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**The vulnerable carotid plaque.  
Identification of endothelial markers predictive of plaque  
weakness, rupture predisposition and neoangiogenesis.**

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**Esame finale anno 2014**

*Ancora una volta, a Debbie, con tanto amore...*

*...e alla mia famiglia, che mi ha sempre sostenuto  
in questo lunghissimo percorso!*

## ABSTRACT

**Background.** Neoangiogenesis is crucial in plaque progression and instability. Previous data from our group demonstrated that intra-plaque neovessels show both a Nestin+/WT+ and a Nestin+/WT1- phenotype, the latter being correlated with complications and plaque instability.

**Aims.** The aims of the present thesis are: (i) to confirm our previous results on Nestin/WT1 phenotype in a larger series of carotid atheromatous plaques, (ii) to evaluate the relationship between the Nestin+/WT1- neoangiogenesis phenotype and plaque morphology, (iii) to evaluate the relationship between the immunohistochemical and histopathological characteristics and the clinical instability of the plaques.

**Materials and Methods.** Seventy-three patients (53 males, 20 females, mean age 71 years) were consecutively enrolled. Symptoms, brain CT scan, 14 histological variables, including intraplaque hemorrhage and diffuse calcifications, were collected. Immunohistochemistry for CD34, Nestin and WT1 was performed. RT-PCR was performed to evaluate Nestin and WT1 mRNA (including 5 healthy arteries as controls).

**Results.** Diffusely calcified plaques (13 out of 73) were found predominantly in females ( $P=0.017$ ), with a significantly lower incidence of symptoms (TIA/stroke) and brain focal lesions ( $P=0.019$  and  $P=0.013$  respectively) than not-calcified plaques, but with the same incidence of intraplaque complications ( $P=0.156$ ). Accordingly, both calcified and not calcified plaques showed similar mean densities of positivity for CD34, Nestin and WT1. The density of Nestin and WT1 correlated with the occurrence of intra-plaque hemorrhage in all cases, while the density of CD34 correlated only in not-calcified plaques.

**Conclusions.** We confirmed that the Nestin+/WT1- phenotype characterizes the neovessels of instable plaques, regardless the real amount of CD34-positive

neoangiogenesis. The calcified plaques show the same incidence of histological complications, albeit they do not influence symptomatology and plaque vulnerability. Female patients show a much higher incidence of not-complicated or calcified plaques, receiving *de facto* a sort of protection compared to male patients.

## OVERVIEW OF THE PHD PROJECT

The principal aims of the current PhD program is the evaluation of the neoangiogenesis in carotid atheromatous plaques, in term both of neovessel density and phenotype, and its correlation with plaque morphology and instability.

We performed a morphological and immunohistochemical study on 73 carotid plaques, in order to evaluate the phenotype of the intra-plaque neovessels, and its relationship with plaque morphology and clinical presentation. In previous studies conducted during the PhD program, we found that specific *vasa vasorum* of normal arteries co-express Nestin and WT1, two markers of progenitor endothelia and vascular proliferative potential. Those results were published in 2012 [Vasuri F, Fittipaldi S, Buzzi M, Degiovanni A, Stella A, D'Errico-Grigioni A, Pasquinelli G. Nestin and WT1 expression in small-sized vasa vasorum from human normal arteries. *Histol Histopathol* 2012;27:1195-1202], and gave us a theoretical basis for the evaluation of Nestin and WT1 in plaque neovessels. During the second year of the PhD program, we evaluated CD34, Nestin and WT1 in 49 specimens, and we found that intra-plaque neovessels express both the Nestin+/WT1+ phenotype and the Nestin+/WT1- phenotype, *i.e.* the WT1/Nestin ratio was inferior than 1. Moreover, the Nestin+/WT1- phenotype correlated with the occurrence of intra-plaque histological complications (and plaque vulnerability) regardless the density of CD34-positive vessels. These results are under revision [Fittipaldi S, Vasuri F, Degiovanni A, Pini R, Mauro R, Faggioli G, D'Errico-Grigioni A, Stella A, Pasquinelli G. Nestin and WT1 expression in atheromatous plaque neovessels: association with vulnerability. *Histol Histopathol*].

The results exposed in the present thesis are the progression of our study, with an enlargement of the carotid specimen series (n=73), and special focus on the different neoangiogenetic profiles in neovessel of plaques with different morphology, and on the correlations with clinical data.

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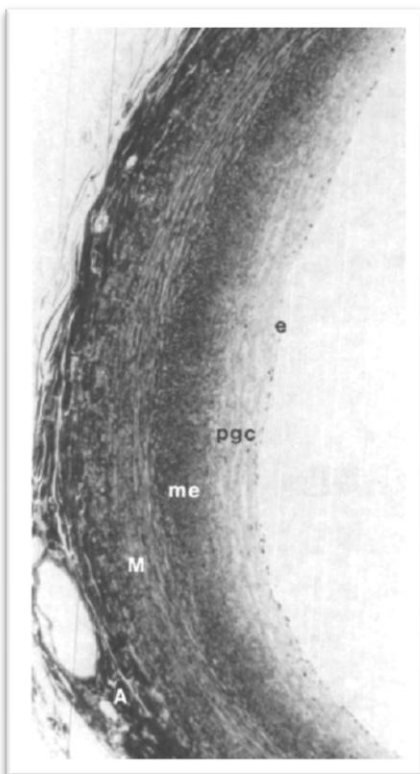
# **1. INTRODUCTION**

## 1.1 FUNCTIONAL ANATOMY OF THE NORMAL ARTERIAL INTIMA

(from Stary 1992)

The intima is defined as the portion of the arterial wall from the endothelium to the media margin (generally the internal elastica lamina, albeit it can be missing in branches and bifurcations). The intima/media ratio can vary from 0.1 to 1 according to the anatomical site. The arterial intima is composed by two layers, whose identification is not always easy at light microscopy (Stary 1992):

- (1) An inner layer called proteoglycan layer, containing finely reticulated non-fibrous connective tissue (proteoglycan) and very few elastic fibers. The proteoglycan layer contains both RER-rich (synthetic) and myofilament-rich (contractile) smooth-muscle cells, and it was firstly described as an alcianophilic inner region of the intima in the nonhuman primates (Wight 1975).
- (2) An outer layer called musculoelastic layer, with abundant contractile smooth-muscle cells and elastic fibers, and the presence of more collagen.



*Figure 1 (Reprinted by Stary et al. 1992). Coronary artery from a 16-month-old boy, showing the different layers composing a healthy vessel wall.*

*e: endothelium; pgc: proteoglycan-rich layer; me: musculoelastic layer; M: media; A: adventitia.*

*1.1.1 Prevalent cell types in the normal intima*

Endothelial cells of normal arteries (both muscular and elastic) are flattened and elongated cells forming a continuous layer. Studies on animal cultured cells demonstrated that the long axis of each endothelial cell is oriented in the direction of the blood flow (Flaherty 1972; Levesque 1985). Polysaccharides, glycosaminoglycans and glycoproteins form the glycocalix that covers the luminal portion of endothelium; some of these molecules are known to bind lectins, lipoprotein lipase, and other enzymes. Moreover, endothelial cells surface shows membrane receptors for LDL (with lipoprotein-lipase activity), insulin, and histamine, among others (Dicorleto 1975; Simionescu 1981; Bar 1982; Heltianu 1982; Wang-Iverson 1982; Jaffe 1987). Endothelial cells carry out several roles, such as the transport of macromolecules (lipoproteins above all) from the blood flow to the intima, the anti-thrombotic homeostasis, the regulation of inflammation and of the response to immunity, as well as the maintenance of the vascular tone and vasoconstriction. Endothelial cytoplasm contain thrombomodulin, whose binding with thrombin inactivates the coagulation factor Va (Esmon 1988). Furthermore, the endothelial-derived prostacyclin PGI<sub>2</sub> inhibits the platelet aggregation and cause vasodilatation (Moncada 1977). Other endothelial-derived factors are likely to promote both vasodilatation and vasoconstriction (Brenner 1989), while the same effects are obtained by soluble mediators with direct action on endothelial receptors, as for serotonin (Vanhoutte 1987), acetylcholine (Furchgott 1980), and endothelin (the latter produced by the endothelium itself) (Yanagisawa 1988).

Two types of smooth muscle cells are usually recognizable in the normal intima of humans and other species:

- (1) The myofilament-rich smooth muscle cells, also called contractile or adult-type (Dilley 1987), are the main type in human intima.
- (2) The rough endoplasmic reticulum (RER)-rich smooth muscle cells, also called synthetic, immature, modulated or ergastoplasm-rich (Dilley 1987), contain few contractile filaments, and they are more frequently visible in proteoglycan-rich intimal layer, since RER-rich smooth muscle cells can synthesize a wide set of matrix molecules (Burke 1979).

Apart from the contractile and synthetic functions, intimal smooth muscle cells are likely to play a role in the removal *via* phagocytosis of LDL and other lipoproteins from the intima, as it is demonstrated by the expression of LDL receptors on smooth muscle cells surface (Stary 1992).

Inactivated macrophages are physiologically present in the human intima, and their number increases from the childhood to adult life. Like in several healthy tissues, macrophages are present in intima as isolated mononuclear cells: they carry out several functions, such as the production of enzymes for matrix remodeling (elastases, collagenases), the secretion of growth factors for smooth muscle, neoangiogenesis, lymphocytes chemotaxis, phagocytosis of lipoproteins, bacteria, necrotic cells and immune complexes (Werb 1975; Nucera 2011).

### ***1.1.2 Extracellular matrix in the normal intima***

The extracellular matrix constitutes up to 60% of the intima, both in healthy condition and during intimal thickenings. Apart from molecules responsible for the cell-cell and cell-matrix adhesion, like fibronectin and laminin, the most represented molecule types in intimal extracellular matrix are proteoglycans, collagen and elastin.

Proteoglycans production by the endothelium cells has been demonstrated on bovine aorta cultured cells, and in particular heparan sulfate proteoglycan and, in a minor amount, dermatan sulfate proteoglycan (Kinsella 1988). In human aortic media, dermatan sulfate proteoglycan resulted to be more associated with collagen fibers, and heparan sulfate proteoglycan with elastic fibers and smooth muscle cells. The different proteoglycans in their different localizations play an important role in maintaining the viscoelastic properties of the vessel wall, and therefore in controlling the homeostasis of vascular wall cells (Wight 1989).

Collagen is involved in the vascular wall integrity by constituting the attachment site of the endothelial cells with the intimal extracellular matrix. The most represented collagen types in the artery wall are type I and type III. In children, the type III collagen (of endothelial derivation) is the prevalent type of collagen present in the subendothelial region, but with aging the synthesis of collagen type I increases, probably due to the concomitant increasing of smooth muscle cells, capable to synthesize both collagen type I and type III *in vitro* (Sage 1981; Sankey 1981).

Elastin is produced by endothelial cells and smooth muscle cells: in the intimal musculoelastic layer elastic fibers are prominent, similar to those present in the media layer. With aging there is a decreasing in elastin content and an increasing in collagen content (Hosoda 1984).

## **1.2 PATHOBIOLOGY OF ATHEROSCLEROSIS**

### ***1.2.1 Atherosclerosis as an inflammatory disease***

(from Ross 1999)

After the birth a progressive increase of the intimal thickness was described in some districts. In studies conducted on rabbits by means of tritiated thymidine, this increase is likely to be due to a mitotic numerical growth of the pre-existing smooth muscle cells (Stary 1974). Anyway it is not still clear whether the migration of smooth muscle cells from the media layer might play a role (Grotendorst 1981).

A part these adaptive changes characterizing some specific human vascular districts, the onset of atherosclerotic lesions is correlated to specific events. According to the historical definition, the first event triggering atherosclerotic lesions is a damage of the endothelial surface (Ross 1973), although more recent theories emphasized an endothelial dysfunction, rather than damage, to play a key role in the lesion initiation. The causes of endothelial dysfunction that were identified and described in the years are multiple, from LDL or homocysteine plasma levels, to diabetes and genetic diseases, from cigarette smoking to viruses such as herpesvirus, Chlamydia, etc. (Ross 1999). According to Ross, after the initial damage, the endothelium acquires pro-coagulant properties (instead of anti-coagulant), and starts the production and secretion of vasoactive factors, cytokines and growth factors. The result is an actual inflammatory response to the initial stimulus, characterized mainly by lymphocytes and monocytes/macrophages (Jonasson 1986; van der Wal 1989), which is soon followed by a migration of the medial smooth muscle cells. With the maintaining of the inflammation, the artery wall thickens and dilates, resulting in a remodeling of the wall, without modifications of the lumen.

Different cycles of inflammatory and muscle cell accumulation, more serum fat entrapment, and the activation of fibrosis by cytokines lead to the formation of a necro-lipidic core and of a fibrous cap, which characterize the so-called advanced lesions. At this point the lesion may protrude in the lumen, leading to stenosis and symptoms.

