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COORDINATORE Prof. Francesco Bernardi

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Cellular and molecular components of the atherosclerotic process: Primary colture and characterization of human vascular smooth muscle cells from carotid artery

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Dottoranda

Dott. Teresa Gagliano

Tutori

Prof. Laura Del Senno

Prof. Giovanna Marchetti

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1.Introduction

1.1 Normal vasculature

The basic constituents of the walls of blood vessel are endothelial cells, smooth muscle cells, and extracellular matrix, including elastic elements, collagen and proteoglycans. They are arranged in concentric layers:

- Intima (adjacent to the lumen)
- Media
- Adventitia (externally)

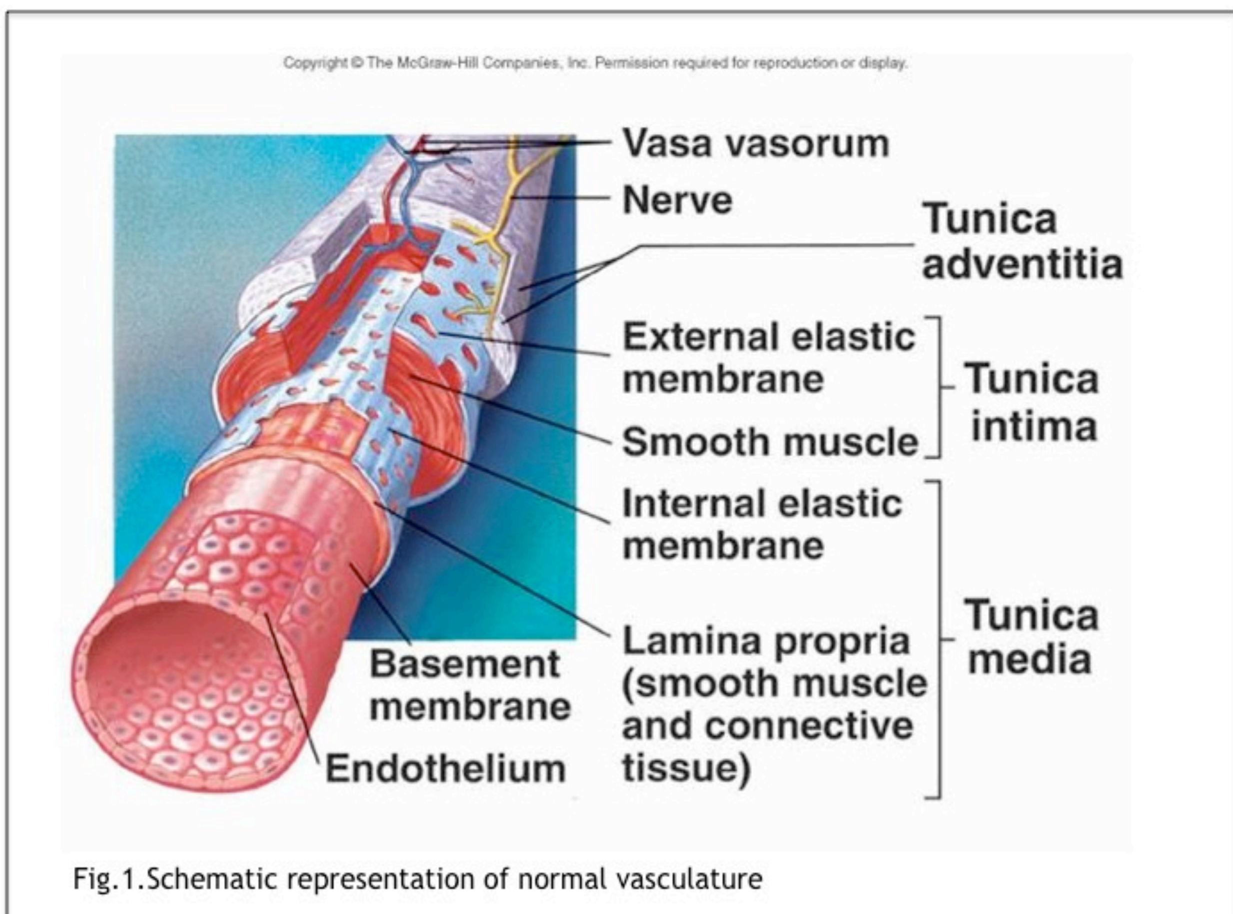


Fig.1.Schematic representation of normal vasculature

In normal arteries, the intima is composed of the lining endothelial cells with minimal underlying subendothelial connettive tissue. It is separated from the media by a dense elastic membrane called internal elastic lamina.

The outer limit of the media of most arteries is marked by a well-defined external elastic lamina.

In the large and medium-sized arteries, the smooth muscle cell layer of the media near the vessel lumen depend primarily on direct diffusion of oxygen from the vessel for their nutritional needs. Diffusion is facilitated by holes (fenestration) in the internal elastic membrane (Robins-Pathologic basis of disease, 1999)

1.1.1 Endothelial cells

Endothelial cells form a monolayer that lines the entire vascular systems (the endothelium). Vascular endothelium is a versatile multifunctional tissue having many synthetic and metabolic properties. Endothelial cells can respond to various abnormal stimuli by adjusting some of the constitutive function, by expressing newly acquired (induced) properties (*Sumagin et al,* 2008). The term endothelial dysfunctions often used to describe several types of potentially reversible changes in the functional state of endothelial cells that occur in response to environmental stimuli (*Selwyn AP et al*, 1997).

1.1.2 Vascular Smooth Muscle Cells

Vascular smooth muscle cells (SMCs) are capable of many function including vasoconstriction and dilatation in response to normal or pharmacologic stimuli; synthesis of collagen, elastin, and proteoglycans; elaboration of growth factors and cytokines; and migration to the intima and proliferation: As the predominant cellular element of the vascular media, smooth muscle cells constitute an important element not only normal

vascular repair, but also pathological such us atherosclerosis. Resting vascular smooth muscle cells are spindle shaped, with single, elongated nuclei resembling fibroblast. Cytoplasm tic filaments that contain actin and myosin mediate the contractile function of SMCs.

During the healing response, smooth muscle cells undergo changes that resemble dedifferentiation (*Bochaton-Piallat ML, Gabbiani G,* 2005). SMCs migrating from the media to the intima lose the capacity to contract, gain the capacity to divide, and increase the synthesis of extracellular matrix molecules, often designated a shift from that contractile phenotype to the proliferative synthetic phenotype. Structurally, there is a decrease in the thick myosin-containing filaments and an increase in the amount of organelles involved with protein synthesis, such us rough endoplasmic reticulum and Golgi apparatus. The contractile state of vascular smooth muscle influences arterial blood pressure and regulates organ blood flow. The contractile apparatus of vascular smooth muscle is composed of thin and thick filaments, and that force generated between these two filaments provides the mechanism for cell shortening. The molecular events that initiate the interaction between these filaments are dependent upon the free sarcoplasmic concentration of activator calcium, which is regulated by the cell membrane and at subcellular sites. Changes in electrical activity of the cell membrane and interaction of pharmacologic agents with membrane receptors alter the cell, causing either a decrease or increase in sarcoplasmic calcium concentration and thus changing the contractile state of the vascular smooth muscle cell. Alterations in the cellular mechanisms that regulate intracellular calcium concentration may contribute to abnormal vascular function in pathologic states (*Bohr DF, Webb RC,* 1984)

1.2 Atherosclerosis

Atherosclerosis is a condition in which patchy deposits of fatty material develop in the walls of medium-sized and large arteries, leading to reduced or blocked blood flow.

Atherosclerosis is caused by repeated injury to the walls of arteries. Many factors contribute to this injury, including high blood pressure, tobacco smoke, diabetes and high levels of cholesterol in the blood (Besler et al 2008). Often, the first symptom is pain or cramps at times when blood flow cannot keep up with the tissues' need for oxygen. To prevent atherosclerosis, people need to stop using tobacco, improve their diet, exercise regularly, and maintain control of their blood pressure and diabetes (Taylor DA & Zenovich AG, 2008). If atherosclerosis causes complications, such as a heart attack or stroke, these are treated. In the most developed countries, atherosclerosis is the leading cause of illness and death.

Arteriosclerosis literarily means hardening of arteries; more accurately, however, it is a generic term for three patterns of vascular disease that have in common thickening and loss of elasticity of arterial walls:

- The dominant patter is ***atherosclerosis***, characterized by the formation of intimal fibrous plaques that often have a central grumos core rich in lipid

- Monckeberg's arteriosclerosis, also called medial calcific sclerosis, is a form of arteriosclerosis or vessel hardening, where calcium deposits form in the middle layer of the walls of medium sized vessels (the tunica media)

- Arteriolosclerosis: sclerosis and thickening of the walls of arterioles. The hyaline form may be associated with nephrosclerosis, the hyperplastic with malignant hypertension, nephrosclerosis and scleroderma.

Atherosclerosis is characterized by intimal lesion called atheromas or fibrofatty plaques that protrude into the lumen, and undergo a series of complication.

