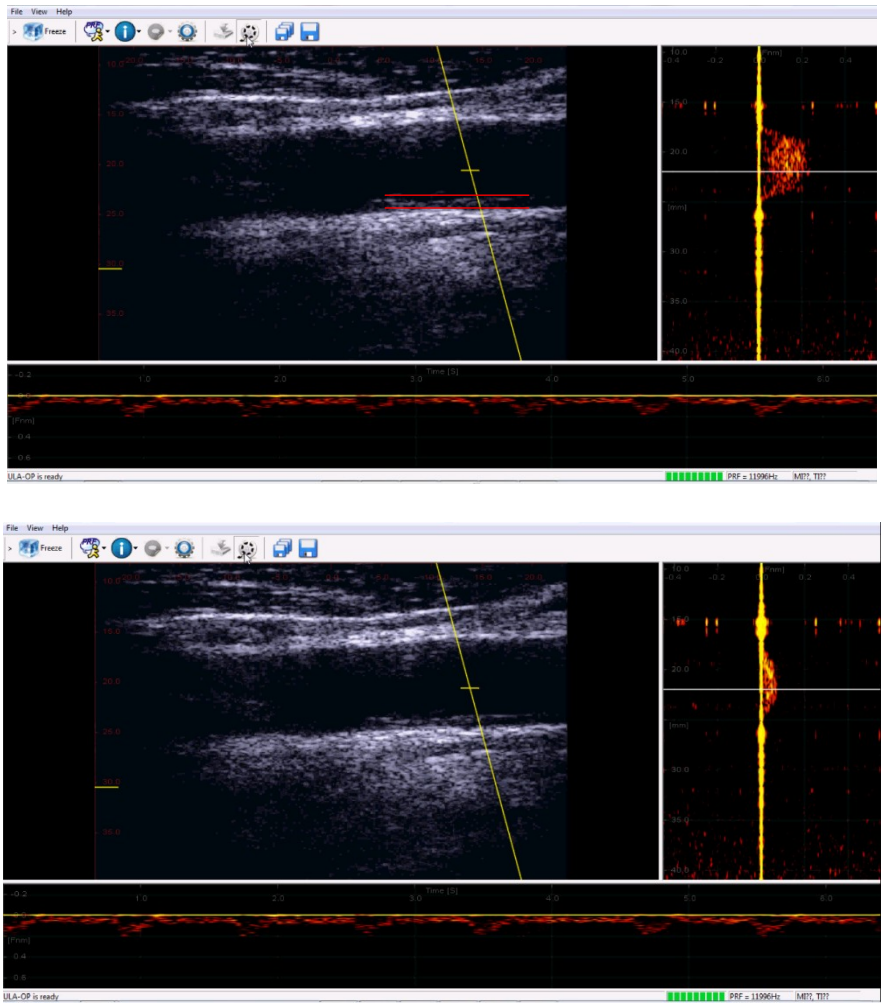


**Figure 6.11: Common carotid blood velocity profiles across a thin plaque (between the two red lines) in systole (upper panel) and late diastole (lower panel). Flow is from right to left in echo images.**