### *1.2.2 Main risk factors*

- LDL. Circulating LDL represent the major cause of endothelium and arterial wall injury, and they are commonly found in the modified forms: oxidized, proteoglycan-associated or glycated forms, the latter most commonly in diabetes (Morel 1983; Griendling 1997). The phagocytosis by the macrophages lead to LDL oxidation, the formation of lipid peroxides and the accumulation of esters of the cholesterol; in these phase the macrophage becomes a "foam cell". The pro-inflammatory cytokines tumor necrosis factor- $\alpha$  and interleukin-1, among others, have shown a facilitative role in LDL binding to the endothelium surface, increasing the expression of the gene encoding LDL receptors (Stopeck 1993).
- Hypertension. Hypertension is connected to the pathogenesis of atherosclerosis through different mechanisms: the activation of the renin-angiotensin system leads to systemic vasoconstriction, and a proliferative stimulus on muscle cells has been described to occur with time. The binding of angiotensin II to specific receptors on the surface of the smooth muscle cells leads also to the activation of phospholipase C, that causes muscle hypertrophy and hyperoxidation of LDL (Gibbons 1992).
- Homocysteine. Homocysteine is toxic for the endothelium, and according to several studies it shows pro-thrombotic and fibrogenic activities (Harker 1993; Hajjar 1993). When serum homocysteine is elevated in patients with no deficit of homocysteine metabolism, there is an increasing risk of symptomatic



atherosclerosis (Verhoef 1995), while in patients with congenital enzymatic deficits which lead to an impaired homocysteine metabolism, a severe atherosclerosis can develop also in childhood (Nygard 1997).

- C-Reactive Protein (CRP). Cardiovascular diseases and diabetes are inflammatory diseases, and many serum inflammatory markers have been studied in the past years (Devaraj 2009). Among them, CRP is considered the best marker to predict the severity and course of the metabolic syndrome (Ridker 2000). High-sensitivity CRP has been found to interact with a number of pro-inflammatory processes *in vitro*, and it is able to induce the expression of the adhesion molecules VCAM-1 and ICAM-1 in humans (Corrado 2010). C-reactive protein levels rise with increasing levels of visceral adipose tissue, and together with other adipose-derived modulators of inflammation evoke the production of acute phase reactants in the liver, implicated in thrombogenesis (Libby 2010).
- Other risk factors include smoking, insulin resistance/diabetes, lack of physical activity, etc. All these factors result in direct or indirect endothelial dysfunction and inflammation of the vessel wall. The same concept is applicable for Lupus Erythematosus Systemicus and other systemic disease which may cause vasculitis.

### ***1.2.3 Plaque progression and neoangiogenesis***

The vulnerable plaques have been originally described as atheromatous plaques with a large lipid core, a thin fibrous cap, a rich infiltrate of macrophages and scarce smooth muscle cells (Kullo 1998). The erosion and rupture of the fibrous cap is the main events triggering the plaque instability. The degradation of the fibrous cap is mediated by

metalloproteases, such as collagenases, elastases and stromelysin (Galis 1994), mainly produced by macrophages in response to cytokines produced by activated T-cells.

More recently, neovascularization stood out as one of the most important pathological processes involved in the plaque progression (Hermus 2010), since neovessel formation was related to increased plaque vulnerability and to the onset of clinical symptoms (Mofidi 2001; Faggioli 2011).

Mofidi et al. have found a higher volume of intraplaque hemorrhage and a higher incidence of symptoms in those plaques with a higher neovessel density, measured with a "simple" immunohistochemistry for CD34 (Mofidi 2001).

The key mechanism triggering plaque neovascularization is the hypoxia of the inner vascular layers due to the lipid accumulation and the myo-intimal thickening up to 500  $\mu\text{m}$  (Moreno 2006). The chronic hypoxia activates different pathways, such as the Hypoxia Inducible Factor and the Toll-like receptor pathways (Frantz 2005), leading to a neovascularization which is thought to originate from the sprouting of the endothelial cells of the post-capillary venules (Carmeliet 2003). Our recent results indicate that the *vasa vasorum* located in specific adventitial "hot spots" are likely to represent the candidate vessels of origin of the neovascularization during atherosclerosis (Vasuri 2012). Anyway, the adventitial origin of the neovascularized vessels is not the only origin described: for example, a previous work from Kumamoto and colleagues identified the luminal endothelium as the source of the 3.5% of the intra-plaque neovessels (Kumamoto 1995).

If the density of neovessels is likely to be directly related with plaque growth and progression (Moreno 2006), also the morphology of the neovascularized structures is important for the onset of plaque instability: indeed, it was described that plaque neovessels lack extracellular junctions (Sluimer 2009), and that symptomatic plaques

showed larger and more irregular neoangiogenetic structures compared to the neovessels of asymptomatic plaques (McCarthy 1999).

The neoangiogenesis has been involved also in the plaque regression mechanisms, since the neovessels are responsible for the lipid transport outside the plaque core. After lipid removal, the plaque becomes fibrotic (type Vc or type VIII according to AHA, see the next chapter) or calcified (type Vb or type VII), and in these regressed plaques a minor neovessel density was described (Moreno 2006).

### **1.3 PATHOLOGIC ANATOMY OF THE INTIMAL LESIONS: THE AMERICAN HEART ASSOCIATION (AHA) CLASSIFICATION**

The original classification of the American Heart Association (AHA), after a paper by Stary et al. in 1994, sorted the intimal lesions into six bio-pathological entities, which in turn were pooled in two main groups: the so-called precursor lesions (types I, II, III), and the advanced lesions (types IV, V, VI) (Stary 1994; Stary 1995). The progression from one type to another is not demonstrated for every lesion, and studies on animal models have shown that a specific sequence of steps is required to progress from the "simple" lipid intimal accumulation (which characterize the precursor lesions) to the intimal thickening and related disorders, with the consequent deformation of the whole arterial wall (advanced lesions).

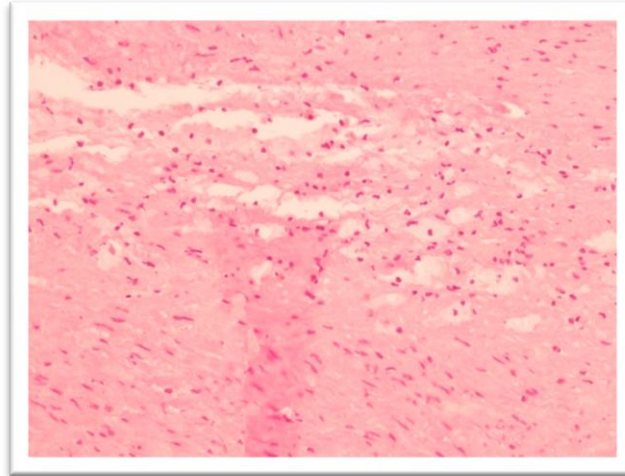
The first step of the whole process is represented by the entrapment of serum lipoproteins in the matrix intima, and their internalization by the macrophages and smooth muscle cells (Schwenke 1989; Steinberg 1990). The following oxidation of the lipoproteins represents a chemotactic stimulus for monocytes (Quinn 1987).

In the study of the atheromatous disease, a particular kind of "gelatinous lesion" has been proposed as precursor lesion: according to some authors, this lesion appears as oval-shaped intimal elevation, pink to pale grey, and soft. At histology, these thickening areas contain gelatinous scarcely-cellulated material, with little or no lipids, and constituted by abundant matrix with little collagen and elastin content (Movat 1959; Haust 1971).

#### ***1.3.1 AHA classification of intimal lesions: initial and intermediate lesions***

- Type I lesions ("initial lesions"). It represents the most frequent intimal lesion in childhood (Stary 1994), and it is constituted by isolated groups of macrophages

with cytoplasmic lipid droplets ("foam cells") (Stary 1989). These findings are observed in the same arterial regions generally prone to the hyperplasia of the eccentric type.



**Figure 2.** *Isolated clusters of intimal macrophagic foam cells.*

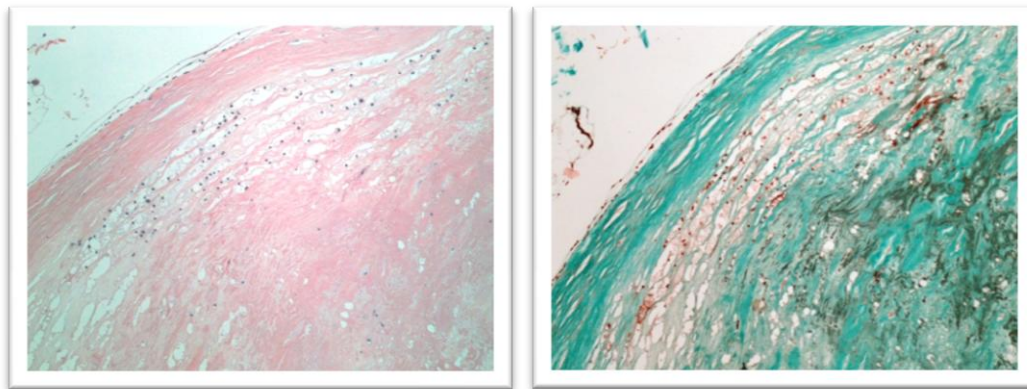
*Haematoxylin-Eosin stain; magnification 20x.*

- Type II lesions (including fatty streaks). In this step, the foam cells are not grouped in isolated clusters, but they form stratified layers in the intima. If the layers are located immediately under the subendothelial surface, the lesion is macroscopically visible as a yellow "fatty streak", but if the foam cell layers are deeper in the intima, the macroscopical appearance will be less defined (Stary 1994). In type II lesions, also intimal smooth muscle cells contain lipid droplets. At any chance, most of the lipids are still contained within cells.

A small subgroup of type II lesions are called type IIa: they raise in specific and predictable arteries with adaptive intimal thickenings, and they are called progression-prone or advanced-prone lesions. However, the majority of type II lesions belong to the type IIb group: they are also called "progression-resistant",

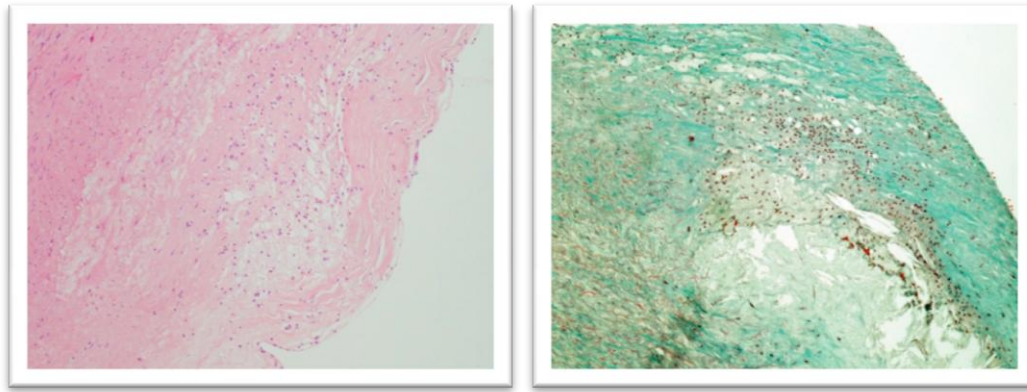
since their progression, when occurring, is slow, and occur only in subjects with very high serum lipoprotein levels (Stary 1994).

On histopathological grounds, type IIa lesions contain more smooth muscle cells than type IIb, together with more extracellular matrix and macrophages, which constitute the adaptive thickening.



**Figure 3.** *Fatty streak. The macrophagic foam cells form stratified intimal layers. Haematoxylin-Eosin (left) and Trichrome stains (right); magnification 10x.*

- Type III lesions ("intermediate lesions" or "preatheroma"). They are placed between type II and type IV lesions, and their morphological hallmark is the deposition of extracellular lipids, which replace the proteoglycan of the intimal matrix, but without a true lipid core. Type III lesions have different lipid content than types I and II: indeed they have more free cholesterol, triglycerids, sphingomyelin and lysolecithin (Katz 1976; Small 1988).



**Figure 4.** A type III lesion, characterized by the presence of extracellular lipids. Haematoxylin-Eosin (left) and Trichrome stains (right); magnification 10x.

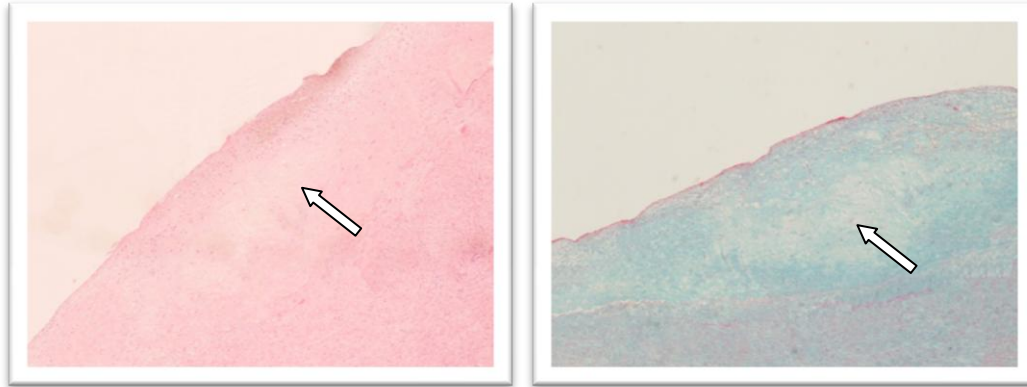
### 1.3.2 AHA classification of intimal lesions: advanced lesions

The so-called advanced lesions are generally associated to a higher incidence of clinical manifestations. In these phases, the accumulation of lipids, matrix, and cellular elements cause a distortion of the arterial wall. The subsequent narrowing of the lumen may reveal itself both angiographically and clinically (Stary 1995).

- Type IV lesions ("atheromas"). The extracellular lipid accumulation occupies a well-defined region of the intima, of variable extension, called *lipid core*. The atheroma does not show complications such as hemorrhages or surface defects, but the presence of the lipid core itself causes a distortion of the arterial walls and a narrowing of the lumen, which might cause stenosis and clinical manifestations: this is why type IV lesions are included among the advanced lesions. In younger subjects, atheromas initially appear in the same anatomical regions of eccentric arterial thickenings, thus they are considered adaptive changes.