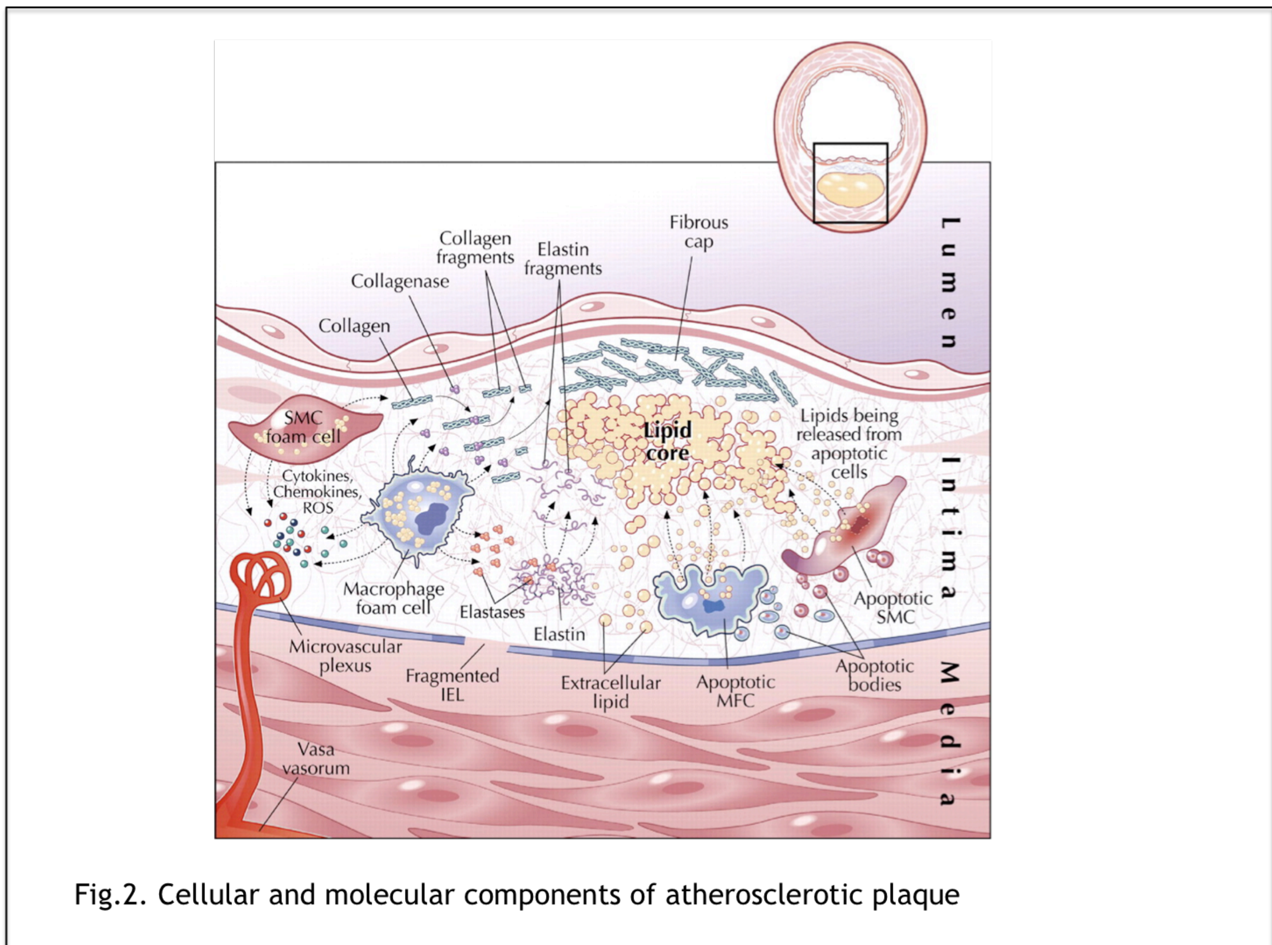


Fig.2. Cellular and molecular components of atherosclerotic plaque

Atherosclerosis primarily affects elastic arteries (e.g., aorta, carotid and iliac arteries) and large and medium sized muscular arteries (e.g. coronary and popliteal arteries). The disease often begins in childhood, but symptoms are not usually evident until middle age or later when the arterial lesions precipitate organ injury (*Milei,* 2003).

Symptomatic atherosclerotic disease is most often localized to arteries supplying the heart, brain, kidneys, lower extremities, and small intestine. Myocardial infarction (heart attack), cerebral infarction (stroke), and aortic aneurysms are the major consequences of this disease. Thus, epidemiologic data on atherosclerosis are expressed largely in terms of the incidence of or the number of death caused by ischemic heart disease (Robins-Pathologic basis of disease, 1999).

1.2.1 Pathogenesis of atherosclerosis

The development of atherosclerosis is complicated, but the primary event seems to be repeated, subtle injury to the artery's wall through various mechanisms. These mechanisms include physical stresses from turbulent blood flow (such as occurs where arteries branch, particularly in people who have high blood pressure) and inflammatory stresses involving the immune system, certain infections, or chemical abnormalities in the bloodstream (high cholesterol, diabetes)(Arora S & Nicholls SJ, 2008).

The pathogenesis of atherosclerosis has not been well defined, however, association of fatty degeneration and vessel stiffing was the first definition of atherosclerosis. Principal events of atherosclerosis are the follow:

- The development of focal regions of chronic endothelial injury, usually subtle, with resultant endothelial permeability and increased leukocyte adhesion (*Crowther MA*, 2005)
- Insudation of lipoproteins into vessel wall, mainly LDL with its high cholesterol content and also very-low-density lipoprotein (VLDL), and modification of such lipoprotein by oxidation *(*Torzewski M, Lackner KJ.2006)

- Adhesion of blood monocytes (and other leukocytes) to endothelium, followed by migration of monocytes into the intima and their transformation into macrophages and foam cells (*Doran AC et al*, 2008)
- Adhesion of platelets to focal areas of denudation (when present) or to adherent leukocytes (*DiCorleto PE*, 1993)
- Release of factors from activated platelets, macrophage, or vascular cells that cause migration of smooth muscle cells from media in to the intima (*Bochaton-Piallat ML, Gabbiani G*, 2005)

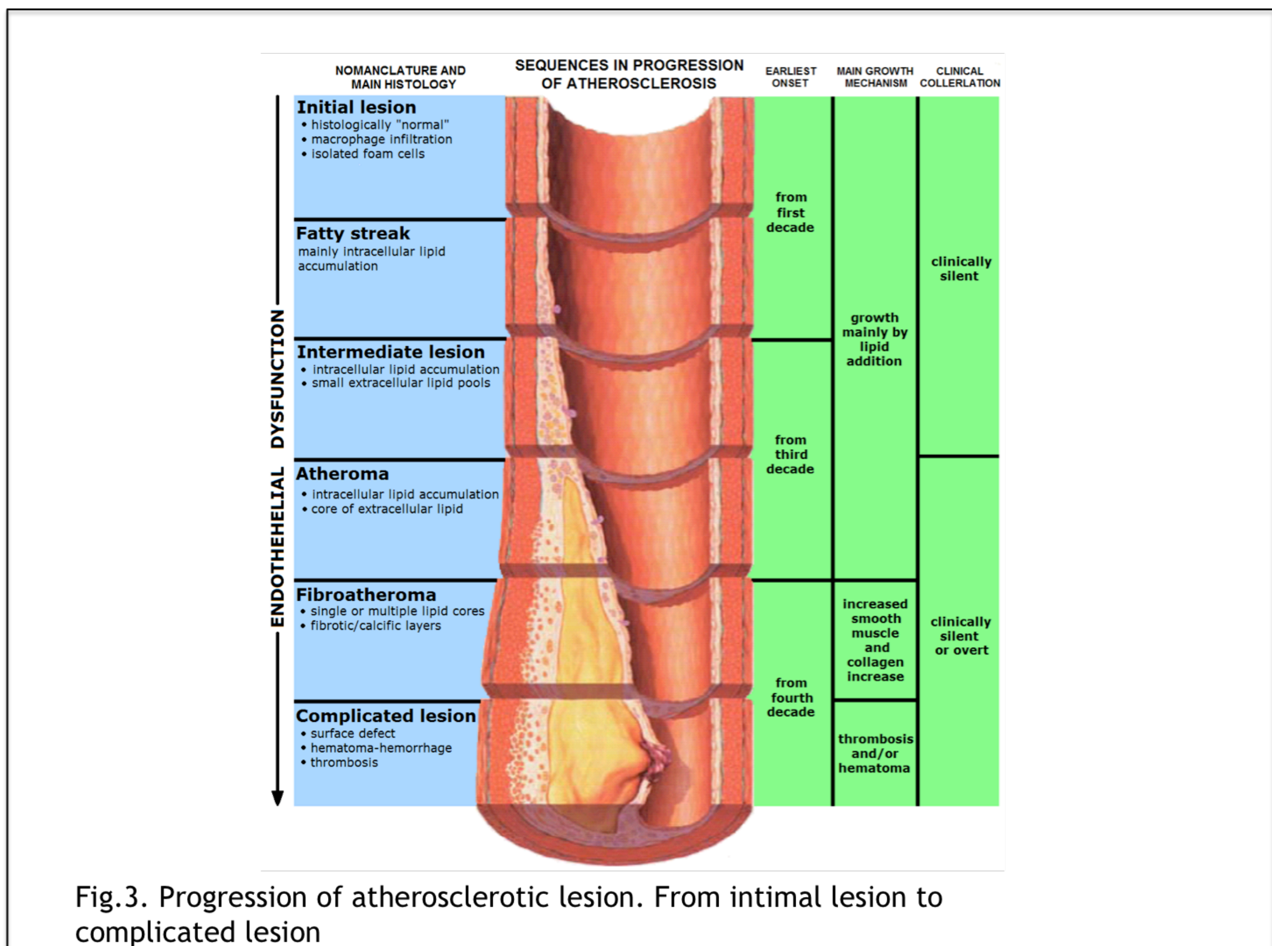


Fig.3. Progression of atherosclerotic lesion. From intimal lesion to complicated lesion

1.2.2 Classification of type lesion

Type I Lesions

Type I lesions consist of the first microscopically and chemically detectable lipid deposits in the intima and the cell reactions associated with such deposits. These lesions have been characterized in studies in which the sequence of lesions was deduced from examining many persons who died at different ages (*Stary et al,* 1994).

Type II Lesions

Type II lesions include fatty streaks, which on gross inspection may be visible as yellow-colored streaks, patches, or spots on the intimal surface of arteries. Fatty streaks stain red with Sudan III4958 or Sudan IV.

The precursors of advanced lesions are divided into three morphologically characteristic types. Both type II lesions and I represent small lipid deposits in the arterial intima, and type II includes those lesions generally referred to as fatty streaks.