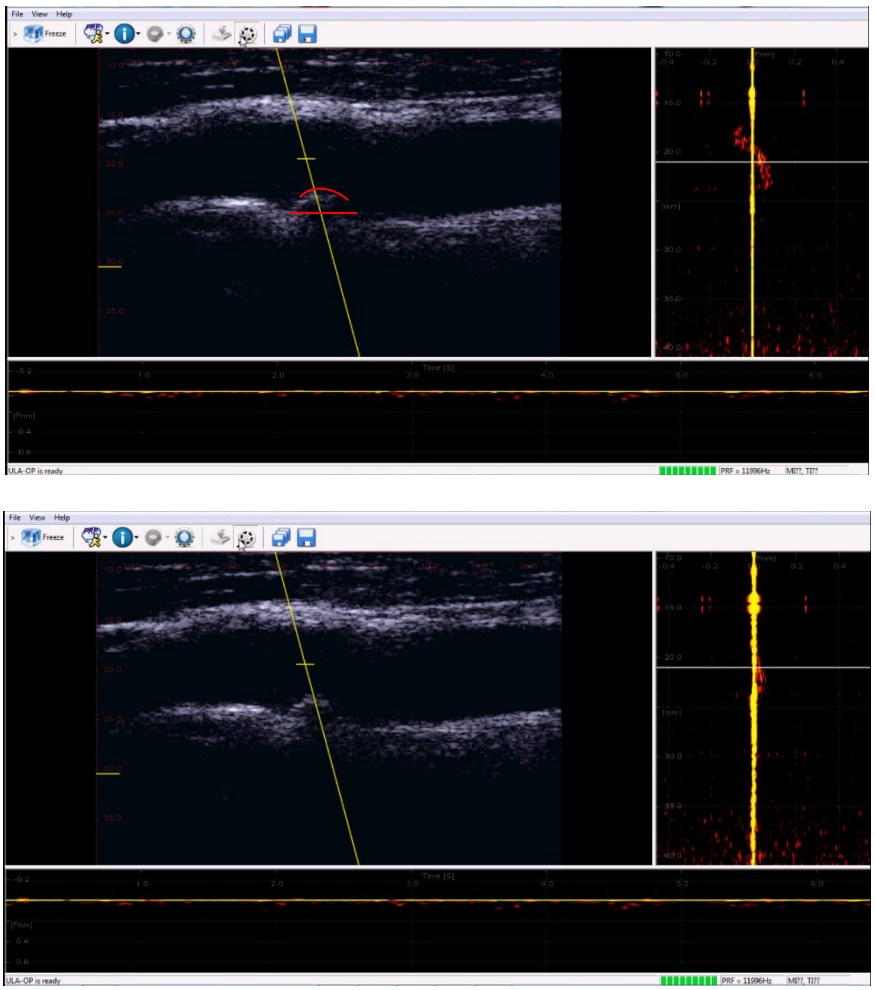


**Figure 6.12: Velocity profiles at the carotid bulb where a plaque encroaching the lumen (between the two red lines) is present. The upper panel corresponds to systole and the lower panel to late diastole. Flow is from right to left in echo images.**

In summary, the potential use of a multigate Doppler system for measurement of blood flow profiles in an atherosclerotic carotid artery has been shown. In order to obtain far wall shear rates in diseased common carotid arteries, measured velocity profiles should be integrated with the corresponding vessel diameter measured along a cardiac cycle. Furthermore, suitable mathematical functions need to be used to fit the measured velocity profiles in order to calculate the velocity gradient at the wall. All these must be validated in vitro or in vivo using magnetic resonance, before being applied to various clinical conditions.

### *6.2.5. Summary*

Although simple, fast and economical, haemodynamic measurements based on the paraboloid flow model in the study of common carotid arteries have several limitations. The key solutions addressed here, via some examples of applications, relate to: taking into account the pseudoplastic behaviour of the blood; mapping the arterial diameter changes during a cardiac cycle, directly verifying the paraboloid blood flow velocity shapes.

In the literature, the use of 1) shear rate dependent blood viscosity, 2) the measurements of central blood pressure, 3) the Quemada model fitted to shear rate-specific measured blood viscosities and implemented in a computational fluid dynamics model, would allow better estimations of carotid haemodynamics. Vice versa, the use of spatial blood flow velocity profiles acquired using multigate Doppler still needs validation beyond healthy common carotids.

# Appendix 1 - Achievement during the project

*Publications – Journal papers submitted from 2010 to 2015 and accepted: 27 English peer reviewed papers, 119 citations, 7 h-index*

*Main articles on haemodynamics:*

**Carallo, C.,** Tripolino, C., De Franceschi, M.S., Irace, C., Xu, XY., Gnasso, A. Carotid endothelial shear stress reduction with aging is associated with plaque development in twelve years. (2016) A**therosclerosis**. 251, p. 63-69.

Tripolino, C., Gnasso, A., **Carallo, C.,** Scavelli, F.B., Irace, C. Difference in carotid artery elasticity in subjects with different brachial artery kinetic of vasodilatation. (2016) **Journal of Human Hypertension**, 30 (8), p. 493-497.

**Carallo, C.,** De Franceschi, M.S., Tripolino, C., Iovane, C., Catalano, S., Giudice, A., Crispino, A., Figliuzzi, M., Irace, C., Fortunato, L., Gnasso, A. Periodontal treatment elevates carotid wall shear stress in the medium term. (2015) **Medicine** (United States), 94 (42), p. e1724.

**Carallo, C.,** Loprete, A., Mazza, G., Bellotti, G., De Siena, M., Serrao, P., Vuoto, E.S., De Franceschi, M.S., Irace, C., Gnasso, A. Biphasic hemodynamic effects of LDL-apheresis in common carotid artery. (2015) **Clinical Hemorheology and Microcirculation**, 60 (3), pp. 297-307.