Most important (at least for the purposes of the present thesis) is that in the type IV lesions neoangiogenic capillaries appear for the first time, initially located

in the peripheral margin of the lipid core ("shoulder"). A mild macrophagic infiltrate may follow the neovessels.



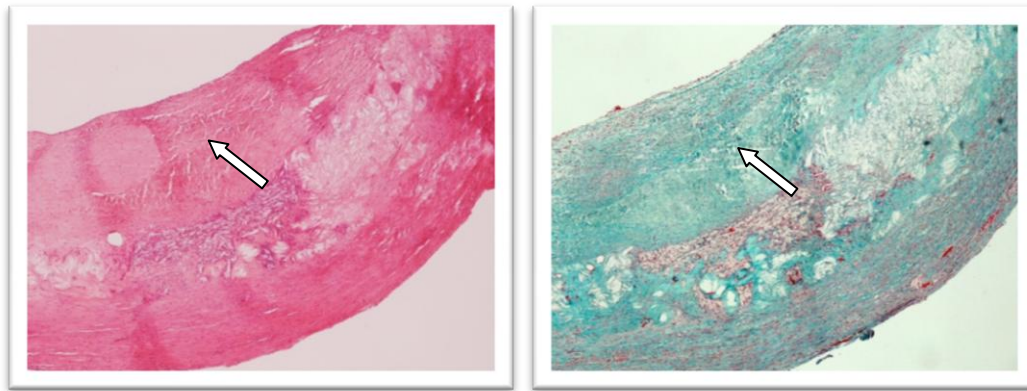
**Figure 5.** A type IV lesion, or atheroma, characterized by a well-visible lipid core (arrow), without a formed fibrous cap yet.

*Haematoxylin-Eosin (left) and Trichrome stains (right); magnification 10x.*

- Type V lesions ("fibroatheromas"). In type V lesions a prominent fibrous cap appears over the lipid core, constituted by newly formed connective tissue with muscle cells rich in rough endoplasmic reticulum. The neovessels are more numerous and diffuse, and they also spread in the fibrous tissue. The type V lesions, which are also classified as type Va in 1995 classification, can be multilayered, due to the overlapping of multiple lipid cores and fibrous caps in time.

Fibroatheromas, as type IV atheromas, can undergo to histological complications, and become type VI lesions, or can regress in two different ways. Lesions showing a massive calcification of the lipid core was defined as type Vb in the 1995 classification, and type VII in the more recent; lesions showing a prominent fibrotic involution of the lipid core was defined as type Vc in the 1995 classification, and type VIII in the more recent.

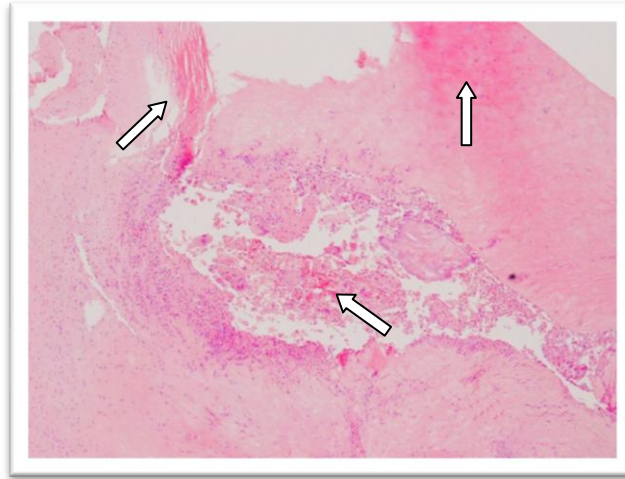




**Figure 6.** *Fibroatheroma (type Va). A thick fibrous cap (arrow) is present above the lipid core. Haematoxylin-Eosin (left) and Trichrome Stains (right); magnification 4x.*

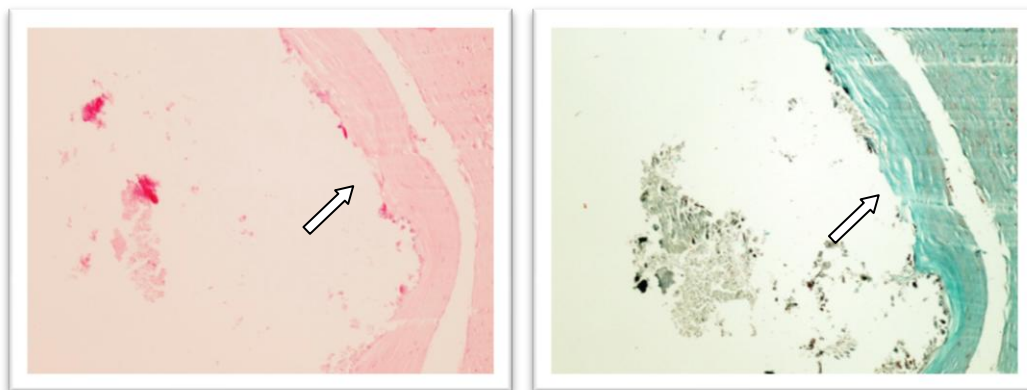
- Type VI lesions ("complicated lesions"). The type VI lesions are defined as atheromatous lesions with features of type IV or V, but with (type VIa) erosion or ulcerations of the endothelial surface (type VIb), hemorrhage/hematoma or (type VIc) luminal thrombosis.

All these complications represent the morphological base of the plaque vulnerability, and will be referred in the present thesis as *histological complications*. Evidences exist of a close relationship between plaque hemorrhage and neoangiogenesis (Mofidi 2001): newly formed vessels show a weak wall, without extracellular junctions (Sluimer 2009), and their growth in the lipid content of the plaque core can easily lead to rupture. Moreover, there is evidence that the neovessels of symptomatic plaques are larger and more irregular compared to the neovessels of asymptomatic plaques (McCarthy 1999).

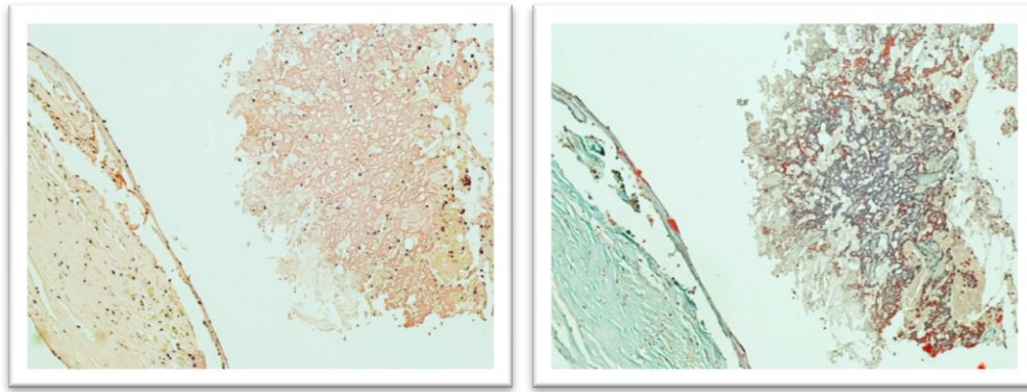


**Figure 7.** An example of type VI plaque, characterized by multiple hemorrhagic foci (arrows).

*Haematoxylin-Eosin stain; magnification 4x.*



**Figure 8.** An example of type VI plaque, characterized by erosion of the endothelial surface (arrows). Haematoxylin-Eosin (left) and Trichrome Stains (right); magnification 4x.



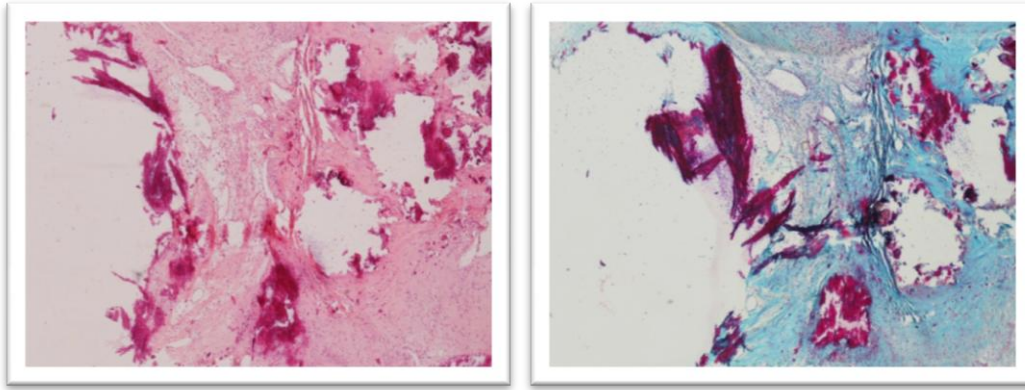
**Figure 9.** An example of type VI plaque, with the presence of an organizing intraluminal thrombus.

*Haematoxylin-Eosin (left) and Trichrome Stains (right); magnification 4x.*

### ***1.3.3 Modifications to the original classification***

In 2000 Sary modified his own previous classification of the atheromatous lesions, in order to better define the progression (or regression) of plaques from one type to another (Sary 2000). According to Sary, type IV and type V lesions can undergo to histological complications, becoming type VI lesions or they can regress into lesions with predominant calcifications or fibrosis; on the other hand, type VI lesions can regress in the same way as well. Complying with these possible different modifications of the plaques, the previous sub-classes Vb and Vc became types VII and VIII respectively.

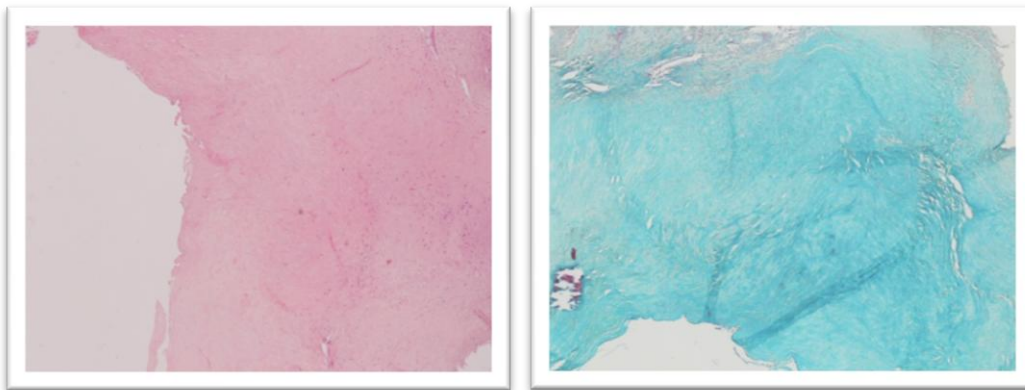
- *Type VII lesions (calcified lesions).* This type of atheromatous lesions replaced the type Vb of the old nomenclature. They are characterized by a core almost fully replaced by a massive deposition of calcification.



**Figure 10.** A heavily calcified uncomplicated plaque, classifiable as type VII (or Vb according to 1995 classification). The calcium deposits completely deform the vessel's architecture.

*Haematoxylin-Eosin (left) and Trichrome Stains (right); magnification 4x.*

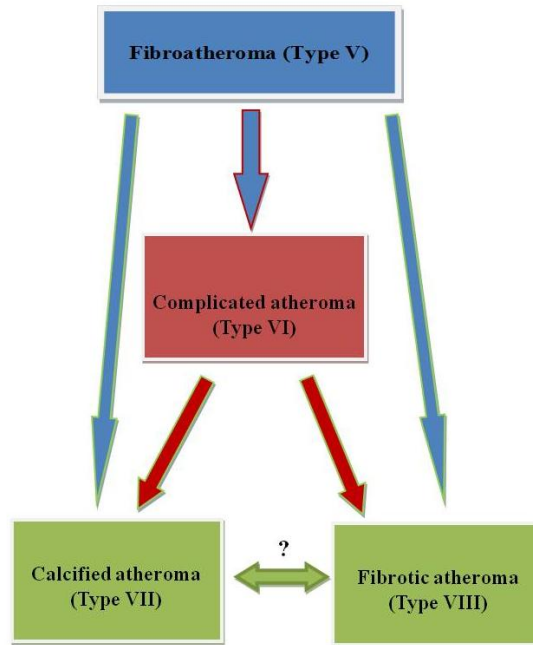
- Type VIII lesions (fibrous lesions). It replaced the type Vc of the old nomenclature. The lipid core is completely replaced by collagen.



**Figure 11.** A type VIII (or Vc according to 1995 classification) plaque. The lipid core is not visible, replaced by collagen.

*Haematoxylin-Eosin (left) and Trichrome Stains (right); magnification 4x.*

The types VII and VIII plaques represent the two major ways of regressive involution to which a atheromatous plaque can undergo. This regression can involve either directly a type V uncomplicated lesion or a type VI lesion, according to the following pathway:



**Figure 12.** Proposed pathway of progression from a plaque type to another.

## **1.4 PREVIOUS RESEARCH OF OUR GROUP**

### ***1.4.1 Nestin and WT1 as markers of active neoangiogenesis***

Nestin is an embryonic intermediate filament protein expressed in neural and mesenchymal stem cells, and it was recently described by Wagner and colleagues to be expressed in a variety of progenitors cells (Wagner 2006). In that paper, the role of Nestin as an angiogenic marker, expressed in newly formed microvessels, was demonstrated in endothelial cells of newly forming blood vessels after myocardial infarction (Wagner 2006), but later it was demonstrated also in both tumoral and not-tumoral neoangiogenesis (Ramasamy 2011). Afterwards numerous Nestin-positive microvessels were described also in tumoral neoangiogenesis, mainly in mouse models (Teranishi 2007; Ramasamy 2011). Moreover, a previous study showed that medial vascular smooth muscle cells in the developing arteries potently express Nestin, but its expression is abolished in adult arteries (Oikawa 2010). It is therefore believed that Nestin expression, representing an early endothelial differentiation or an endothelial progenitor phenotype, could be a reliable marker of “progenitor-committed” endothelial cells, or of a “young” endothelium, during active neoangiogenesis.