Fatty streaks are not significantly raised and thus do not cause any disturbance in blood flow. They may be precursor however of the most ominous atheromatouse plaque (*Herbert et al*, 1995).

Type III Lesions

The designation "type III lesion" applies only to lesions that form the morphological and chemical bridge between type II lesions and atheromas.

The type III lesion is also known as the intermediate lesion, the transitional lesion, and as preatheroma. Its characteristic histological features are microscopically visible (*Stery et al,* 1994).

Type IV

In type IV lesion a dense accumulation of extracellular lipid occupies an extensive but well-defined region of the intima. This type of extracellular lipid accumulation is known as the lipid core. A fibrous tissue increase is not a feature, and complications such as defects of the lesion surface and thrombosis are not present. The type IV lesion is also known as atheroma. Type IV is the first lesion considered advanced in this classification because of the severe intimal disorganization caused by the lipid core. The characteristic core appears to develop from an increase and the consequent confluence of the small isolated pools of extracellular lipid that characterize type III lesions (*Stary HC,* 1989).

Type V

Type V lesions are defined as lesions in which prominent new fibrous connective tissue has formed. When the new tissue is part of a lesion with a lipid core (type IV), this type of morphology may be referred to as fibroatheroma or type Va lesion. A type V lesion in which the lipid core and other parts of the lesion are calcified may be referred to as type Vb. A type V lesion in which a lipid core is absent and lipid in general is minimal may be referred to as type Vc. With these lesions, arteries are variously narrowed, generally more than with type IV (*Herbert et al*, 1995).

Type VI

Type IV or V lesions with one or more of these additional features are classified as type VI and may also be referred to as complicated lesions. The superimposed features may subdivide type VI:

- ***Surface Defects and Haematoma***

  Disruptions of the lesion surface include fissures and ulcerations, but their extent and severity may differ greatly (*Arbustini et al*, 1999). The smallest ulcerations consist of focal loss of a part of the endothelial cell layer and are visible only under the microscope. Deep ulcerations may expose and release lipid from a lipid core. Fissures or tears of the lesion surface are of variable depth and length.

- ***Thrombosis***

  Capillary haemorrhages within lesions could conceivably cause sufficient disruption to precipitate thrombosis. (*Paterson JC*, 1936; *Barger AC et al,* 1984)

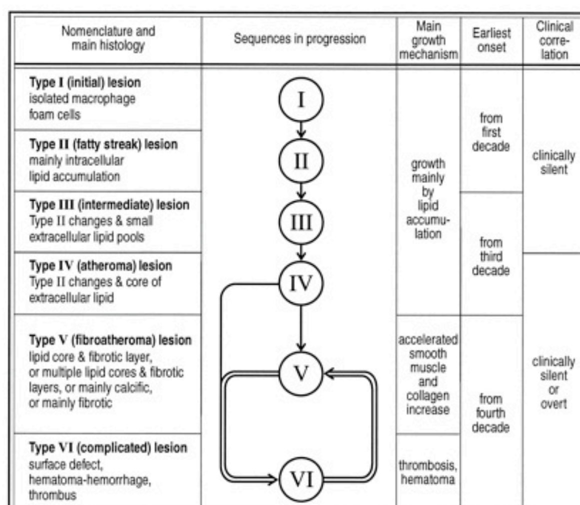


Fig.4. Type lesion

1.2.3 Role of vascular smooth muscle cells in atherosclerotic plaque

The initiation of atherosclerosis results from complex interactions of circulating factors and various cell types in the vessel wall, including endothelial cells, lymphocytes, monocytes, and smooth muscle cells (SMCs). Human autopsy studies, in vitro mechanistic studies, and in vivo correlative data suggest an important role for SMCs in the initiation of atherosclerosis (Orlandi et al, 2006). SMCs are the major producers of extracellular matrix within the vessel wall and in response to atherogenic stimuli can modify the type of matrix proteins produced. In turn, the type of matrix present can affect the lipid content of the developing plaque and the proliferative index of the cells that are adherent to it(Bijianet al 2005).

SMCs are also capable of functions typically attributed to other cell types. Like macrophages, SMCs can express a variety of receptors for lipid uptake and can form foam-like cells, thereby participating in the early accumulation of plaque lipid (Doran et al, 2008). Like endothelial cells, SMCs can also express a variety of adhesion molecules such as vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 to which monocytes and lymphocytes can adhere and migrate into the vessel wall (Doran et al, 2008).

If the hypercholesterolemia (or other inciting event) persist, smooth muscle cells proliferation and extracellular matrix deposition in the intima continue and are the major processes that convert a fatty streak into a mature fibrofatty atheroma, accounting for the progressive growth of atherosclerotic lesion (Rosenfeld ME, 2000). Arterial smooth muscle cells synthesize collagen elastin and glycoproteins. Several growth factors have been implicated in the proliferation of SMCs: PDGF, which is release by platelets adherent to the focus of endothelial injury, macrophage, endothelial cells,

smooth muscle cells; FGF; and TGF-a. SMCs proliferation is modulated by inhibitors, including heparin-like molecules present in endothelial cells and smooth muscle cells and TGF-b derived from endothelial cells or macrophages (Robbins- Pathologic basis of disease, 1999).

1.2.3.1 SMC distinct phenotype

Normally, adult medial SMCs (termed "contractile") are arranged in concentric layers and are filled with myofilaments and dense bodies but contain a relatively poorly developed Golgi apparatus and rough endoplasmatic reticulum. In contrast, SMCs characteristic of the intimal lesion of atheroma atherosclerosis (termed "synthetic") have lost this appearance and are characterised by an abundance of rough endoplasmatic reticulum and sometime no evident myofilaments (*Campbell GR, Campbell JH,* 1990). During atherosclerosis progression SMCs switching from a contractile to a synthetic phenotype, the concept is that a predisposed SMC subpopulation is responsible for the production of intimal thickening (IT). This possibility has been raised on the basis of original work by Beneditt and Beneditt (*Beneditt EP, Beneditt JM*, 1973) who reported that human atheromatouse plaque have been featured of a monoclonal lesion, recently it has been demonstrate that human plaques are at least oligoclonal (Swarts Sm, Murry CE, 1998). The concept of SMC heterogeneity has been established by the description of contractile and synthetic phenotypes in vivo and in vitro (Thyberg J et al1995).

The contractile phenotype is typical of the differentiated artery, and the synthetic one is typical of developing and pathologic arteries. A further step was the characterization in vitro of morphologically distinct SMC populations, which has been observed in many species, including humans (Thomas WA, 1983).

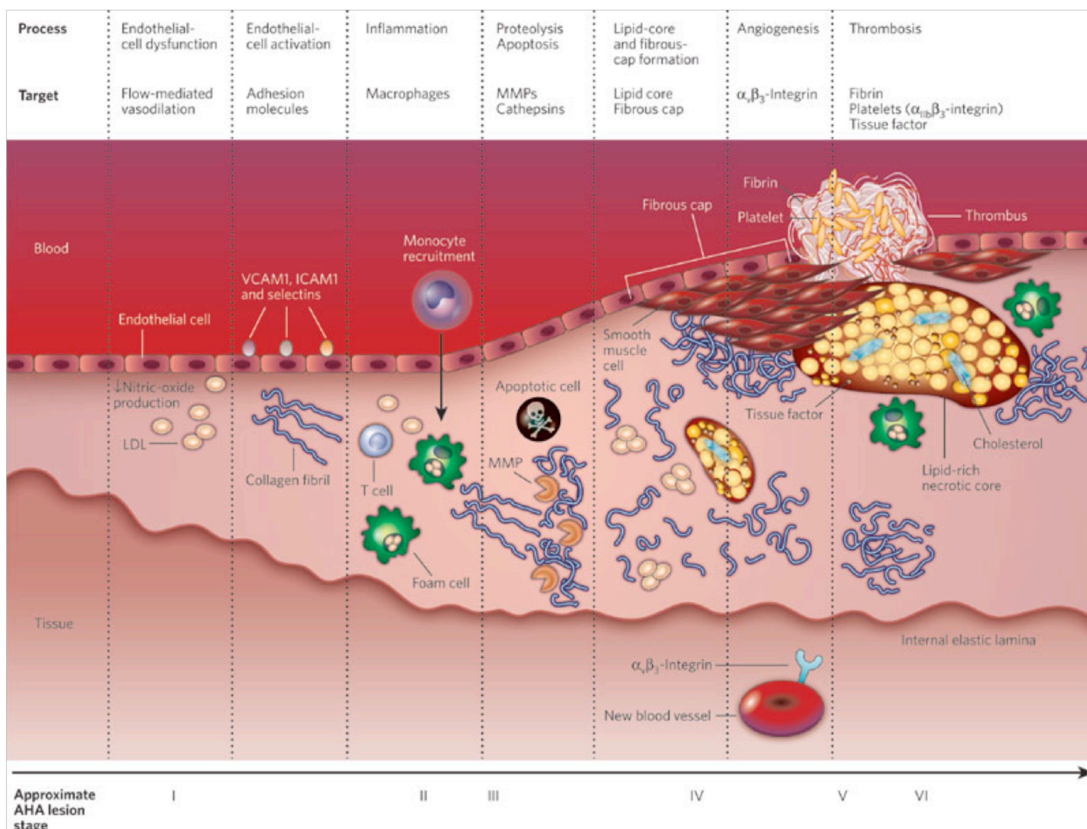
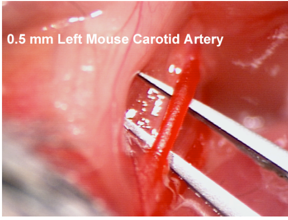


Fig.5 Cellular components involved in atherosclerotic progression

1.3 Animal model of atherosclerosis



Several animals have been used for the study of atherosclerosis, such as the non-human primates, swine, mice, guinea pigs and hamsters. However there is no one perfect animal model that completely replicates the stages of human atherosclerosis, cholesterol feeding and mechanical endothelial injury are two common features shared by most models of atherosclerosis (*Dhanya SP, Hema CG*, 2008).