Tripolino, C., **Carallo, C.,** Irace, C., Scavelli, F., De Franceschi, M.S., Gnasso, A. Plasma viscosity is increased in subjects with elevated ankle brachial index. (2015) **Clinical Hemorheology and Microcirculation**, 60 (3), pp. 291-296.

Tripolino, C., Irace, C., **Carallo, C.,** De Franceschi, M.S., Della Valle, E., Gnasso, A. Blood urea impairs brachial artery flow mediated dilation. (2015) **International Angiology**, 34 (4), pp. 392-397.

Irace, C., Tripolino, C., Scavelli, F., Messiniti, V., Tassone, B., Della Valle, E., **Carallo, C.,** Gnasso, A. Blood viscosity but not shear stress associates with delayed flow-mediated dilation. (2015) **European Journal of Applied Physiology**, 115 (4), pp. 747-753.

De Franceschi, M.S., Palange, A.L., Mancuso, A., Grande, L., Muccari, D., Scavelli, F.B., Irace, C., Gnasso, A., **Carallo, C.** Decreased platelet aggregation by shear stress-stimulated endothelial cells in vitro: Description of a method and first results in diabetes. (2015) **Diabetes and Vascular Disease Research**, 12 (1), pp. 53-61.

Irace, C., **Carallo, C.,** Scavelli, F., De Franceschi, M.S., Esposito, T., Gnasso, A. Blood viscosity in subjects with normoglycemia and prediabetes. (2014) **Diabetes Care**, 37 (2), pp. 488-492.

**Carallo, C.,** Irace, C., Tripolino, C., De Franceschi, M.S., Procopio, A., Crispino, A., Fortunato, L., Gnasso, A. Time course analysis of brachial artery flow mediated dilatation in subjects with gingival inflammation. (2014) **International Angiology**, 33 (6), pp. 565-572.

Tripolino, C., Irace, C., Scavelli, F.B., De Franceschi, M.S., Esposito, T., **Carallo, C.,** Gnasso, A. Triglyceride glucose index and common carotid wall shear stress. (2014) **Journal of Investigative Medicine**, 62 (2), pp. 340-344.

Irace, C., Padilla, J., **Carallo, C.,** Scavelli, F., Gnasso, A. Delayed vasodilation is associated with cardiovascular risk. (2014) **European Journal of Clinical Investigation**, 44 (6), pp. 549-556.

Irace, C., **Carallo, C.,** Scavelli, F., Esposito, T., De Franceschi, M.S., Tripolino, C., Gnasso, A. Influence of blood lipids on plasma and blood viscosity. (2014) **Clinical hemorheology and microcirculation**, 57 (3), pp. 267-274.

**Carallo, C.,** Irace, C., De Franceschi, M.S., Esposito, T., Tripolino, C., Scavelli, F., Merante, V., Gnasso, A. The effect of HDL cholesterol on blood and plasma viscosity in healthy subjects. (2013) **Clinical Hemorheology and Microcirculation**, 55 (2), pp. 223-229.

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Irace, C., De Luca, S., Shehaj, E., **Carallo, C.,** Loprete, A., Scavelli, F., Gnasso, A. Exenatide improves endothelial function assessed by flow mediated dilation technique in subjects with type 2 diabetes: Results from an observational research. (2013) **Diabetes and Vascular Disease Research**, 10 (1), pp. 72-77.

Irace, C., **Carallo, C.,** Loprete, A., Tripolino, C., Scavelli, F., Gnasso, A. Delayed flow-mediated vasodilation and carotid atherosclerosis. (2013) **European Journal of Clinical Investigation**, 43 (1), pp. 49-55.

Irace, C., **Carallo, C.,** De Franceschi, M.S., Scicchitano, F., Milano, M., Tripolino, C., Scavelli, F., Gnasso, A. Human common carotid wall shear stress as a function of age and gender: A 12-year follow-up study. (2012) **Age**, 34 (6), pp. 1553-1562.

**Carallo, C.,** Irace, C., De Franceschi, M.S., Coppoletta, F., Tiriolo, R., Scicchitano, C., Scavelli, F., Gnasso, C. The effect of aging on blood and plasma viscosity. An 11.6 years follow-up study. (2011) **Clinical Hemorheology and Microcirculation**, 47 (1), pp. 67-74.

**Carallo, C.,** Fortunato, L., de Franceschi, M.S., Irace, C., Tripolino, C., Cristofaro, M.G., Giudice, M., Gnasso, A. Periodontal disease and carotid atherosclerosis: Are hemodynamic forces a link? (2010) **Atherosclerosis**, 213 (1), pp. 263-267.