The known transcription factors regulating Nestin gene expression are: Pou, Sox, TTF-1 and WT1 (Wagner 2006; Pelizzoli 2008). The Wilms tumor suppressor (WT1) was originally described in the paediatric Wilms’ tumor of the kidney (or nephroblastoma), but its involvement has been demonstrated in a variety of other tumors and tumor cell lines (Hohenstein 2006; Wagner 2008). Some recent studies showed the involvement of WT1 as an activator of the Nestin gene (Hohenstein 2006), and the co-localization of WT1 and Nestin during the different development steps in embryogenesis (Wagner 2006).

***1.4.2 Physiology of vasa vasorum and neoangiogenesis in diseased arteries***

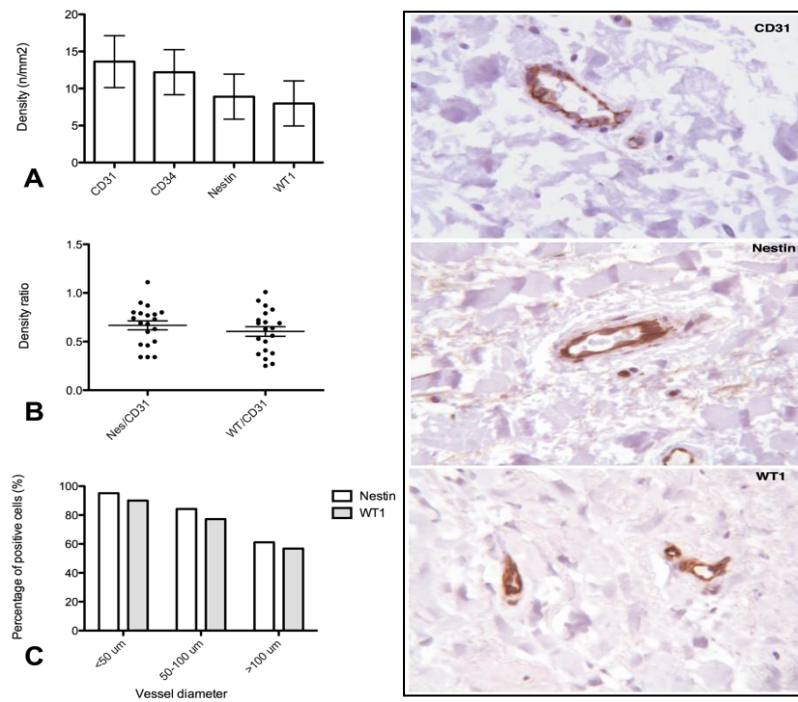
*Vasa vasorum* are small arteries present in the adventitia of the vessels of major calibre. The principal functions of *vasa vasorum* are nutrient and oxygen delivery to the deeper vascular layers and waste removal (Ritman 2007; Mulligan-Kehoe 2010). In the largest vessels the so-called first-order *vasa vasorum* take origin directly from other arteries and run longitudinally along the adventitia (Kwon 1998). The second-order *vasa vasorum* derive from a branching of the first-order *vasa vasorum* and reach the deeper adventitia and media layers. In animal models the application of microscopic computed tomography in healthy vessels demonstrated that *vasa vasorum* form a plexus around the adventitia, with generally a lower number of second-order *vasa vasorum* rather than first-order. This condition changes in pathological conditions, where a dramatic increase in second order *vasa vasorum* was observed (Kwon 1998). In diseased arteries the vessel wall hypoxia unleashes the neoangiogenesis starting from the adventitial *vasa vasorum*. In these pathological conditions *vasa vasorum*, and especially the second-order *vasa vasorum* proliferate quickly, also regulated by local and circulating growth factors such as vascular endothelial growth factor and hypoxia inducible factor, among others (Moreno 2006). This neovasculogenic potential has been largely studied in the last decades, and the existence of endothelial progenitor cells (EPCs) within the layers of the larger vessels has been widely accepted, although their location, phenotype and roles in the vascular damage and angiogenesis are still debated (Zengin 2006; Pasquinelli 2007; Torsney 2011). Most authors agree that a population of progenitor cells is localized in the adventitia, characterized by positivity for different markers of vascular stem potential, such as CD34, c-Kit, CD105, Notch-1, SCA1, and KDR (Alessandri 2001; Hu 2004; Pfister 2005; Zengin 2006; Pasquinelli 2010; Leri 2011).

***1.4.3 Nestin and WT1 expression in healthy vasa vasorum***

In the first study that we published during the PhD program, we performed a histological and immunohistochemical analysis of *vasa vasorum* in 20 not-diseased arteries from 16 healthy multiorgan donors (Vasuri 2012). Our aim was to verify Nestin and WT1 expression in endothelial cells of normal adult *vasa vasorum*, in order to localize and quantify these microvessels that could be responsible for neovascularization in diseased arteries. In these 20 specimens immunohistochemistry for CD34, CD31, Nestin and WT1 was performed manually and the microvessel “densities” of positivity were calculated for each antibody, dividing the vascular adventitia in 1-mm<sup>2</sup> fields with an ocular micrometer in each slide. Double immunofluorescence was used to investigate Nestin and WT1 co-localization in *vasa vasorum*.

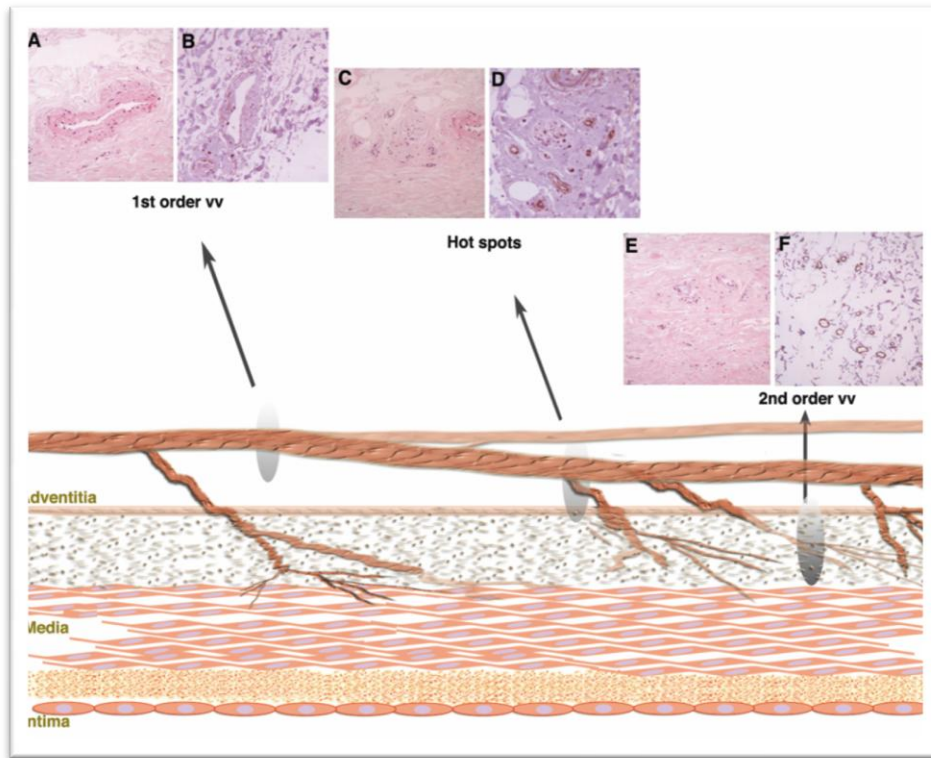
As results, the mean positivity “densities” for CD31, CD34, Nestin and WT1 were 13.63/mm<sup>2</sup>, 12.20/mm<sup>2</sup>, 8.90/mm<sup>2</sup> and 7.98/mm<sup>2</sup> respectively. Mean Nestin/CD31 and WT1/CD31 ratios were 0.69 and 0.63 respectively, which means that 69% and 63% of the CD31-positive neovessels also expressed Nestin and WT1 respectively. As expected, the Nestin:WT1 ratio was close to 1:1, as also confirmed by immunofluorescence, which indentified Nestin and WT1 coexpression in the same endothelial cells.





**Figure 13** (reprinted from Vasuri 2012). On the left, schematic quantitative representation of CD31, CD34, Nestin and WT1 expression in healthy arteries, and their relationships. On the right, the immunohistochemical positivity of CD31, Nestin and WT1 in vasa vasorum.

Finally, *vasa vasorum* <50 μm in diameter showed a higher percentage of Nestin/WT1-positive cells than the larger *vasa vasorum*, especially in “hot spots”, characterized by several small-sized arteriolar *vasa vasorum*, often together with *nerva vasorum*. Our experience indicated that WT1 and Nestin colocalize with a 1:1 ratio in the *vasa vasorum* endothelium from adult healthy arteries.

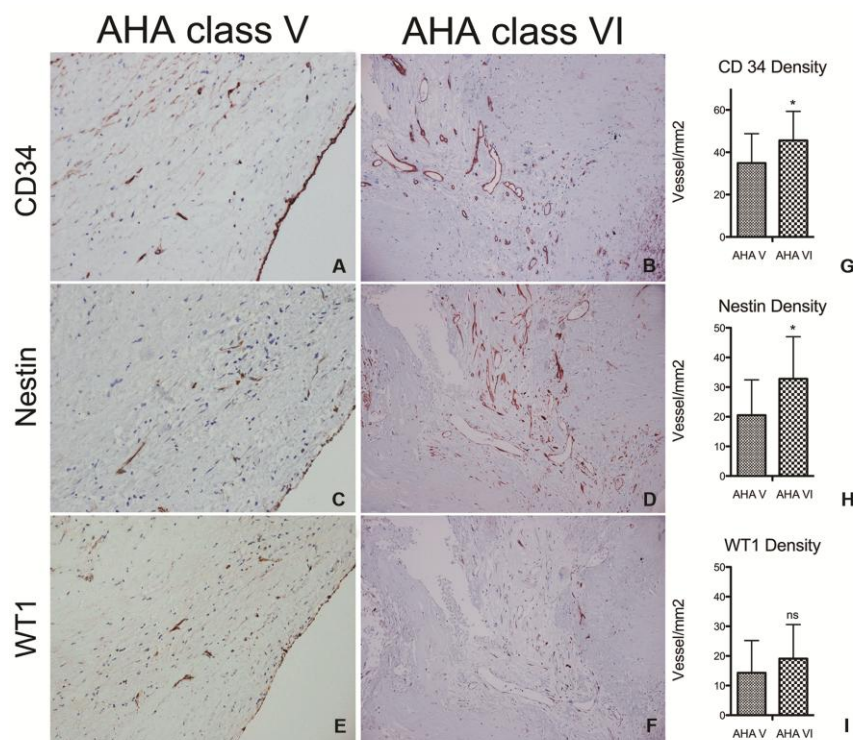


**Figure 14** (reprinted from Vasuri 2012). Schematic picture of vasa vasorum, and their different Nestin/WT1 phenotypes.

#### **1.4.4 Nestin and WT1 in neoangiogenesis of atheromatous plaques**

In the second step of our research, we performed a study on the expression of Nestin and WT1 in the neoangiogenic component of the atheromatous plaques, in order to describe and quantify the protein and mRNA levels of WT1 and Nestin in the plaque neovessels and to investigate the role of neovessel phenotype in the occurrence of the histological complications correlated with plaque instability (Fittipaldi et al., under revision). For these purposes we prospectively evaluated 49 consecutive carotid endarterectomy specimens, collected the main histopathological characteristics (particularly the occurrence of histological complications) and carried out immunohistochemistry for CD34, Nestin and WT1; the density of positivity was evaluated for each marker in "regions of interest". RT-PCR was performed to assess Nestin and WT1 mRNA levels on the first 10 plaques and on 10 control arteries.

As results, the mean immunohistochemical densities of CD34, Nestin, and WT1-positive structures were 41.88/mm<sup>2</sup>, 28.84/mm<sup>2</sup> and 17.68/mm<sup>2</sup> respectively. Among the CD34+ neovessels, 68% and 42% expressed Nestin and WT1 respectively, i.e., nearly 36% of the neovessels showed a Nestin+/WT1- phenotype. Furthermore, complicated plaques (n=30) showed significantly more CD34 and Nestin-positive vessels than uncomplicated plaques (n=13; P=0.045 and P=0.009), while WT1 was less increased (P=0.139). RT-PCR confirmed that WT1 gene expression was 3-fold lower than Nestin gene in plaques (p=0.001). we concluded that plaque neovascularization shows both a Nestin+/WT1- and a Nestin+/WT1+ phenotype. The Nestin+/WT1- neovessels are significantly more represented in plaques with histological complication (hemorrhage above all).



**Figure 15** (reprinted from Fittipaldi, under revision). Different Nestin/WT1 phenotypes in complicated and uncomplicated atherosclerotic plaques.