It is important to note that not all animal models for atherosclerosis develop intimal thickening as a precursor to lesion formation. While intimal thickening is observed in primate and chicken models, it is not found any of the commonly used rodent models (*Stary et al*, 1992). Although several works have attempted to study atherosclerosis in rodent models by injuring vessels and disrupting internal elastic lamina to induced SMC proliferation, the plaque that results from this approach differ significantly from spontaneous human atherosclerosis (*Doran et al*, 2008).

However, until now, the most-studied species has been the rat. The initial description of SMC heterogeneity was made in the rat carotid artery injury model, wherein 2 SMC populations were identified: a spindle-shaped phenotype, with the classic "hill-and-valley" growth pattern, obtained from the normal media, and an epithelioid phenotype, in which cells grow as a monolayer and exhibit a cobblestone morphology at confluence, isolated from the IT 15 days after endothelial injury (*Orlandi A et al,* 1994). The findings of 2 SMC distinct phenotype have been confirmed by many laboratories (*Bochaton-Piallat ML et al,* 1996; *Yan ZQ, Hansson GK*, 1998).

Irrespective of the species studied, epithelioid and rhomboid phenotypes, when compared with spindle-shaped SMCs, have in common several features, among which the most relevant are (1) enhanced proliferative activity, including serum-independent growth in some species; (2) enhanced migratory activity; (3) increased proteolytic activity; (4) poor level of differentiation, as defined by citoscheletal and contractile protein expression and (5) high sensitivity to apoptotic stimuli. In all species studied, epithelioid and rhomboid SMCs show a higher proliferative activity than do spindle-shaped SMCs; however, contrary to spindle-shaped SMCs, they stop growing at confluence as a result of cell contact inhibition (Hao et al, 2003).

1.4 Citoscheletal proteins

1.4.1 Alpha-Smooth muscle actin

Alpha-Smooth Muscle Actin (a-SMA), an isoform typical of smooth muscle cells (SMC) and present in high amounts in vascular SMC.

Despite the structural similarities between different actin isoform, there is evidence for functional diversity. Alpha-Smooth Muscle Actin also appears during vascular development but, during maturation, becomes the single most abundant protein in adult vascular smooth muscle cells (Gabbiani et al 1984). Likewise, a-SMA is the major isoform of vascular tissue, such as the aorta, while γ-smooth muscle actin predominates in the gastrointestinal and genital tracts (J. Vandekerckhove, K. Weber; 1979).

Stress fibers, which have been studied mainly in cultured cells, are considered to be contractile organelles and are associated with the generation of isometric tension (Wang et al 2006).

In smooth muscle cells, SMA comprises a very large proportion of total cell protein, while the content of SMA in non-muscle cells such as myofibroblasts is much lower (P.D. Arora and C.A. McCulloch. 1994).

1.4.2 Desmin

Desmin is a type III intermediate filament found near the Z line in sarcomeres. It was first purified in 1977, the gene was characterized in 1989, and the first knock-out mouse was created in 1996(Costa M, et al 2004). Desmin is only expressed in vertebrates, however homologous proteins are found in many

organisms. It is a 52kD protein that is a subunit of intermediate filaments in skeletal muscle tissue, smooth muscle tissue, and cardiac muscle tissue.

1.4.3 Vimentin

Vimentin is a member of the intermediate filament family of proteins. Intermediate filaments are an important structural feature of eukaryotic cells. They, along with microtubules and actin microfilaments, make up the cytoskeleton. Although most intermediate filaments are stable structures, in fibroblasts, Vimentin exists as a dynamic structure (*Tang DD,* 2008).

1.4.4 Smooth muscle myosyn heavy chain

Myosin II is a major contractile protein of skeletal, cardiac and smooth muscles. It is composed of two heavy chains (approximately 200 kDa) and two pairs of light chains (approx. 20 kDa). The myosin heavy chains form a dimer consisting of two globular amino-terminal heads and carboxy-terminal c (-helical coiled coil tails). The heads bind the light chains and contain the ATP- and actin-binding sites whereas the tails are involved in the assembly of myosin molecules into filaments. Four smooth muscle specifc myosin heavy chain isoforms are known (*Quevillon-Chéruelet al,* 1999). Two isoforms (named SMB and SMA) are defined by the presence or the absence of an insert of seven amino acids in the N-terminal globular head region.

microtubules

actin filaments

intermediate filaments

Fig.6. Microtubules and filaments of cytoskeleton

The study of citoscheletal proteins, which are accepted as reliable differentiation markers, has allowed characterization of the contractile versus the synthetic phenotype. It should be noted that when placed in culture, all SMCs tend to show a dedifferentiated phenotype. With this limitation, the phenotypic variation of cultured SMCs furnishes important information concerning the influence of many factors on their biologic features. α-SMA is expressed in vascular SMCs, even at early stages of development, and thus represents the most general marker of SMC lineage. Although α-SMA is permanently expressed in SMCs, it is more abundant in spindle-shaped SMCs than in epithelioid or rhomboid SMCs. Desmin, an intermediate filament protein, and SMMHC are expressed in well-differentiated SMCs are relatively well studied. In cultured rat SMCs, Desmin

generally disappears. In larger animals, Desmin is maintained at a significant level of expression in spindle-shaped SMCs, whereas it is hardly detectable in epithelioid and rhomboid SMCs. SMMHC are clearly expressed more importantly in spindle-shaped SMCs than in epithelioid and rhomboid SMCs *(Schwartz et al,* 1995; *Shanahan CM et al*, 1998; *Owens GK,* 1998; *Sartore S et al,* 1999).In general, SMCs isolated from larger animals, including humans, are more differentiated than are those isolated from rodents. Porcine spindle-shaped SMCs maintain appreciable expression of α-SMA, SMMHC, Desmin, and Smoothelin. SMMHC, calponin, h-caldesmon, and metavinculin are abundantly expressed in bovine spindle-shaped SMCs (F*rid MG et al*, 1997; *Hao H et al,* 2002).

Taken together, the data obtained in different species suggest that the degree of differentiation of SMCs changes with the phenotype; this integrates well into a view that reconciles the heterogeneity of SMCs with the modulation concepts. Once distinct populations have been defined, the ultimate aim is to identify genes and/or proteins that are differentially expressed and to test whether they are involved in the phenotypic changes that occur in vivo. Studies on the possibility that an SMC phenotype can evolve into another have shown that the results depend on the species used. The specific phenotype of SMC observed in vitro is maintained when they are placed back into an in vivo environment. The evolution of the lesion in vivo appears to depend on the relative replicative activity and/or susceptibility to apoptosis of these phenotypes. Moreover, in the rat, an agent that influences the biologic behaviour of epithelioid SMCs, i.e., retinoic acid, inhibits IT formation. In the pig, the situation is different, in that spindle-shaped SMCs can modulate into rhomboid SMCs and, if the stimulus ceases, can return to their original phenotype, at least in vitro (*Hao et al,* 2003).

1.5 Carotid endarterectomy (CEA)

Carotid endarterectomy (CEA) is an operation to clean out an artery and restore normal blood flow through the artery. An endarterectomy is basically a "Rotorooter" procedure. It removes diseased material from the inside of an artery, and also removes any occluding atheromatouse deposits, the aim being to leave a smooth lining within the vessel, so the blood can flow normally. In endarterectomy, the surgeon opens the artery and removes the plaque. The aim of CEA is to prevent the adverse sequelae of carotid artery stenosis secondary to atherosclerotic disease, i.e. stroke. As with any prophylactic operation, careful evaluation of the relative benefits and risks of the procedure is required on an individual patient basis. Peri-operative combined mortality and major stroke risk is 2 – 5%.

Carotid stenosis is diagnosed with ultrasound Doppler studies of the neck arteries or magnetic resonance arteriography (MRA). Symptoms have to affect the other side of the body; if they do not, they may not be caused by the stenosis, in which case endarterectomy will be of minimal benefit (Adams HP Jr. 2009)

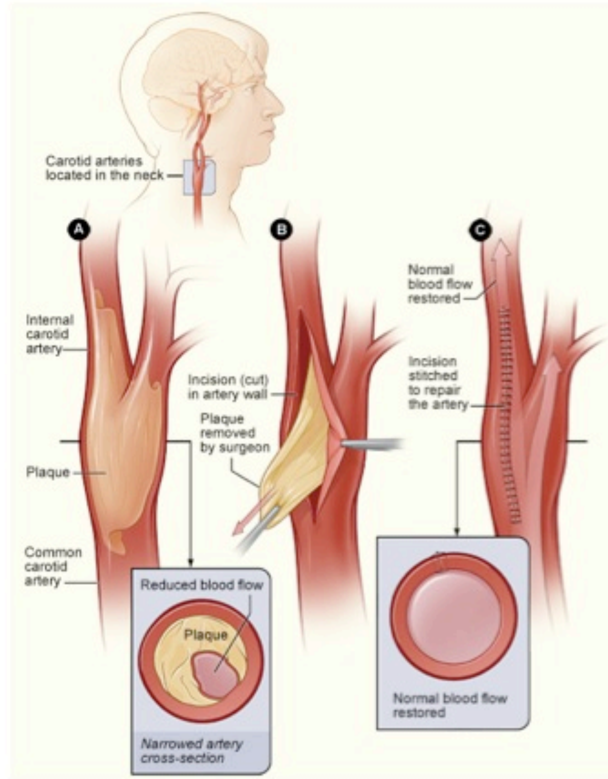


Fig.7. Carotid endarterectomy

2. Aim of the study

The last years were characterized by several studies proceeding to understand SMC heterogeneity. Animal models of rat or rabbit IT after endothelial lesion are presently the most studied models for atherosclerotic plaque formation and have been useful in order to understand several biological features of SMCs. The disadvantages of animal models are that there is no possibility to reproduce the same history of pathogenesis in animals as that in man. There is no perfect animal model that completely replicates all stages of human atherosclerosis.