# Appendix 2 - Description of the chosen statistical approaches

**Student t tests** for paired and unpaired data were frequently used in the Thesis to compare continuous variables. In particular, the paired t-test was used to compare the means of two samples where the observations in one sample can be paired with observations in the other sample, in order to compare whether two groups have different average values (for example, the blood viscosity measured in the same group at baseline and follow-up). Moreover, a t-test asks whether a difference between two groups' averages is likely to have occurred because of random chance in sample selection. It is known that a difference is more likely to be meaningful and "real" if it is large, the sample size is large, and responses are consistently close to the average values and not widely spread out (i.e. the standard deviation is low). Indeed, the statistical significance and the effect size are two primary outputs of the t-test. Statistical significance indicates whether the difference between sample averages is likely to represent an actual difference between populations, and the effect size indicates whether that difference is large enough to be practically meaningful.

Another test used here was **Pearson's correlation coefficient**. The Pearson product-moment correlation coefficient (also called PPMC) is a measure of the strength of a linear relationship between two sets of data and is denoted by r. It was measured to attempt to draw a line of best fit through the data of two variables, for example between age and diameter, or between velocity and shear stress. The Pearson coefficient, r, indicates how far away all these data points are to this line of best fit (i.e., how well the data points fit this new model/line of best fit). It can take a range of values from +1 to -1. A value of 0 indicates that there is no association between the two variables. A value greater than 0 indicates a positive association; that is, as the value of one variable increases,

so does the value of the other variable. A value of less than 0 indicates a negative association; that is, as the value of one variable increases, the value of the other variable decreases. Achieving a value of +1 or -1, therefore, means that all the data points are included in the line of best fit – there are no data points that show any variation away from this line. Values for *r* between +1 and -1 (for example, $r = 0.8$ or -0.4) indicate that there is variation around the line of best fit. The closer the value of *r* to 0 the greater the variation around the line of best fit.

In this Thesis, **multiple linear regression** was used as a predictive analysis to explain the relationship between one continuous dependent variable (for example, the percentage change in intima-media thickness/) and two or more independent continuous or categorical variables (traditional cardiovascular risk factors plus the percentage change of haemodynamic forces). Multiple regression analysis was also used to assess whether confounding exists. Since multiple linear regression analysis allows us to estimate the association between a given independent variable and the outcome holding all other variables constant, it provides a way of adjusting for (or accounting for) potentially confounding variables that have been included in the model.

**Multiple logistic regression** was also performed in the Thesis, for example using carotid atherosclerosis development as a binary categorical dependent variable and both the appearance of cardiovascular risk factors and haemodynamic variations during follow-up as independent variables. Like all regression analyses, logistic regression is a predictive analysis. Logistic regression is used to describe data and to explain the relationship between one dependent binary variable and one or more metric (interval or ratio scale) independent variables. Multiple regression can be used for a) controlling for other explanatory variables when assessing relationships between a dependent variable and several independent variables b) predicting outcomes of a dependent variable using a linear combination of explanatory (independent) variables.

Looking at the Section 5.1.3, “Statistical analysis of haemodynamic variations and the development of atherosclerotic plaques”, the model building strategy was structured as follows: in order to correct for the presence of risk factors at baseline, the first block (mode: enter) included baseline values of age, male gender, smoking, obesity, hypertension, diabetes, dyslipidaemia, plus each individual haemodynamic measurement; the second block (mode: stepwise forward) included the development at the follow-up visit of smoking, obesity, hypertension, diabetes, dyslipidaemia, plus the percentage variation of the individual haemodynamic measurements included in the first block. Using the output of this multiple logistic regression, the odds of the development of carotid atherosclerosis could be predicted, and how these predicted odds changed later; the second block variables were added to the model and controlled for the influence of first block variables. The addition of explanatory variables should increase the percentage of correct classification significantly if the model is good. Block 2 shows the results after the addition of the selected explanatory variables. The omnibus Tests of Model Coefficients table gives the result of the Likelihood Ratio test, which indicates whether the inclusion of this block of variables contributes significantly to model fit. A p-value of less than 0.05 for the block means that the block 2 model is a significant improvement on the block 1 model.