## **1.5 AIMS OF THE THESIS**

The aims of the present thesis are:

- 1. To confirm our previous results on Nestin/WT1 phenotype in a larger series of carotid atheromatous plaques.**

Histopathological analysis and immunohistochemistry for CD34, Nestin and WT1 will be carried out on a series of 73 consecutive carotid plaques. The plaque morphology and the immunohistochemical densities of positivity will be correlated in order to confirm our previous data on Nestin+/WT1+ and Nestin+/WT1- phenotypes in plaque neoangiogenesis.

- 2. To evaluate the relationship between the Nestin+/WT1- neoangiogenesis phenotype and the main histopathological plaque characteristics (especially complications).**

The Nestin+/WT1+ and Nestin+/WT1- phenotypes will be analyzed according to the separate atheromatous types (V, VI, VII, and VIII). Special attention will be paid to histological complications (hemorrhage, surface defects, thrombosis) that identify type VI plaques, and massive calcifications, that define class VIII (ex class Vb) plaques.

- 3. To evaluate the relationship between the immunohistochemical and histopathological characteristics and the clinical instability of the plaques.**

The clinical data of the 73 patients who underwent endarterectomy will be collected, with focus on symptomatology (stroke and/or transient ischemic attack) and on the presence of brain lesions at computerized tomography. These data will be related to the plaque type and the neoangiogenesis phenotype.

## **2. MATERIALS AND METHODS**

## **2.1 PATIENTS SELECTION AND CLINICAL DATA**

### **2.1.1 Indications for endarterectomy**

According to the European Society for Vascular Surgery (ESVS) and the Society of Vascular Surgeons (SVS) recommendations (Hobson 2008; Liapis 2009), the patients were submitted to carotid endarterectomy (CEA) for either symptomatic carotid plaques  $\geq 50\%$  or asymptomatic carotid stenosis  $\geq 70\%$ . Symptomatic carotid stenosis was defined as the occurrence of ipsilateral cerebral ischemic events (major or minor stroke, transient ischemic attack, or *amaurosis fugax*) in the last 6 months.

### **2.1.2 Clinical data and follow-up**

For each patient the following clinical data were collected:

1. Occurrence of symptoms related to the carotid disease, i.e. transient ischemic attack (TIA) and stroke.
2. Evidence of brain focal lesions by means of CT.
3. Association with chronic ischemic cardiopathy.
4. Association with chronic obstructive broncopneumonia.
5. Association with chronic renal failure.
6. Concomitant therapy with acetylsalicylic acid (asa), oral anticoagulant, or statins.
7. Percentage of carotid stenosis, and percentage of the eventual contralateral stenosis, if present.

As follow-up, TIA, stroke, myocardial infarction, hematoma or death related to these causes were evaluated as patients poor outcome after endarterectomy.

## **2.2 HISTOPATHOLOGICAL ANALYSIS**

Endarterectomy specimens were sent to our Pathology Unit in formalin (Formaldehyde 4%), from gross examination, which normally includes the description and the measurement of the specimen (carotid segment or carotid bifurcation, including the common, the inner and the outer branches), the description of all plaques and visible intimal thickenings, the eventual evidence of macroscopic erosions and thrombosis. Specimens were therefore cut in cross sections, and included *in toto* in one or two specimen blocks.



**Figure 16.** *An example of endarterectomy specimen. Note the deformation of the vessel wall architecture, and the endothelial irregularities.*

Blocks were routinely processed and paraffin embedded. From these formalin-fixed paraffin-embedded (FFPE) samples, 2  $\mu\text{m}$ -thick slices were cut for routine histopathological diagnosis with Hematoxylin-Eosin stain. The most representative sample was also stained with Trichrome stain, which briefly evidences the elastic fibers in red (fuchsin) and the collagen in green (Light Green).

The histopathological routine analysis and the collection of the histopathological variables were performed by means of an Olympus<sup>®</sup> BX50 microscope. For given

variable we used an Olympus® ocular micrometer: one length unit of the micrometer is equal to 5 µm at 20x magnification, which means that an area of 100 x 100 units is equal to 0.25 mm<sup>2</sup>, and four are equal to 1-mm<sup>2</sup>.

For each case, 14 histopathological features were collected as single variables:

1. Maximum size of the fibrous cap, evaluated by means of the ocular micrometer.
2. Minimum size of the fibrous cap, evaluated by means of the ocular micrometer.
3. Extension of the lipid core, from one fourth to four fourth of the vessel section.
4. Extension of calcifications, from one fourth to four fourth, then simplified in low-grade calcification (if absent or 1-2/4) and high-grade calcification (if 3-4/4)
5. Amount and localization of the inflammatory infiltrate. The intraplaque flogosis was semi-quantified from 0 (absent) and 1+ (mild) to 2+ (moderate) and 3+ (severe). Flogosis localization was assessed as well, whether prevalently in the shoulder, in the fibrous cap, or diffuse throughout the section.
6. Amount and localization of the neoangiogenesis. As for the flogosis, the intraplaque neoangiogenesis was semi-quantified in 0 (absent), 1+ (mild), 2+ (moderate) and 3+ (severe), with the same localizations as above.
7. Minimum diameter of the plaque neovessels, evaluated by means of the ocular micrometer.
8. Maximum diameter of the plaque neovessel, evaluated by means of the ocular micrometer.
9. Evidence of "large and winding" neovessels, by Haematoxilin-Eosin or with CD34.
10. Evidence of "dot-like" structures, exclusively by means of CD34.



11. Evidence of histopathological complications, which put the plaque into the AHA type VI. The complications were therefore sorted into three main groups, as follows:

12. Hemorrhage, with or without fissurations.

13. Thrombosis.

14. Endothelial erosion or ulceration.

## 2.3 IMMUNOHISTOCHEMISTRY

The monoclonal antibodies used in this study are listed in Table 1.

Antibody	Clone	Manufacturer
Nestin	<i>10C2 (mouse IgG)</i>	Millipore
WT1	<i>6F-M2 (mouse IgG)</i>	Roche Ventana
CD34	<i>QBEnd/10 (mouse IgG)</i>	Roche Ventana

**Table 1.** Technical characteristics of the antibodies used for immunohistochemistry.

### 2.3.1 Manual procedure

Immunohistochemistry for Nestin was performed manually; the first step consisted in the dewaxing of the histological slides by means of three baths in BioClear (Bio-Optica, Milan), 10 min each, followed by alcohol in decreasing dilution (100°, 96°, 70°) and rehydration with distilled water for 5 min. A citrate buffer chelating the bivalent ions, pH 6.0, was applied as antigen retrieval: slides are put in citrate buffer for four cycles 5 min each, in a 750 W microwave. Endogenous peroxidases were blocked in a solution of 3% H<sub>2</sub>O<sub>2</sub> in methanol for 10 min. After the antigen retrieval and the peroxidases inhibition, slides were equilibrated in a TBS buffer (Tris Buffered Salin pH 7.6) 1x for 10 min before the incubation with the primary antibody.

Slides were incubated with the anti-Nestin primary antibody in a wet chamber at room temperature for 1 h, and therefore washed three times or 5 min each in TBS buffer. Finally, histological sections were incubated with the NovoLink Polymer Detection System (Leica Biosystems, Newcastle Ltd Balliol Business Park, West Benton Lane, Newcastle Upon Tyne NE12 8EW, United Kingdom) as follows: (1) a first incubation lasting 30 min in the Novocastra Post Primary Block reagent, used in order to imply the

penetration of the second polymeric reagent; (2) a second incubation (spaced out by a 5-min bath in TBS), lasting 30 min, in NovoLink Polymer, which links mouse and rabbit immunoglobulins.

Afterwards, sections were incubated in DAB chromogen 3'-3' diaminobenzidine) for 10 min. The staining reaction was blocked by means of distilled water for 5 min, and therefore the slides were counterstained with Heamatoxylin for 2 min, dehydrated with alcohol in increasing dilution (70°, 96°, 100°) and BioCLear, and then mounted with Eukitt (O.Kindler, Freiburg, Germany).

### ***2.3.2 Automatic procedure***

Immunohistochemistry for CD34 e WT1 was performed automatically, by means of Benchmark XT (Ventana Medical System), using the XT ultraView DAB v3 program. After the automatic dewaxing, the protocol included a pretreatment with the Cell Condition 1 Ventana (CC1), an EDTA buffer, pH 8.0, for 36 min at 95°C. Afterwards the slides were treated with an inhibitor at 37°C to avoid aspecific signals, and then washed. The primary antibodies were incubated at 37°C for 28 min (WT1) or 16 min (CD34). After the bath, the polymer and the chromogen (UV HRP UNIV MULT e UV DAB, Ventana Medical Systems), needed for the amplification of the antigen-antibody reaction, were added on the slides, followed by further reagents and baths in order to block the development reaction (UV DAB H<sub>2</sub>O<sub>2</sub>, UV COPPER). The last automatic step consists in the counterstaining with Haematoxilin II (Roche-Ventana Medical Systems); afterwards slides are dehydrated and mounted.

### ***2.3.3 Evaluation of the "density" of positivity with Immunohistochemistry***

The microvessel “density” per section was obtained by dividing the sum of all CD34-positive vascular structures observed by the number of counted fields in the section of

the total areas analyzed in all fields. Together with the microvessel density, we identified specific Regions of Interest (ROI), characterized by a major density of CD34-positive structures, and we counted the number of vascular structures expressing Nestin and WT1 in each ROI. Briefly, at 20x magnification we divided each ROI in 1-mm<sup>2</sup> fields, using a Olympus<sup>®</sup> ocular micrometer (1 length unit = 5 μm, which means that an area of 100 x 100 units is equal to 0.25 mm<sup>2</sup>).

With these data, we could therefore evaluate the following variables:

- The number of fields, positive structures and density of positivity for CD34, Nestin, WT1.
- The ratio between the densities of positivity of Nestin/CD34: this value represents how many CD34-positive neovessels concomitantly express Nestin.
- The ratio between the densities of positivity of WT1/CD34: this value represents how many CD34-positive neovessels concomitantly express WT1.
- The ratio between the densities of positivity of WT1/Nestin: this value represents how many Nestin -positive neovessels concomitantly express WT1.

Moreover, as for the previous analysis on diseased carotid arteries, the presence of "dot-like" structures and "large winding" neovessels was assessed. The former represent small neovessels with no visible lumen, generally packed in groups; the latter are neovessel with winding and irregular lumen, of large diameter, resembling the immature neovessel of malignant tumors (Sluimer 2009). The positivity for the three antibodies by the "dot-like" and "large and winding" structures was recorded as well.

## **2.4 RT-PCR**

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) for the products of the Nestin and WT1 genes was carried out on 5 type V and on 5 type VI plaques, in order to evaluate the different expression in complicated and uncomplicated carotid plaques.

A pool composed by 5 healthy arteries was used as control. Healthy arteries were kindly provided by the Cardiovascular Tissue Bank of S.Orsola-Malpighi Hospital of Bologna.

### ***2.4.1 Trizol RNA extraction***

Tissues were homogenized with an Ultraturax and incubated with 800  $\mu$ l of Trizol reagent (TRIreagent, Ambion Cat #AM9738) for 5 min at RT. RNA extraction with Trizol was performed following manufacturer instructions. Mainly chloroform was added to the sample (0.2 ml: 1 ml trizol). The sample are shaken vigorously for 15 seconds and incubated for 2 min at room temperature, left for 2 min in ice, centrifuged at 12000g, 15 min at 4°C. The RNA phase was gently collected in a new Eppendorf and 500  $\mu$ l of isopropyl alcohol was added with 5  $\mu$ l of glycogen (2 $\mu$ g/ $\mu$ l). To allow RNA precipitation, samples were incubated at room temperature for 10 min and centrifuged at 12000g for 10 min, 4°C. Supernatant was discarded and RNA pellet was washed with 75% ice ethanol, then centrifuged at 7500g for 5 min and air dried for 20 min. Finally RNA was eluted in 15  $\mu$ l RNase free-water, heated at 65°C for 5 min in order to denature RNA and to inactivate RNases. RNA quality and concentration were measured by using an ND-1000 spectrophotometer (NanoDrop, Fisher Thermo, Wilmington, DE, USA). To compare efficiency of RNA extraction from FFPE we used as a positive control RNA extracted from fresh tissue.

**2.4.2 RT-PCR analysis**

Reverse transcription assay was performed using 2.0 µg of starting total RNA quantity per 25 µl of mix, following the manufacturer's protocol (High capacity cDNA Archive kit, Applied Biosystem). The cDNA was stored at -20°C until RT-PCR was performed. RT-PCR was carried out following MasterMix TaqMan<sup>®</sup> Protocol (TaqMan Univ PCR MasterMix, Applied Biosystems). Four µl of neat cDNA was amplified using specific probes for WT1 (NC\_000011.9), Nestin (NC\_000001.10) and GUSB (NM\_000181.3) in the RT-PCR mix (TaqMan<sup>®</sup> Gene Expression Assay, Applied Biosystems, respective ID assay: Hs01103751\_m1, Hs04187831\_g1, Hs00939627\_m1). Reactions were run on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Cycling conditions were as follows: 10 min at 95°C, 50 cycles at 95°C for 15 s and 60°C for 60 sec. Each assay was carried out in triplicate and the transcription level was normalized using βActin as a reference gene.

## **2.5 STATISTICAL ANALYSIS**

All statistical analyses in the present study were carried out with SPSS<sup>®</sup> software for Windows, version 20.