However, identification of particular SMCs subpopulation in human arteries represents a difficult challenge for understandable reason, such us material availability and experiment standardization. The relevance of SMC heterogenity to human disease still remains to be demonstrated.

Thanks to collaboration between our laboratory and the Vascular Surgery's group of Sant'Anna Hospital (University of Ferrara) leading by Francesco Masculine, we have had the possibility to obtain samples of atherosclerotic and no-atherosclerotic human carotid artery from patients undergone to carotid endarterectomy.

Aim of this study is to obtain primary culture of human vascular smooth muscle cells (SMCs) from both atherosclerotic and no-atherosclerotic carotid artery, to investigate human SMC heterogeneity.

The possibility to isolate o SMC subpopulations, from a single human artery, could be useful to verify the hypothesis that distinct SMC subtypes are involved in atherosclerosis pathogenesis and progression.

3.Materials and Methods

3.1 Tissue explantation

Specimens collect during endarterectomy were conserved in cold (4°C) sterile RPMI plus 2% Hepes, containing 100 U/ml of penicillin and 100 µg/ml of streptomycin and 400mM l-glutamine (All reagent from Gibco, Invitrogen). In about 1 hour from surgery, specimens were taken to the laboratory. The specimens were washed several times with fresh RPMI to remove blood.

The abluminal surface of tissue were gently scraped to remove any connettive tissue, whereas the luminal surface undergone the same treatment to remove endothelial cells.

For tissue explantation, CEA specimens were cut into 3x3x3-mm pieces. The abluminal side of the explants was carefully placed in contact with the culture dish (15 to 20 tissue pieces per 60-mm dish). After 40 minutes, during which specimens dried, RPMI (10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 400mM l-glutamine) was added to petri dish. The times requested for emergence of the first cells from the explants and growth to confluence was recorded. Explanted tissue pieces were removed after 10 days.

Population isolated by tissue explantation were studied between second and sixth passage.

3.2 Cells Culture

Once cells outgrowth from explanted tissue rinse confluences, were passed by a 1:2 spit ration and cultured in RPMI plus 10% FBS, containing 100 U/ml of penicillin and 100 µg/ml of streptomycin and 400mM l-glutamine (All reagent from Gibco, Invitrogen), and cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% of CO_2.

In all experiment cells were used from 2nd to 6th passage.

3.3 Co-culture experiments

Co-culture of Plaque and Media was performed by using the Transwell system (24-mm-diameter Transwell clear polyester membrane, 0.4-μm pore size, Costar). Media specimens were plated into the upper compartment in RPMI plus 10% FBS, plaque were plated in the lower compartment (position of media and plaque specimens was inverted for control). Specimens were removed as above, and cells were cultured as above-mentioned.

3.4 Immunofluorescence

Cells from 3rd to 6th passage were growth on glass cover slips. Cells were fixed using a solution of RPMI 2% Hepes (Gibco, Invitrogen corporation) and 1% paraformaldeide (Sigma Aldrich) for 20 Minutes at room temperature. Cells were, then, washed with Phosphate Buffered Saline (PBS), and methanol fixation (for citoscheletal components) for 8 minutes. Then Incubation with primary Antibody was performed for 1 hour. Antibodies used were:

Anti-SMA, IgG_{2a} mouse monoclonal antibody, dilution 1:50, generously provided by Gabbiani-Bochaton-piallat group

Anti-Vimentin, IgG_{1a} mouse monoclonal antibody. dilution 1:200(Dako Inc.)

Anti-desmi, IgG_{1a} mouse monoclonal antibody. dilution 1:10(Dako Inc.)

Anti-SMMHC, IgG_{H+L} (data produced in Gabbiani-Bochaton-Piallat Laboratory, department of pathology University de Geneva)

Anti-S100A4, IgM mouse monoclonal antibody dilution 1:10 (generously provided by Gabbiani-Bochaton-Piallat group)

Anti-TFPI, rabbit polyclonal antibody, dilution 1:10 (Santa Cruz Biotechnology, Inc)

If it is desirable to examine the co-distribution of two different antigens in the same cell, a double immunofluorescence procedure may be used. Cells may be incubated simultaneously with two primary antibodies, provided they are monospecific and can be distinguished with secondary antibodies conjugated to different fluorochromes.

After incubation with primary antibody cells were washed 3 times with PBS and than incubate with DAPI (4', 6-diamidino-2-phenylindole) and secondary antibody conjugate with FITCH or Rodhamin:

Goat anti-mouse IgM rhodamine (TRITC) conjugate dilution 1:50 (SouthernBiotech)

Goat anti-mouse IgG_{2a} fluorescein (FITC) conjugate dilution 1:50(SouthernBiotech)

Goat anti-mouse IgG_1 fluorescein (FITC) conjugate dilution 1:50(SouthernBiotech)

Mouse anti-rabbit IgG rhodamine (TRITC) conjugate dilution 1:50 (Dako)

After 3 wash with PBS, coverslips with cells are mounted in PVA (polyvinyl alcohol) and observe at microscopy.

Images of immunofluorescence staining were acquired and overlaid by using Adobe Photoshop.

3.5 Semiquantitative RT-PCR

From 5x 10^6cells, at 3rd passage, RNA was extract by using *Illustra RNAspin Isolation Kit* (GE Healthcare) was used. Total RNA (1 g) was used for each 20 l reverse transcription reaction (0,05mM oligo dT, 0,5mM dNTPs, 50mM Tris HCl pH 8.3, 75mM KCl, 3mM $MgCl_2$, 100mM DTT) with 200 units of Superscript III reverse transcriptase (Invitrogen).

For PCR reaction 1 l of cDNA obtained from reverse transcription reaction was used. Each vials contain: 25l of a solution 10mM Tris HCl pH 9, 50mM KCl, 3mM $MgCl_2$, 30nM dNTPs, 200ng of each Reverse and Forward Primers. Semiquantitative PCR was performed by using *Taq DNA Polymerase* (Fisher Scientific), with 25 cycles each consisting of 1 minutes at 95°C, 30s at Annealing temperature (depends on the couple of primer used) and 1 minutes at 72°C. PCR products were visualised on 2% agarose gel.

Tab. Primers

Primers Name	Gene Name	Sequence
SMA-F	alpha-smooth muscle actin	5' CCGGGAGAAAATGACTCAAA 3'
SMA-R		5' AAGTCCAGAGCTACATAACA 3'
DES-F	Desmin	5' AAGTCGAAGGTGTCAGACCT 3'
DES-R		5'GGGCTGGTTTCTCGGAAGTT 3'
SMMHC-F	Smooth muscle myosin heavy chain	5' TTCAACAACTACACCTTCCT 3'
SMMHC-R		5' CCAAAGCCTCTACAGCAAAG 3'
VIM-F	Vimentin	5' GACCTCTACGAGGAGGAGAT 3'
VIM-R		5' GTGCCAGAGACGCATTGTCA 3'
S100A4F	S100A4	5' GCATGCTGAAGCTGGTGAC 3'
S100A4R		5' GCGATGCAGGACAGGAAGAC 3'
IL-6F	IL-6	5'TGAGGAGACTTGCCTGGTGAA 3'
IL-6R		5 'GCGCAGAATGAGATGAGTTGTC 3'
SAA2F	SAA2	5' TGAGAAATACTGGCTTCCT 3'
SAA2R		5' CCACCTCTTAAGCATTTATT 3'

3.6 Microarray assay

RNA from 3 couple of cultured cells from both NAA and AA carotid artery was extract as previously described.

Whole human genome expression detection by oligo microarray.

RNAs from 6(3 NAA and AA cells population) samples were hybridized on Agilent whole human genome oligo microarray (Agilent Technologies, Palo Alto, CA). This microarray consists of 60-mer DNA probes synthesized in situ, which represent 41,000 unique human transcripts. One-colour gene expression was performed according to the manufacturer's procedure. RNA quality is assessed by the use of Agilent 2100 Bioanalyzer (Agilent Technologies). Low quality RNAs (RNA integrity number below 7) were excluded from microarray analyses. Labelled cRNA is synthesized from 500 ng of total RNA using the Low RNA Input Linear Amplification Kit (Agilent Technologies) in the presence of cyanine 3-CTP (Perkin-Elmer Life Sciences, Boston, MA). Hybridizations were performed at 65°C for 17 hours in a rotating oven. Images at 5 um resolution were generated by Agilent scanner and the Feature Extraction 9.1 software (Agilent Technologies) was used to obtain the microarray raw-data.

Microarray data analysis

Microarray results were analyzed by using the GeneSpring GX software (Agilent Technologies). Data files were pre-processed using the GeneSpring plug-in for Agilent Feature Extraction software results. Data transformation was applied to set all the negative raw values at 5.0, followed by on-chip and on-gene median normalization. A filter on low gene expression was used so that only the probes expressed (flagged as Present) in at least one sample were kept; the probes that do not change between

samples, identified as having an expression value across all samples between median ± 1.5, were removed. Then, samples were grouped in accordance to their differentiation status and compared. Differentially expressed genes were selected as having a 2-fold expression difference between their geometrical mean in two or more groups of interest and a statistically significant p-value (<0.05) by ANOVA (analysis of variance) statistic, followed by the application of the Benjamini and Hoechberg correction for false positives reduction. Differentially expressed genes were employed for Cluster Analysis of samples, using the Pearson correlation as a measure of similarity.

GO and Pathway analysis

The Gene Ontology (GO) and enriched Pathway analysis, on differentially expressed genes, was performed with the GeneSpring tools and by using the FatiGO+ algorithm.

4. Results

4.1 Carotid endarterectomy

Carotid endarterectomy involves a longitudinal arteriotomy extending to internal carotid distal to the lesion, and a dissection plane established between media and the external elastic lamina of artery. The plaque (with underlying media) is then resected and arteriotomy closed.