In the same Section 5.1.3, the **infraclass correlation coefficient** was calculated in order to exclude a cluster effect derived by the analysis of both carotids together. To measure the bivariate relation of variables representing different measurement classes, one must use an interclass correlation coefficient, and there is only one such coefficient in common use, the Pearson r. When one is interested in the relationship between variables of a common class, however, which means variables that share both their metric and variance, infraclass correlation coefficients (also called ICCs) are alternative statistics for measuring homogeneity, not only for pairs of measurements but for larger sets of measurements as well.

The most fundamental interpretation of infraclass correlation coefficients is that it is a measure of the proportion of a variance (variously defined) that is attributable to objects of measurement, often called targets.

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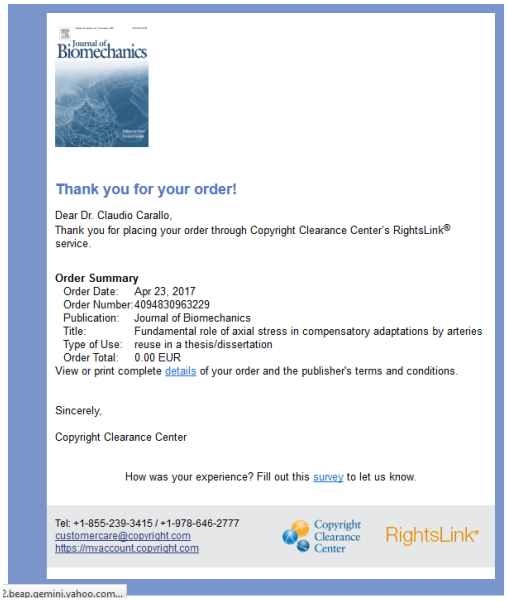

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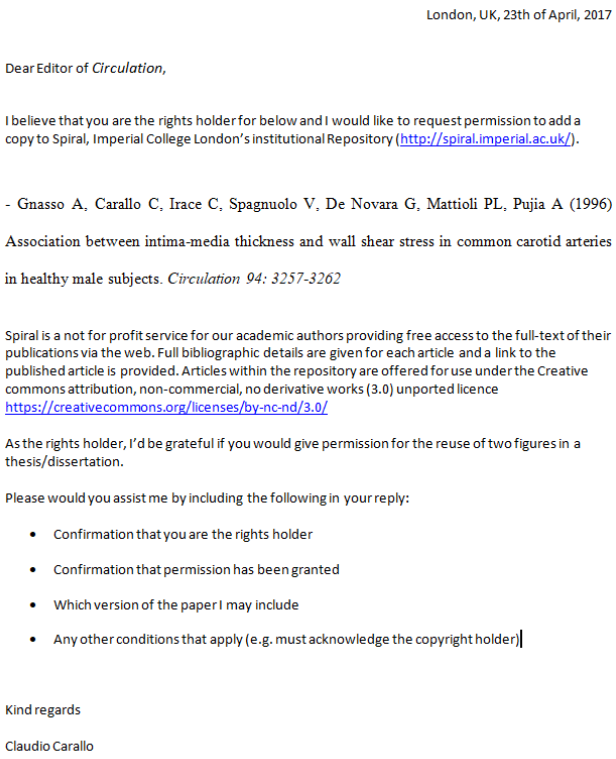

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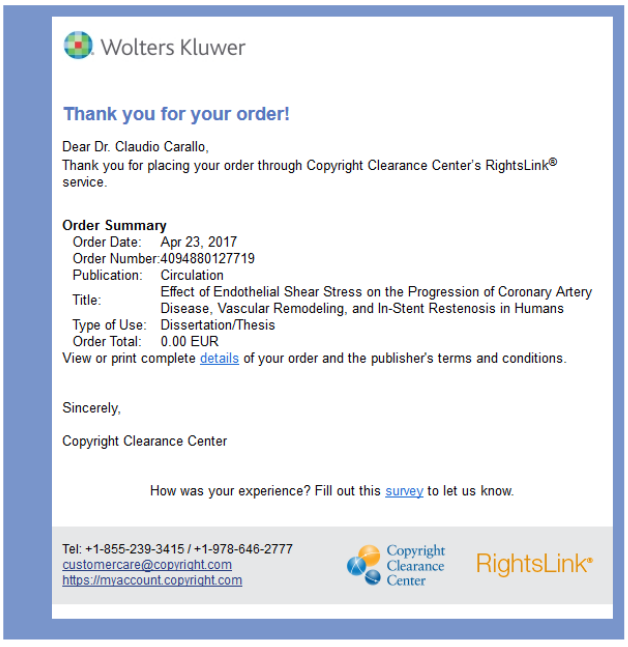

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