All continuous variables are expressed as means, standard deviations and ranges; all categorical variables (both nominal and ordinal) are expressed as number of cases and percentages.

According to the kind of independent and dependent variable, the following statistical tests were applied:

- The Spearman's test was used to compare continuous variables. It was also applied in all cases to evaluate the Spearman coefficient.
- The chi-square test was used to compare categorical variables.
- The Mann-Whitney test was used to compare a categorical dichotomous independent variable (e.g. type V versus type VI plaques, or calcified *versus* not-calcified plaques) with continuous dependent variables.
- The Kruskal Wallis test was used to compare a categorical ordinal independent variable (e.g. the four different kinds of plaques) with continuous dependent variables.

As for RT-PCR analysis, a threshold at 0.2 was selected in order to be in the exponential phase. The expression values for atheromatous carotid plaques are presented as fold expression in relation to healthy arteries; the actual values were calculated using the  $2^{-\Delta\Delta CT}$  equation (Yuan et al. 2006), where  $\Delta\Delta CT = [CT \text{ Target} - CT \beta\text{Actin}] (\text{atheromatous sample}) - [CT \text{ Target} - CT \beta\text{Actin}] (\text{healthy sample})$ . Due to the technical difficulty to detect WT1 gene expression, Human immortalised myelogenous leukemia line cell line (K562) were used as a positive control (Yuan et al. 2006).

## **3. RESULTS**



### 3.1 PATIENTS AND CLINICAL DATA

Seventy-three patients were finally enrolled, 53 (72.6%) males and 20 (27.4%) females, mean age at the time of surgery  $70.8 \pm 8.7$  years (range 42-86).

	<b>Number of patients</b>	<b>Percentage</b>
Stroke	11	15.1%
TIA	18	24.6%
TC positivity	25	34.2%
Hypertension	65	89.0%
Dyslipidemia	51	69.9%
Diabetes	20	27.4%
Smoke	6	8.5%
Chronic Ischemic Cardiopathy	25	34.2%
BPCO	4	5.5%
Chronic Renal Failure	4	5.5%
Aspirin use	64	87.7%
Statins use	42	57.5%

**Table 2.** Summary of the main clinical characteristics of the 73 patients.

Of note, 11 (15.1%) patients had a stroke as clinical presentation, and 18 (24.6%) a transient ischemic attack. So a total of 29 (39.7%) plaques were symptomatic.

At follow-up, 2 patients had a TIA, 2 had a stroke, and 3 more patients had a myocardial infarction. Two patients died at follow-up, one with stroke and one in absence of post-surgical acute comorbidities. Due to the low number of events at follow-up, no survival studies will be performed further.

### 3.2 HISTOPATHOLOGICAL ANALYSIS

Here are listed the rough results about the morphological variables that were analyzed separately in the 73 plaques:

1. Maximum cap size:  $1132.8 \pm 485.6 \mu\text{m}$  (range 120-2500)
  
2. Minimum cap size:  $284.3 \pm 199.7 \mu\text{m}$  (range 40-1125)
  
3. Extension of the lipid core: not evident in 8 (11.0%) cases
  - 1/4 in 16 (21.9%)
  - 2/4 in 18 (24.7%)
  - 3/4 in 23 (31.4%)
  - 4/4 in 8 (11.0%)
  
4. Calcifications: absent in 11 (15.1%) cases
  - 1/4 in 21 (28.8%)
  - 2/4 in 20 (27.4%)
  - 3/4 in 16 (21.9%)
  - 4/4 in 5 (6.8%)

According to this semi-quantitative assessment of the calcification extent (expressed as fourths of the lumen occupied by calcification), the plaques were therefore subdivided in two major groups: low-grade calcification (which included the plaques with up to 2/4 of extension) and high-grade calcification (including plaques with calcifications in 3/4 and 4/4 of the wall circumference). In our series, 52 (71.2%) plaques showed low-grade calcifications, and 21 (28.8%) high-grade calcifications.

It should be remembered that the high-grade calcification does not imply a placement in the AHA type VII, since calcified plaques with histological complications were considered as type VI.

- 5. Inflammation:
  - absent in 4 (5.5%) cases
  - mild in 10 (13.7%)
  - moderate in 20 (27.4%)
  - severe in 39 (53.4%)

As expected, the inflammatory infiltrate was mainly composed by monocytes, macrophages (as "foam cells" in proximity if the lipid core) and lymphocytes; the presence of granulocytes was rarely observed. The flogosis was localized in the plaque shoulder in 14 (19.2%) cases, within the cap in 19 (26.0%), intra-plaque in 11 (15.1%), and in was diffuse in all artery wall in 25 (34.2%). In 4 cases, 5.5%, flogosis localization was not evaluable.

- 6. Neoangiogenesis (semi-quantitative assessment):
  - absent in 2 (2.7%) cases
  - mild in 19 (26.0%)
  - moderate in 21 (28.8%)
  - severe in 31 (42.5%)

Neoangiogenesis was localized in the plaque shoulder in 19 (26.0%) cases, within the cap in 17 (23.3%), intra-plaque in 13 (17.8%), and in was diffuse in 21 (28.8%). One (1.4%) case was not evaluable.

- 7. Minimum diameter of the plaque neovessels:  $15.3 \pm 24.0 \mu\text{m}$  (range 1-125)

8. Maximum diameter of the plaque neovessel:  $224.1 \pm 210.4 \mu\text{m}$  (range 50-1500)
9. Evidence of large and winding neovessels: this kind of neovessel, very resembling the neovessel of tumoral neoangiogenesis, was recorded in 32 (43.8%) of cases.
10. Evidence of "dot-like" structures: they were seen in 31 (42.5%) plaques.
11. Histopathological complications: the presence of at least one histopathological complication, which define the type VI plaque of the AHA classification, was recorded in 52 (71.2%) plaques. In particular, plaque hemorrhage/fissuration was present in 41 (56.1%), endothelial erosion or ulceration in 22 (30.1%), and thrombosis in 4 (5.5%) cases.

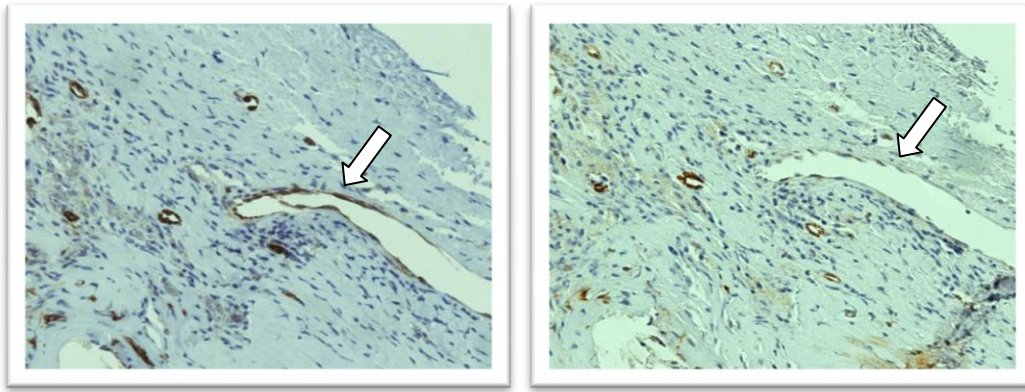
### 3.3 IMMUNOHISTOCHEMISTRY

IHC for the three antibodies was evaluated in 71 cases, i.e. excluding the two cases with no visible neoangiogenesis at CD34. We initially evaluated the intraplaque neoangiogenesis by CD34, and we assessed specific areas named region of interest (ROI). ROI were defined as areas with CD34-positive neoangiogenesis, and the microvessel counting was performed for each slide in ROI, by means of an ocular micrometer. The Nestin- and WT1-positive vessels were counted in the same ROI where CD34 was evaluated.

The microvessel “density” of each antibody was obtained by dividing the sum of all positive structures observed in ROI by the number of the counted fields expressed in  $\text{mm}^2$  (number of positive vessels/ $\text{mm}^2$ ).

	Min	Max	Mean	St. Dev.
CD34 evaluated fields	1	33	12.4	7.3
CD34+ structures	11	391	130.7	91.4
CD34 density	3.5	22.1	10.1	3.9
Nestin evaluated fields	1	34	10.5	6.3
Nestin+ structures	7	296	73.0	59.4
Nestin density	1.4	18.5	6.8	3.7
WT1 evaluated fields	1	37	11.3	6.8
WT1+ structures	4	300	54.7	54.8
WT1 density	0.8	18.8	4.5	3.0
Nestin/CD34 ratio	0.2	1.5	0.7	0.2
WT1/CD34 ratio	0.1	1.0	0.4	0.2
WT1/Nestin ratio	0.2	1.8	0.7	0.3

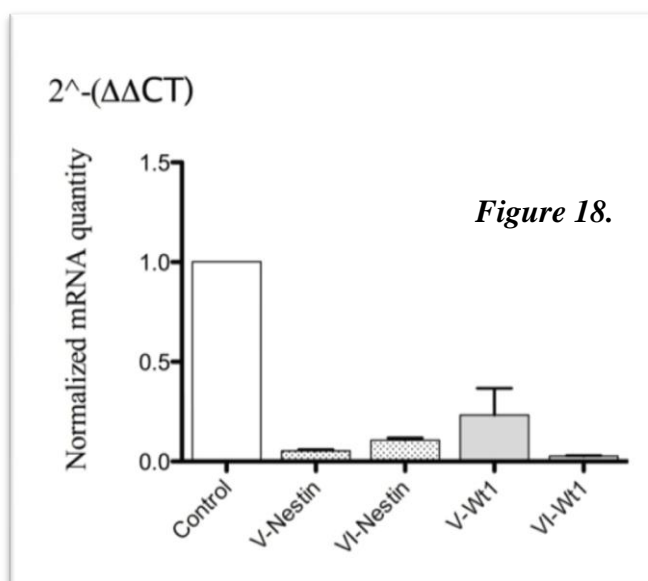
*Table 3. IHC results for each separate variable.*



**Figure 17.** Immunohistochemistry for Nestin (on the left) and WT1 (on the right). Not all Nestin+ vascular structures also expressed WT1 (arrow).

### 3.4 RT-PCR

The total mean extracted mRNA from healthy tissue, type V and type VI plaques was 8764 ng, 5069.4 ng and 2172 ng respectively. The mean CT values of endogenous control GUSB were  $36.28 \pm 0.21$  in healthy tissue,  $31.36 \pm 0.32$  in type V plaques and  $34.20 \pm 0.22$  in type VI plaques. Mean CT for tested gene Nestin were  $33.04 \pm 0.06$  in healthy tissue,  $32.37 \pm 0.12$  in type V plaques and  $34.21 \pm 0.30$  in type VI plaques. Mean CT for tested gene WT1 were  $41.99 \pm 1.10$  in healthy tissue,  $36.61 \pm 1.20$  in type V plaques and  $45.07 \pm 1.15$  in type VI plaques.  $\Delta\Delta CT$  Nestin and  $\Delta\Delta CT$  WT1 were significantly different from 0 ( $P=0.0001$  and  $P=0.0006$ ) thus the null hypothesis was rejected, which indicated a change in Nestin and WT1 gene expression between healthy, type V and type VI plaques. In type V and type VI plaques, the mean  $\Delta\Delta CT$  Nestin was respectively 4.25 and 3.25, and the mean  $\Delta\Delta CT$  WT1 was 2.54 and 5.16. This



corresponds to  $2^{-(\Delta\Delta CT)}$  of 0.05 for nestin gene expression in type V plaques and 0.11 in type VI plaques. This corresponds to  $2^{-(\Delta\Delta CT)}$  of 0.17 for WT1 gene expression in type V plaques and 0.03 in type VI plaques. The type VI plaques showed a 2-fold expression increase for Nestin

gene and a 5-fold expression decrease for WT1 gene (compared to type V plaques). Thus, in complicated plaques Nestin expression increases while WT1 gene expression decreases (see Figure 18).

### 3.5 CORRELATIONS AMONG MORPHOLOGICAL VARIABLES

At cross-correlation among the morphological variables, the mean maximum size of the plaque cap was significantly related with the amount of neoangiogenesis ( $P=0.006$ , Spearman coefficient 0.325). In particular, the mean maximum cap size was  $974.63\pm 402.75$   $\mu\text{m}$  in plaques with low neoangiogenesis (1+/2+) and  $1364.29\pm 510.74$   $\mu\text{m}$  in plaques with high neoangiogenesis (3+/4+).

The extension of lipidic core was correlated with flogosis ( $P<0.001$ , Spearman coefficient 0.464), and intra-plaque hemorrhage ( $P=0.025$ , chi-square test, Spearman coefficient 0.339), since 33/40 plaques with hemorrhage had core extension  $\geq 2+$ .

Moreover, the core extension was inversely correlated with calcifications ( $P=0.005$ , chi-square test, Spearman coefficient -0.324). In particular, 13/20 calcified plaques had 0/1+ of lipid core extension, *versus* 10/53 of the others.

Finally, the plaques with massive calcifications (3+/4+) were characterized by a lower flogosis ( $P=0.004$ , Spearman coefficient -0.332) than other plaques.



### 3.6 CORRELATIONS BETWEEN IMMUNOHISTOCHEMISTRY AND MORPHOLOGY

As expected, the densities of positivity of the three antibodies significantly correlated each others at  $P < 0.001$  (Spearman coefficients: CD34-Nestin 0.803; CD34-WT1 0.588; Nestin-WT1 0.669).