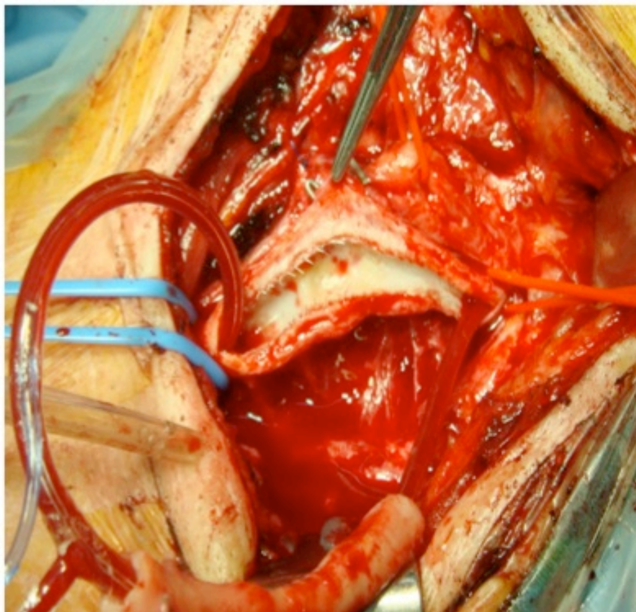


Fig.8
a)Carotid endarterectomy
b) Sample of atherosclerotic lesion obtained by carotid endarterectomy

During endarterectomy, not only atherosclerotic lesion but also a sample of no-atherosclerotic carotid artery is taken from carotid artery, which does not show typical characteristics of atheromatouse plaque.

No-Atherosclerotic Artery (NAA) is composed of a normal media underlying a thickened intima without necrotic core (AHA classification: intermediate or type III lesion) before the proximal end point of carotid endarterectomy (fig.9)

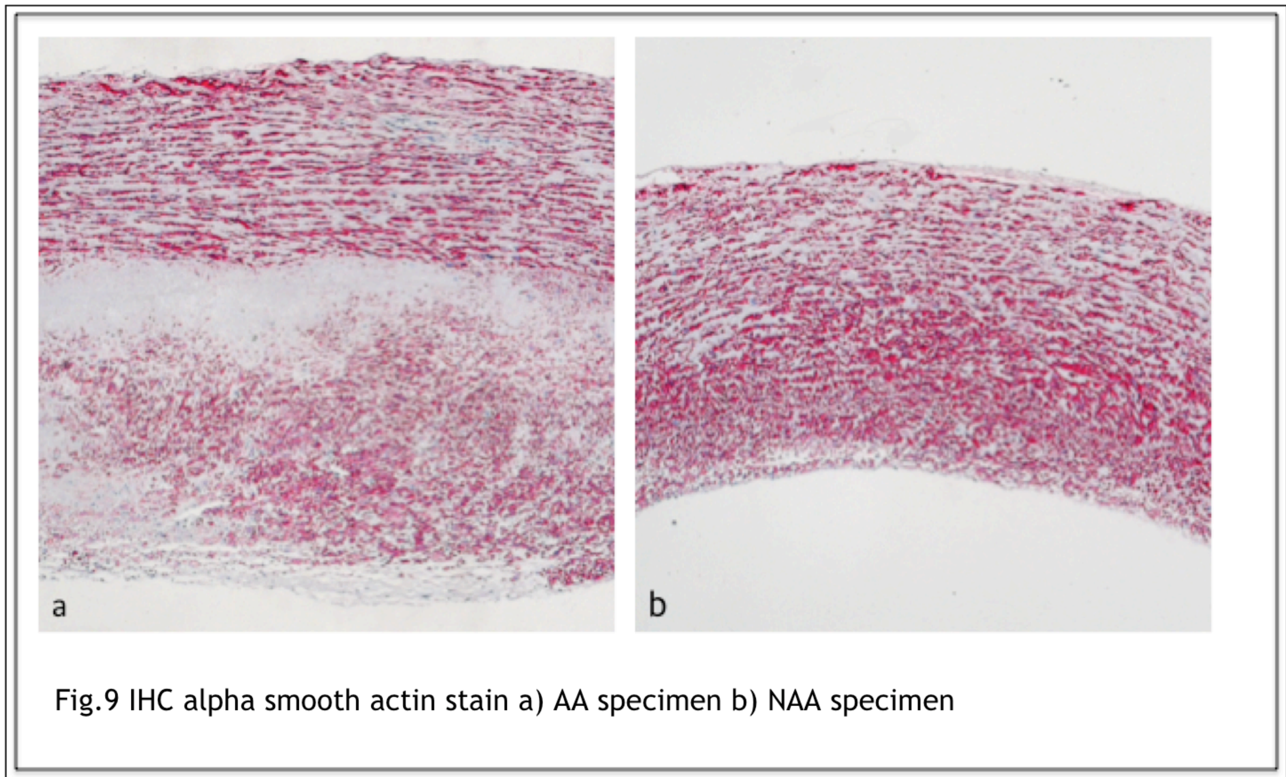


Fig.9 IHC alpha smooth actin stain a) AA specimen b) NAA specimen

Atherosclerotic Artery (AA) is composed of a media underlying an atheromatouse plaque (AHA classification: atheroma-fibroatheromata or type IV-V lesion) at the proximal end-point of carotid endarterectomy (fig.9)

4.2 Isolation of SMCs by tissue explantation

In order to obtain primary culture of SMCs from CEA specimens, of both AA and NAA, tissue explantation technique was used.

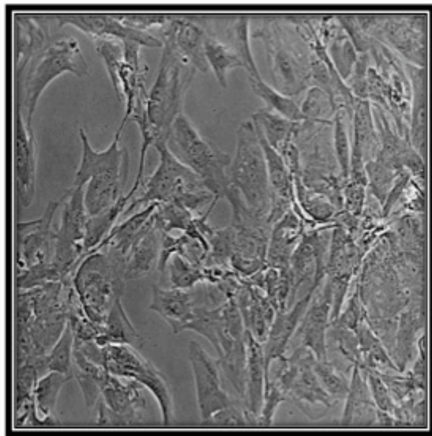
After four days, from tissue explantation, wells contain specimens were observe daily, to record cellular outgrowth.

Cells outgrew from fifth to tenth day after tissue explantation, time for outgrowth was similar for both NAA and AA tissue.

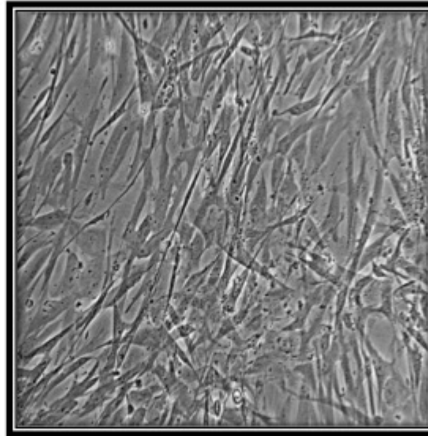
After ten days, independently of cellular outgrowth, specimens were removed from well.

Although cells from NAA and AA outgrew at similar time, confluence was rise at different day, in fact while NAA cells raised the confluence nine days after cellular outgrowth, AA cells took about seven day to raise confluence.

As shown in figure 10 differences between NAA cells and AA cells were immediately clear: In fact, while NAA cells are large and grow in a monolayer, AA cells are smaller and elongated, and grown multilayered with *hills and valley* pattern.



a



b

Fig.10
Phase contrast 20X magnification a) Cells coming from No-Atherosclerotic Artery (NAA) b) Cells coming form Atherosclerotic Artery

4.3 Identification of the proliferative compartment

We were able to obtain cells from both NAA and AA tissue and efficiency was similar as shown in tab below.

	N°Experiment	Outhgrowth ratio(%)
No-atherosclerotic artery	19	94,7
Atherosclerotic artery	19	89,5

From the observation of fig.9, that while NAA is a monolayer of media formed of SMCs, AA is featured by two compartments, media and plaque, that both contain SMCs, even if

In a different percentage. Cells from NAA could outgrow only from media, differently in

AA specimens cells might arise both from media and plaque. To understand which was

The origin layer of cells, we prepared experiments in which media and plaque were separated with a surgical blade taking advantage of the natural cleavage plane that separates them (fig.11)