The correlations of the immunohistochemical densities of the three antibodies with the plaque morphological variables are shown below:

- CD34 (Table 4). It correlated with the lipid core extension ( $P=0.05$ , Spearman coefficient 0.234), the flogosis ( $P=0.002$ , Spearman coefficient 0.353), and it was inversely correlated with the amount of calcifications ( $P < 0.001$ , Mann-Whitney test, Spearman coefficient -0.322). Notably, the density of CD34 did not correlate with the occurrence of histological complications ( $P=0.111$ , Mann-Whitney test, Spearman coefficient 0.189).

	Not-calcified plaques	Calcified plaques	Complicated plaques	Uncomplicated plaques
<b>CD34 density</b>	11.19±3.84	7.52±3.09	10.68±3.79	8.99±4.13
<b>Mann-Whitney</b>	P<0.001		<b><u>P=0.111</u></b>	

*Table 4.*

- Nestin (Table 5). The density of Nestin-positive neovessels was correlated with flogosis ( $P=0.005$ , Spearman coefficient 0.327), and inversely correlated with the occurrence of calcifications ( $P < 0.001$ , Mann-Whitney test, Spearman coefficient -0.280). Unlike CD34, Nestin density correlated with the occurrence of histological complications ( $P=0.015$ , Mann-Whitney test, Spearman coefficient 0.293).

	Not-calcified plaques	Calcified plaques	Complicated plaques	Uncomplicated plaques
<b>Nestin density</b>	7.69±3.77	4.59±2.31	7.45±3.71	5.31±3.09
<b>Mann-Whitney</b>	P<0.001		P=0.015	

**Table 5.**

- WT1 (Table 6). It correlated with flogosis (P<0.029, Spearman coefficient 0.259), the occurrence of histological complications (P<0.033, Mann-Whitney test, Spearman coefficient -0.263), and it was inversely correlated with the amount of the calcifications (P=0.020, Mann-Whitney test, Spearman coefficient -0.280).

	Not-calcified plaques	Calcified plaques	Complicated plaques	Uncomplicated plaques
<b>WT1 density</b>	4.99±3.31	3.16±1.64	4.88±3.19	3.45±2.43
<b>Mann-Whitney</b>	P=0.020		P=0.033	

**Table 6.**

### **3.7 IMMUNOHISTOCHEMICAL PROFILES OF THE DIFFERENT PLAQUE TYPES**

#### ***3.7.1 Complicated plaques***

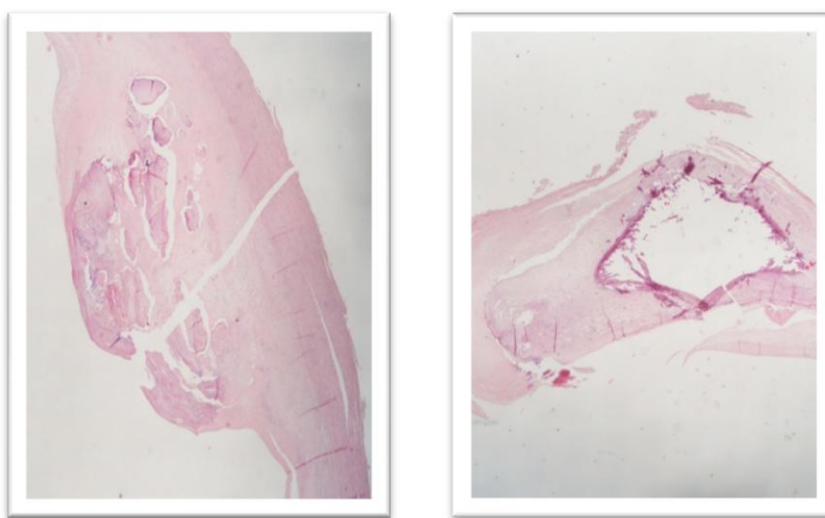
In our series, the plaques with histological complications, classified as type VI according to AHA, were characterized by a larger extension of the lipid core (P=0.029, Spearman coefficient 0.260) and showed a slightly thinner fibrous cap P=0.074, Spearman coefficient -0.217) than uncomplicated plaques.

At immunohistochemistry, as stated previously, the density of CD34-positive neovessel was not correlated with histological complications (P=0.111), while the density of both Nestin and WT1 was (P=0.015 and P=0.033 respectively). This is an important results, since it confirms our previous results, i.e. that it is not the amount of the sole neoangiogenesis to characterize type VI plaques, but the amount of the Nestin-positive neoangiogenesis. Notably, in this enlarged series, also WT1 is correlated with intra-plaque complications (even if with a lower significance), while in our previous results it did not.

#### ***3.7.2 Calcified plaques***

Twenty-one (28.8%) plaques showed extensive calcifications (3+/4+) in our series. Among these, 13 cases (61.9% of the calcified plaques) concomitantly showed one or more histological complications, and were therefore classified as type VI plaques according to AHA. In these cases, hemorrhage or fissuration were recorded in 11 cases, thrombosis in 3 cases, and defects in the endothelial surface in 5. The remaining 8 cases (38.1% of the calcified plaques) did not show histological complications, and they were classified as type VII according to AHA (Stary 2000), equivalent to the type Vb of the previous classification (Stary 1995).

Interestingly, the incidence of histological complications did not differ significantly between calcified and not-calcified plaques ( $P=0.167$ , Spearman coefficient  $-0.117$ ). Moreover, at histopathology, calcified plaques showed significantly less flogosis ( $P=0.002$ , Mann-Whitney test, Spearman coefficient  $-0.332$ ) than not-calcified plaques. At immunohistochemistry, as stated above, the densities of positivity of all three antibodies were less represented in the calcified plaques.



**Figure 19.** *Two examples of calcified atheromatous plaques. The low quality of the slides is due to the objective difficulties in processing and cutting calcified tissue.*

*Magnification 4x.*

### ***3.7.3 The influence of calcifications and neoangiogenesis on intra-plaque complications***

From our immunohistochemical results, it seems that the calcified plaques have a minor amount of flogosis and CD34-positive neovessels, but with the same incidence of histological complications, compared to the other plaques.

If we exclude the 21 calcified cases from the series, we can notice that the correlation between neoangiogenesis and complications becomes more important.

	Cap	Core	CD34	Nestin	WT1
All cases	P=0.074	P=0.029	<b>P=0.114</b>	P=0.014	P=0.029
Not-calcified cases	P=0.048	P=0.012	<b>P=0.026</b>	P=0.002	P=0.011

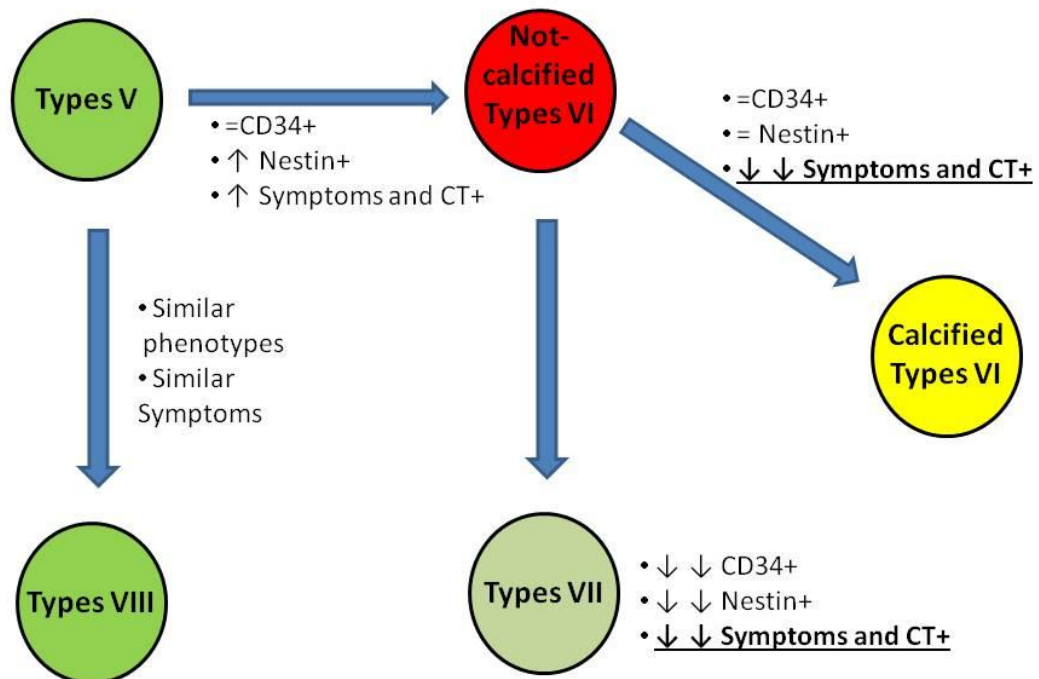
**Table 7.** Statistical significance (Mann-Whitney test) in the morphophenotypical differences between type VI and other plaques, in all cases and in not-calcified cases only.

In "classic" not-calcified plaques, the thickness of the fibrous cap and the extension of the core become more significant between calcified and not-calcified plaques, as well as all immunohistochemical markers of neoangiogenesis. In particular, CD34 density is significantly higher in "classic" plaques than in calcified plaques in the onset of intra-plaque complications.

	Types V-VIII (N=13)	Type VII (N=8)	Type VI not- calc (N=39)	Type VI calc (N=13)	Sig. (K W)
<b>Mean min cap</b>	382.5	328.8	249.1	284.2	<b>P=0.826</b>
<b>Mean max cap</b>	1187.5	952.5	1145.1	1132.8	<b>P=0.215</b>
<b>Mean core</b>	2.0	1.0	2.5	2.1	<b>P=0.014</b>
<b>Flogosis</b>	2.5	1.1	1.2	2.3	<b>P=0.005</b>
<b>Mean neoang.</b>	2.2	1.5	2.4	2.2	<b>P=0.156</b>
<b>CD34</b>	10.1	6.4	11.6	10.1	<b>P=0.002</b>
<b>Nestin</b>	5.9	3.8	8.3	6.8	<b>P=0.001</b>
<b>WT1</b>	3.7	2.9	5.4	4.5	<b>P=0.022</b>
<b>Symptoms</b>	3 (23.1%)	1 (12.5%)	22 (56.4%)	3 (23.1%)	<b>P=0.018*</b>
<b>CT positivity</b>	4 (57.1%)	1 (25.0%)	18 (62.1%)	2 (20.0%)	<b>P=0.092*</b>

**Table 8.** Immunomorphological differences among plaques sorted by AHA type and calcification.

K W: Kruskal Wallis test. \*chi-square test. CT data are available for 50 cases.



**Figure 20.** Progression/regression pathways among different plaque types, and their relative changes in neoangiogenesis phenotype and clinical presentations according to our results.

Figure 20 summarizes the relationship among the different plaque that we found in the present study. While no differences seem to exist between type V and type VIII (fibrotic) plaques, in type VI (complicated) plaques the Nestin-positive neoangiogenesis increases, as well as the incidence of symptoms and focal lesions at CT, as expected. The type VII (uncomplicated) calcified plaques show sensibly lower CD34-positive and Nestin-positive neoangiogenesis, and very few symptomatic cases, confirming that they represent a regressed kind of plaque. Conversely, the calcified complicated plaques show a CD34-positive and Nestin-positive neoangiogenesis comparable to the

neoangiogenesis of type VI not-calcified plaques, and therefore higher than type VII, but with an incidence of symptomatic cases similar to uncomplicated type VII plaques. This latter kind of plaque shares the same histopathological complications and Nestin-positive neoangiogenesis of "classic" type VI plaques, but its clinical presentation is comparable to type VII plaques.

### 3.8 CORRELATIONS AMONG MORPHOLOGICAL PLAQUE TYPES, NEOVESSEL PHENOTYPES AND CLINICAL DATA

#### 3.8.1 *Histological complications correlate with symptoms*

As expected, the plaques of AHA type VI had a significantly higher incidence of symptoms (stroke or transient ischemic attack) than the plaques of types V, VII and VIII ( $P=0.026$ , chi-square test, Spearman coefficient 0.256), since 25 out of 29 (86.2%) patients with symptoms had type VI plaques, *versus* 27 out of 44 (61.4%) patients without symptoms.

In our series, AHA type VI did not correlate with the occurrence of focal lesions at Computed Tomography ( $P=0.500$ , chi-square test).

#### 3.8.2 *Calcified plaques have less symptoms and brain lesions regardless the histological complications*

Among the 21 patients with calcified plaques, only 4 (19.0%) had symptoms at the onset, regardless the occurrence of histological complications, *versus* 25 out of 52 (48.1%) not-calcified plaques ( $P=0.019$ , chi-square test, Spearman coefficient -0.229).

Data about brain Computed Tomography were available in 50 cases, 14 with calcified plaques and 36 not-calcified plaques. Focal brain lesions were detected in 3 out of 14 (21.4%) patients with calcified plaques and 22 out of 36 (61.1%) patients with not-calcified plaques. This difference was significant as well ( $P=0.013$ , chi-square test, Spearman coefficient -0.231).



### 3.9 THE CALCIFIED PLAQUES IN THE FEMALE PATIENTS

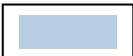

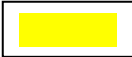
Only 3 (15.0%) of the 20 female patients in our study were symptomatic. Notably, all 3 had type VI atheromatous lesions. Nine had uncomplicated and asymptomatic plaques, which is the 45.0% of female patients, a percentage sensibly lower than in males (15.1%). Twenty-seven (50.9%) out of 53 male patients were asymptomatic and 26 (49.1%) showed symptoms: in any case, the most represented type of atheromatous lesions in males was the type VI not-calcified plaque.

The female sex is likely to represent a protective factor, since it implies a lower incidence of type VI plaques ( $P=0.044$ , chi-square test) and symptoms ( $P=0.007$ ) than male patients. This seems to be related to the higher incidence of calcified plaques ( $P=0.017$ ).

	Type VI plaques	Calcified plaques	Symptoms
Females (N=20)	11 (55.0%)	10 (50.0%)	3 (15.0%)
Males (N=53)	41 (77.4%)	11 (20.8%)	26 (49.1%)
Sig. (chi-square)	$P=0.058$	$P=0.017$	$P=0.007$

**Table 9.** Different incidence in intra-plaque complications and calcifications between male and female patients.

				sex		Total	
				male	female		
asymptomatic	Not-VI	calcif	V-VIII	6	4	10	
			VII	2	5	7	
		Total	8 (15.1%)	9 (45.0%)	17		
	VI	calcif	No	13	4	17	
			Yes	6	4	10	
		Total	19 (35.8%)	8 (40.0%)	27		
				27 (50.9%) m	17 (85.0%) f		
			asymptom	asymptom			
	symptomatic	Not-VI	calcif	V-VIII	3		3
				VII	1		1
Total			4 (7.5%)	0 (0.0%)	4		
VI		calcif	No	20	2	22	
			Yes	2	1	3	
		Total	22 (41.5%)	3 (15.0%)	25		
				26 (49.1%) m	3 (15.0%) f		
		symptom	symptom				
Total	Not-VI	calcif	V-VIII	9	4	13	
			VII	3	5	8	
		Total	12 (22.6%)	9 (45.0%)	21		
	VI	calcif	No	33	6	39	
			Yes	8	5	13	
		Total	41 (77.4%)	11 (55.0%)	52		
		Patients		53 m	20 f	73 total	

-  → High prevalence of uncomplicated plaques in asymptomatic females
-  → High prevalence of not-calcified complicated plaques in males
-  → Higher incidence of symptomatic plaques in males than females

**Table 10.** Multivariable cross-table describing our series, with special emphasis on sex differences.

## **4. DISCUSSION**

The aims of the present thesis were: (1) to confirm our previous results on Nestin/WT1 phenotype in a larger series of carotid atheromatous plaques, (2) to evaluate the relationship between the Nestin+/WT1- neoangiogenesis phenotype and the main histopathological plaque characteristics, and (3) to evaluate the relationship between the immunohistochemical and histopathological characteristics and the clinical instability of the plaques.

On an enlarged consecutive series of 73 carotid endarterectomy specimens, we could confirm our previous results on the different Nestin/WT phenotypes in carotid plaques, as well as some consolidated data present in the literature.

At morphology, our results confirm the main features characterizing the vulnerable plaques: the extension of lipidic core was correlated with flogosis and intra-plaque hemorrhage, and the maximum size of the plaque cap was significantly related with the amount of neoangiogenesis. This confirms that the vulnerable plaque (with hemorrhage) is generally characterized by a large lipid core, flogosis, and a neoangiogenesis which is commonly visible with Haematoxylin-Eosin stain by an expert eye.

At immunohistochemistry, about 70% of the intra-plaque neovessels expressed Nestin, and about 40% expressed WT1, with a mean WT1/Nestin ratio of 0.7. Consequently we can confirm that the Nestin+/WT1- phenotype was the more represented in the carotid atheromatous lesion, and that this phenotype correlated with the occurrence of intra-plaque histopathological complications (which put the plaque in the type VI according to AHA). The main noteworthy difference that we found in this enlarged series compared to our previous results it is that also WT1 positively correlates with the intra-plaque complications. In fact, in our preliminary study (Fittipaldi et al. under revision) WT1 was not significantly different between type VI and not-type VI plaques, while in our 73 patients both Nestin and WT1 were significantly increased. It is comprehensible

that a quote of Nestin+/WT1+ neovessels increases together with the more represented Nestin+/WT1- neovessels: at any chance, the lower statistical significance together with the fact that this data emerged only with a bigger series may indicate that this Nestin+/WT1+ portion is less represented.

In our opinion, the most noteworthy result that emerged from the present study is the particular clinical and pathological profile characterizing the calcified plaques.

The calcification of the atheromatous plaque was historically considered as a passive, degenerative process, and only recently it has begun to be studied, especially in aorta and coronary vessels. Most individual aged more than 60 years have progressively enlarging deposits of calcium in their major arteries, generally starting from the coronary arteries (Allison 2004). The severity and extent of mineralization reflect the atherosclerotic plaque burden and strongly predict cardiovascular morbidity and mortality (Sangiorgi 1998; Vliegenthart 2005): in a relatively recent study, no patients were found to have calcifications confined only to the coronary or carotid beds (Allison 2004). The extent of calcification is associated with worse prognosis, albeit the real impact of calcification within a specific lesion remains unclear (Huang 2001). For example, in the coronary vessels small calcium depositions increase the probability of atherosclerotic plaque rupture, especially on their edges, while with individual, large calcification foci such risk may even decrease (Huang 2001; Vengrenyuk 2006). In a study on 10 stables and 10 ruptured coronary artery post-mortem specimens, calcifications did not significantly affect the stability of the atheroma, in contrast with the significant reduction in stability associated with lipids: removing the calcifications led to a statistically insignificant change in stress (Huang 2001). Anyway, vascular calcification is considered a worsening factor, probably due to the association with the general risk factors: a study by Iribarren et al (2000) found that aortic arch calcification

was associated with coronary heart disease risk both in men and women. Thus aortic arch calcification may reflect the general burden of disease or be a marker of more aggressive disease.

Vascular calcification risk factors are similar to those of atherosclerosis: hypertriglyceridemia, increased LDL, decreased HDL, obesity, hypertension (Polhe 2001). In particular, hypertension in men showed a odd ratio of 3.2 in the carotids, 3.9 in coronaries and 2.7 in proximal aorta (Allison 2004).

As for the pathogenesis, vascular calcification is now recognized as a pathobiological process sharing many features with embryonic bone formation (Demer 2008). Vascular cell differentiation responds to microenvironmental and mechanical cues, since substrates of great stiffness, such as fibronectin, promote osteochondrogenic differentiation, whereas distensible substrates, such as laminin, promote smooth muscle or adipogenic differentiation (Yip 2009). The so-called biomineralization process begins from the crystallization nucleators, which trigger the formation of a primary crystal nucleus, together with the removal of the mineralization inhibitors (ANK, nucleotide pyrophosphatase, matrix Gla protein). The extracellular matrix vesicles contain deposits of calcium, alkaline phosphatase (ALP) and pyrophosphatase, among others, which increase the inorganic phosphates in the vesicles, at least in mouse models (Anderson 2004). They also stimulate the production of osteopontin, another nucleation inhibitor (Speer 2002).

During the vessel calcification there are processes similar to those in the bone biomineralization. In depositions in both tunica interna and media of the vessel wall, matrix vesicles have been identified (Tanimura 1983).

Post-mortem studies have shown that vessel wall may contain a typical bone, cartilage or adipose tissue, with bone as the predominating type of metaplasia (10-15% of samples), appearing in various morphological forms, from amorphous calcium deposits

to mature bone tissue (Karwowski 2012). Another recent theory (Pal 2011) postulated that the osteochondrogenic cells may derive from circulating progenitor cells (probably derived from the bone marrow, according to Sata 2005). These cells migrate and proliferate in the diseased artery as a response to different cytokines such as SDF-1 $\alpha$ . Other cytokines like PDGF and VEGF, produced by platelet activation after the loss of endothelial integrity, are involved (Roberts 2005).

Histologically, arterial calcifications can be classified in (1a) calcifications of the tunica intima, especially related to atherosclerosis and, (1b) calcifications of the tunica media, unrelated to atherosclerosis (Monckeberg's type) (Karwowski 2012). The latter group embraces also the calcific uremic arteriopathy. Etiologically, the calcifications can be divided into metastatic and dystrophic (Karwowski 2012). The first type occurs when the calcium and phosphates increase (hyperthyroidism, neoplasm, vitamin D overdose, etc.), the latter occurs with normal mineral concentrations in damaged or necrotic tissues, as it is the case of atherosclerosis, but also neoplasm, tuberculosis, parasitosis, etc. (Demer 2008).

The intimal (1a) atherosclerotic calcifications are the most common form of calcification. Calcium accumulation is initiated by an increase in the plaque of modified lipids, pro-inflammatory cytokines, phosphate and lipoprotein complexes, as well as foci of necrosis (Demer 2008; Karwowski 2012). *In vitro* studies have shown that pro-inflammatory cytokines, oxidized LDL or other macrophage release products promote osteogenesis and the calcium accumulation (Tintut 1998; Proudfoot 2002; Tintut 2002), while some studies (but not all) correlated the vascular calcification with the so-called "cholesterol-years" (Schmidt 1996) and with inflammation *in vivo* (Aikawa 2007). *In vitro*, the calcifying vascular cells (CVC, a subpopulation of smooth muscle cells with osteoblastic characteristics that spontaneously form bone mineral) may spontaneously produce minerals that cluster locally as small lumps.

The most intense medial artery calcifications (1b) are observed in patients with diabetes and chronic renal disease (Reaven 2005; Okuno 2007). An important role in the medial calcification process is attributed to metalloproteinases (MMP), which are secreted by adventitial/medial inflammatory cells. MMP activity causes an increase in elastin matabolites, which promote further inflammation and may become crystallization nucleators (Lee 2006). MMP inhibition breaks the vicious cycle of monocyte activation and restricts vascular calcification. At any chance, elastin degradation appears early in many forms of elastic calcification.

During aging, medial calcification may develop by unknown etiology, or result from associated conditions such as chronic renal failure, atherosclerosis, insulin resistance, etc. (Demer 2008).

The plaques with a predominant core calcification are classified as AHA type Vb according to the 1995 Stary's classification or AHA type VII according to the 2000 Stary's classification. The idea that led to this modification is that events such as calcification or fibrosis could represent regressive processes, "extinguishing" the type IV, V, or VI plaques. At any chance, the clinical impact of a plaque in which both calcification and histological complications coexist is far from being clarified.

According to our data calcified plaques showed less flogosis, a smaller lipid core, and less neoangiogenesis than other plaques, but with the same incidence of hemorrhage, thrombosis and surface defects. Yet, interestingly, in these plaques complications are not correlated with clinical plaque instability that we evaluated as symptomatology and focal brain lesions, since their incidence is sensibly lower regardless the intra-plaque complications.

This is noteworthy, since the presence of calcifications seems to imply a sort of “protection” towards complications in these plaques, so that they show a much lower



incidence of TIA and stroke at clinical onset, as well as focal brain lesions at CT. For these reasons, in our opinion, these plaques can be classified among the type VII plaques, instead of type VI, at least on clinical grounds. Alternatively, they can be classified as type VI, but the extension of the calcifications should be stated in the pathological report, to highlight their protective nature.

The finding that extensive calcifications protect the plaque can lay in the big dimensions of the calcifications, in accordance with recent papers that showed that small coronary calcifications increase the probability of plaque rupture, while individual, large calcifications make the plaque more stable (Huang 2001; Vengrenyuk 2006). The reason why the neoangiogenesis and the pathological complications do not affect symptomatology remains unclear, but it is possible that the hemorrhages and erosions found in these plaques might have a different pathogenesis. For example, it is possible that they can be due directly by the mechanical stresses of the calcified mass, and not by immature neoangiogenesis and endothelial damage. Another possible explanation is that the "calcified type VI" plaques can represent an early stage of type VII plaques, in which the regressive process is more recent, and the complications are not disappeared yet.

The last result that emerged from our data is that 50% of the female patients had calcified plaques, showing a significantly lower incidence of symptoms, CT positivity and type VI plaques than the male patients. Ten years ago, Allison et al. have found 53% and 30% prevalence of "zero calcification" in female and male patients respectively before age 50: after that age the prevalence of a diffuse vessel calcification increases, in a linear fashion in males and exponentially in females (Allison 2004). Actually, female sex hormones play an important role in bone tissue metabolism, increasing bone density and inhibiting osteoclast activity. Estrogen therapy for post-

menopausal women reduces vascular calcifications, probably due to the effects on the risk factors, but also to a direct genomic and extragenomic effect on macrophages, endothelial cells and smooth muscle cells (Mackey 2005; Manson 2007; Jeon 2010).

Nevertheless, it should be kept in mind that most women in our series were post-menopausal, and their age at the moment of surgery did not differ from males ( $70.2 \pm 9.5$  versus  $71.0 \pm 8.5$  years), so the question whether the post-menopausal hormone therapy might play a role in the pathophysiology of the atherosclerosis is still open.

In conclusion, this thesis confirms that the neovessels of atheromatous plaques express a double Nestin/WT1 phenotype, and that the Nestin+/WT1- phenotype characterizes the plaques with features of instability, regardless the real amount of the neoangiogenesis (expressed as CD34-positive vessels). The plaques with massive calcifications show the same incidence of histological complications, but they do not influence symptomatology and plaque vulnerability. Female patients show a much higher incidence of not-complicated or calcified plaques, receiving *de facto* a sort of protection compared to male patients.

A possible translational application of these findings could be a comparison between the plaque dynamic imaging and the histological assessment of calcification, to evaluate the possibility of a pre-surgical risk stratification of patients, basing on their sex, risk factors and intra-plaque calcification. The pre-surgical identification of those patients at major risk of developing stroke or brain lesions is likely to make more rational the priority for endarterectomy.

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