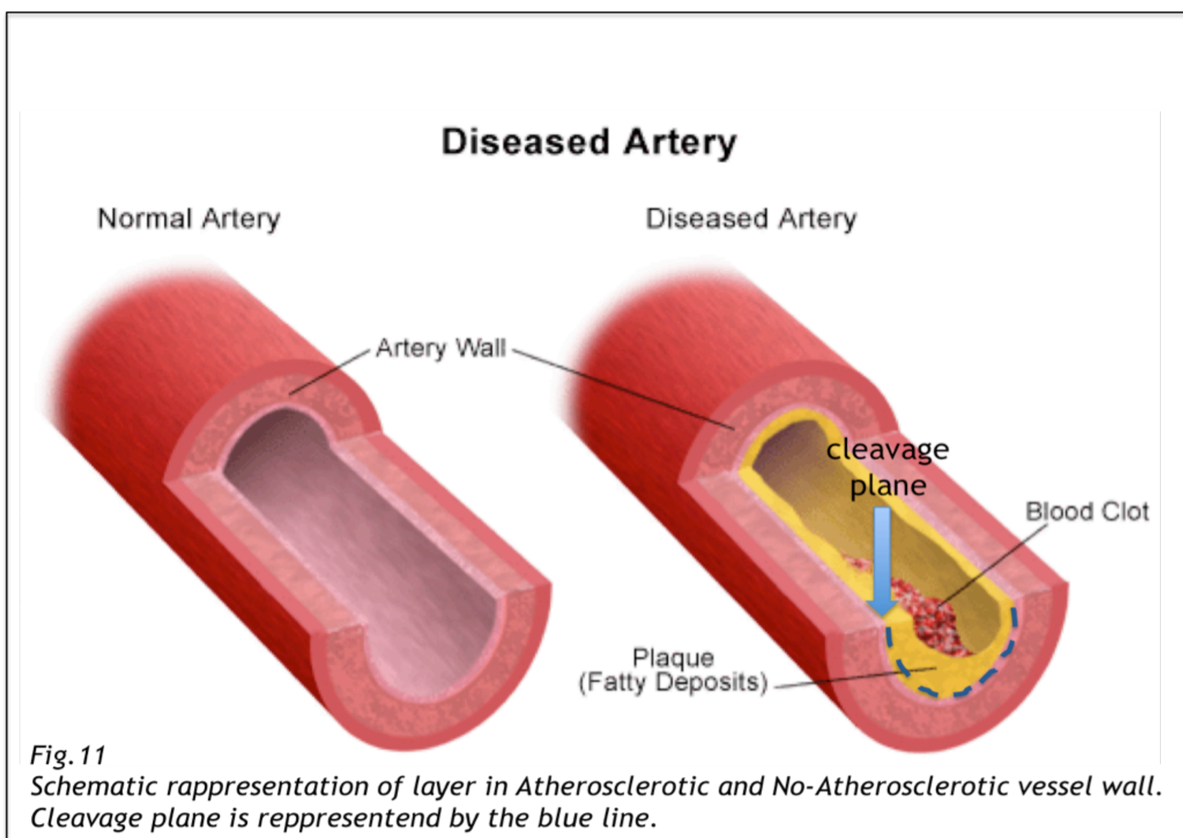


Fig.11
Schematic rappresentation of layer in Atherosclerotic and No-Atherosclerotic vessel wall. Cleavage plane is reppresentend by the blue line.

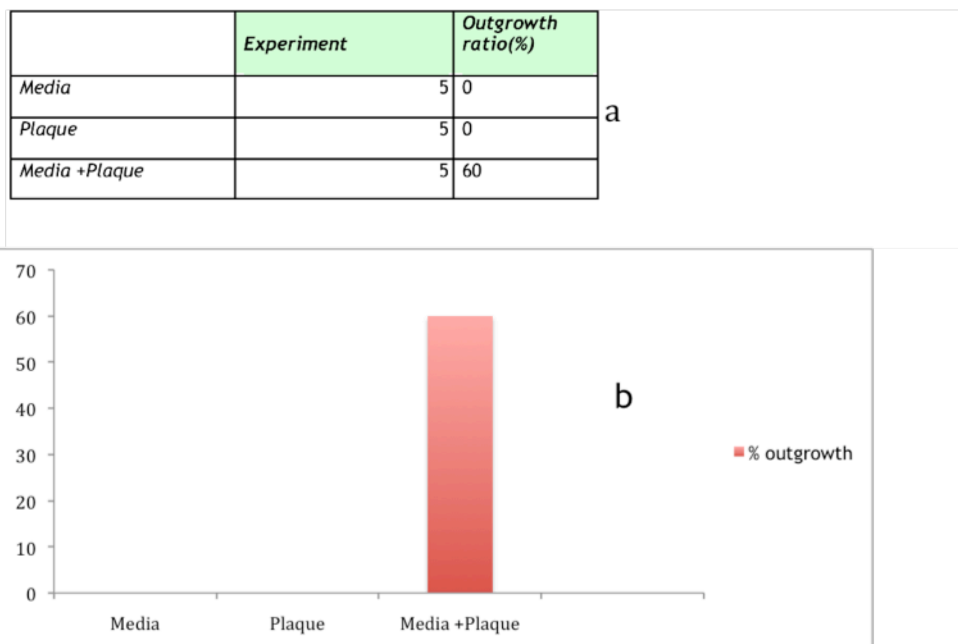
Successively, we set up experiments, in which were used: or fragments of media alone or fragments of plaque alone, or complete lesion. From samples collected from five different patients (undergone endarterectomy), were been prepared experiments in which a part of every lesion was subdivided in media and plaque. Three conditions of culture were therefore created:

1. Tissue explantation of media alone, after plaque overhanging removal

2. Tissue explantation of plaque alone, after removal of media below

3. Tissue explantation of undivided lesion, without separation of the layers

In all experiment served out, cellular proliferation was observed neither from media alone or from plaque alone, while cellular outgrowth were noticed only by complete lesion without separation of layers (fg.12).



	Experiment	*Outgrowth ratio(%)*
Media	5	0
Plaque	5	0
Media +Plaque	5	60

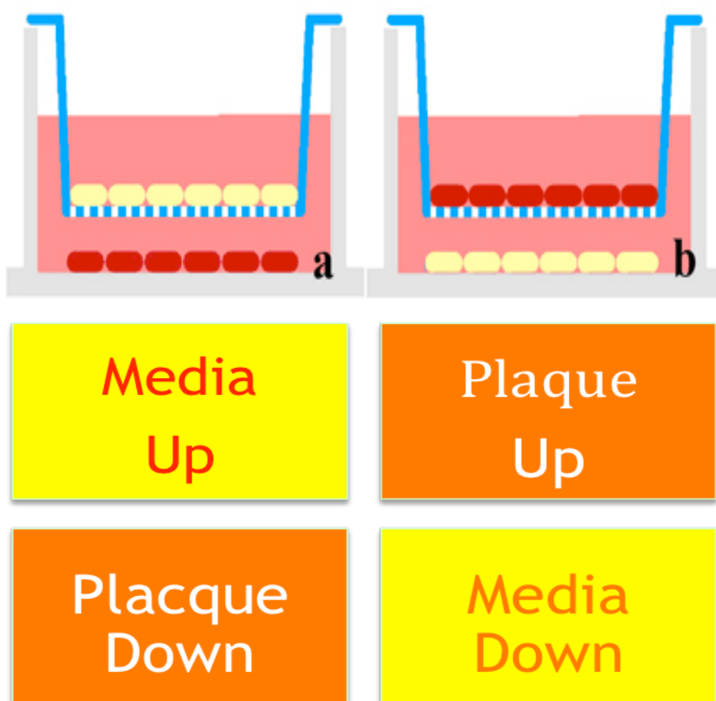
Fig.12
Results of experiments obtained by tissue explantation with separation of AA layers

Data obtained did not permit to identify proliferative layer, therefore, It was necessary to prepare experiments in which media and plaque were physically separate, but shared the same culture medium, to understand from which layer cells outgrew.

4.4 Co-culture experiment

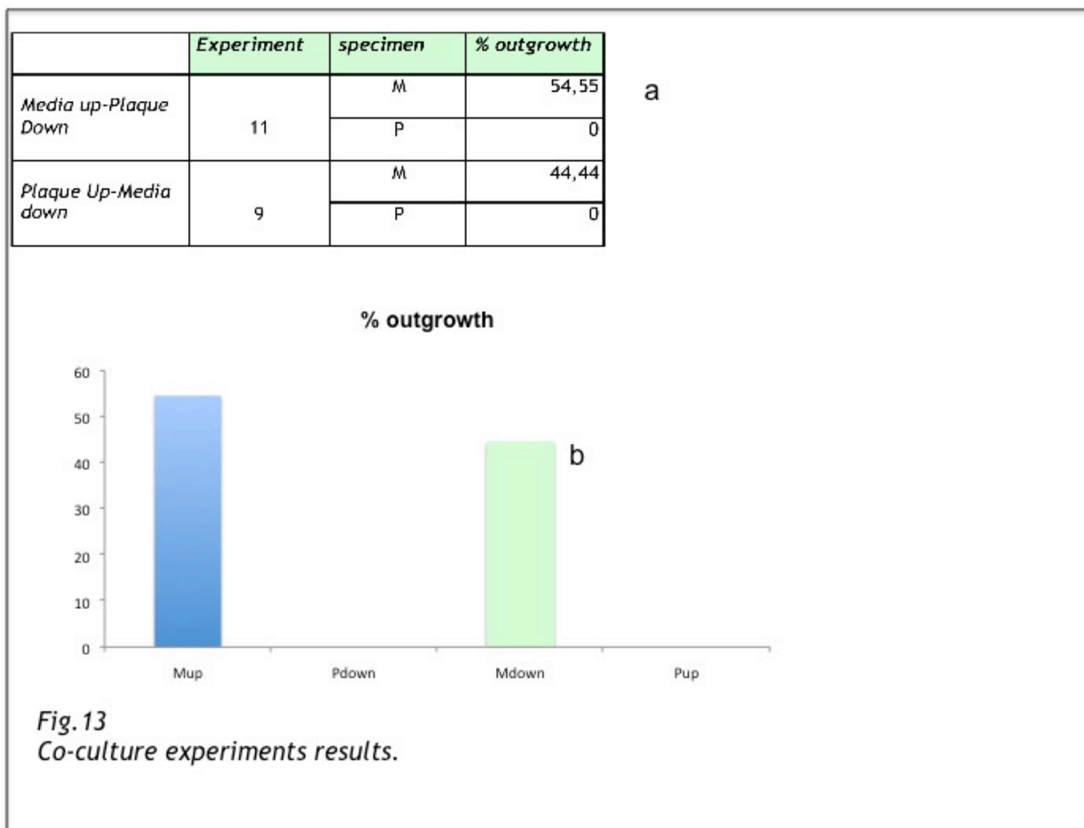
In this experiment media and plaque were placed in two different compartments, thus allows to discriminate from which layer cells outgrew, depending on the compartment in which cells were be found.

Transwell membranes (corning corporation) were used, a systems that let to prepared co-culture experiments. This polyester membrane is supported on a plastic small basket, inserted in wells (made of polystyrene). The membrane has holes of 0,4μ that does not allow the cells to cross it, but permit medium to diffuse from a compartment to other. The transwell system permit to mimic structure of the vessel wall, in which cells of media and plaque are separate by elastic lamina, but they can communicate and influence each other by secreting biological molecules, able to diffuse from a compartment to another.



In order to avoid influence of polymer type on the cellular outgrowth media specimens were placed on the transwell membrane, and plaque on the bottom of wells or vice versa. Seven CEA specimens were used in co-culture experiments.

Data obtained show that cells outgrew always only from media wherever it was situated (on transwell membrane, or on the bottom of the well). From plaque, independently of its position, we never observed cellular outgrowth (fig. 13).



	Experiment	*specimen*	*% outgrowth*
Media up-Plaque Down	11	M	54,55
		P	0
Plaque Up-Media down	9	M	44,44
		P	0

Fig. 13
Co-culture experiments results.

Cells outgrew from fifth to tenth day, once at confluence cells showed a typical grown patter of AA cells, previously observed.

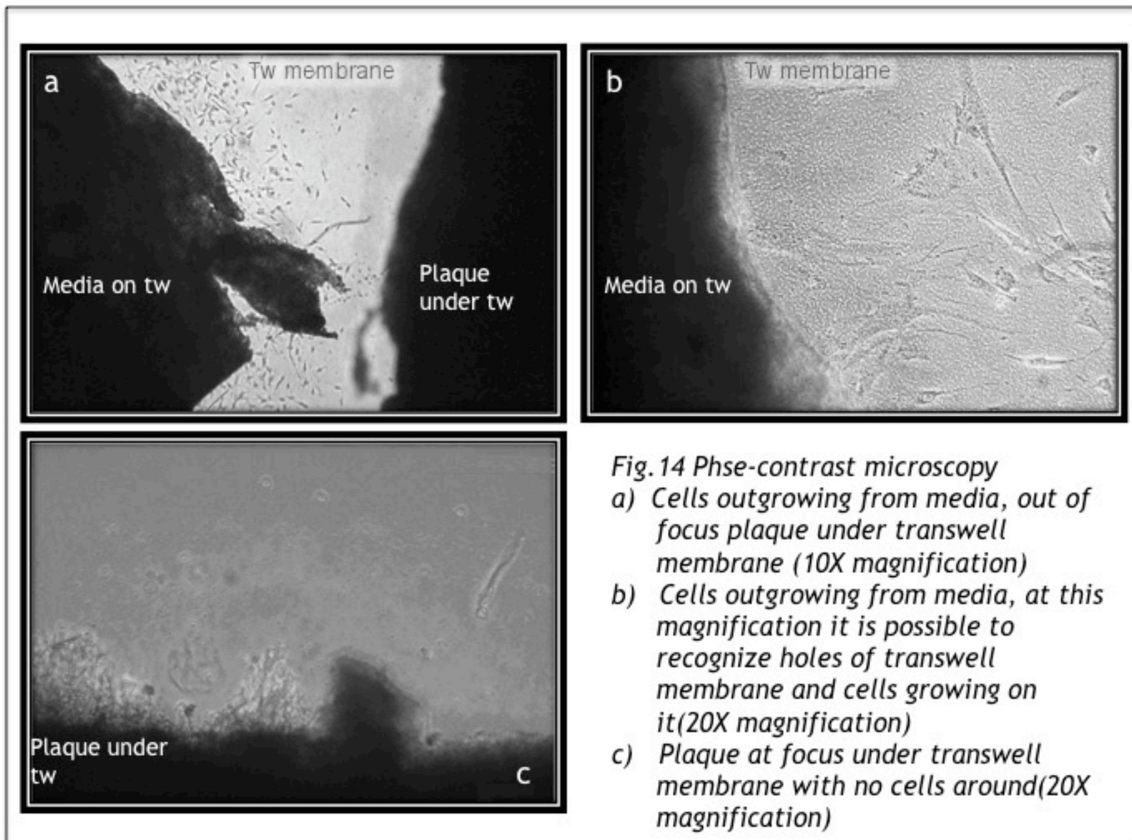


Fig.14 Phse-contrast microscopy
a) Cells outgrowing from media, out of focus plaque under transwell membrane (10X magnification)
b) Cells outgrowing from media, at this magnification it is possible to recognize holes of transwell membrane and cells growing on it(20X magnification)
c) Plaque at focus under transwell membrane with no cells around(20X magnification)

Taken together these data indicate that media is the proliferative compartment, but the presence of plaque is necessary for cellular outgrowth.

This result suggests that the plaque releases factors necessary for SMC migration.

4.5 Distinct SMCs phenotypes

Observation of cells by phase-contrast microscopy clearly showed morphological differences between NAA and AA cells (figure 10)

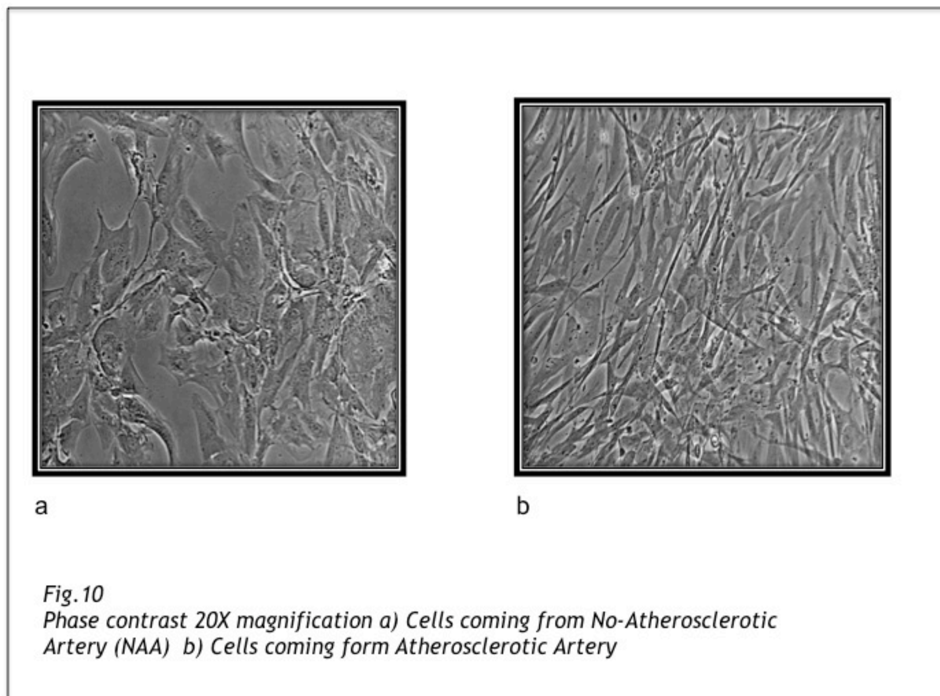


Fig.10
Phase contrast 20X magnification a) Cells coming from No-Atherosclerotic Artery (NAA) b) Cells coming form Atherosclerotic Artery

Cells from NAA are large (L-SMCs), grow as a monolayer and exhibit cobblestone morphology at confluence, while AA cells are small (S-SMCs) and grow multilayer with the classic "hill-and-valley" growth pattern.

In addition, the two distinct types of SMC exhibited differences in the proliferation. After 7 days of culture s-SMCs showed a higher proliferative activity as compared with l-SMC, which stopped growing at confluence because of cell contact inhibition (fig15)

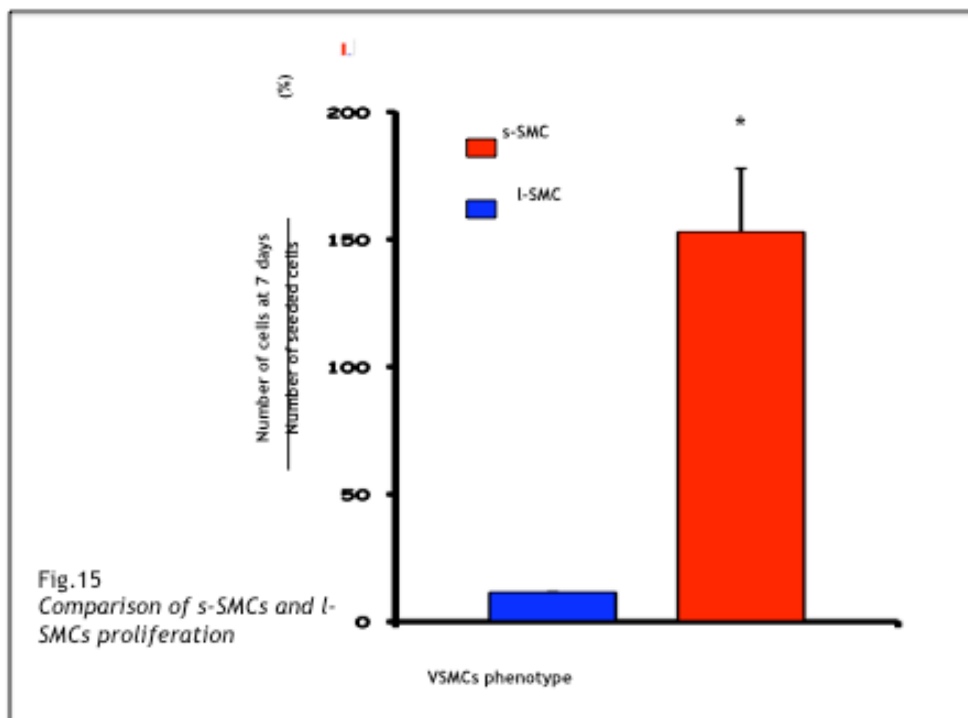


Fig.15
Comparison of s-SMCs and l-SMCs proliferation

4.5.1 Distinctive features of SMC

Immunofluorescence staining

Differences between two kind of cells were further analyzed by investigate expression of characteristics molecular marker of SMCs level. For this purpose Immunofluorescences staining was used.

Since mature SMCs express alpha smooth muscle actin (αSMA), identification of this marker in these cells is necessary to define them as smooth muscle cells.

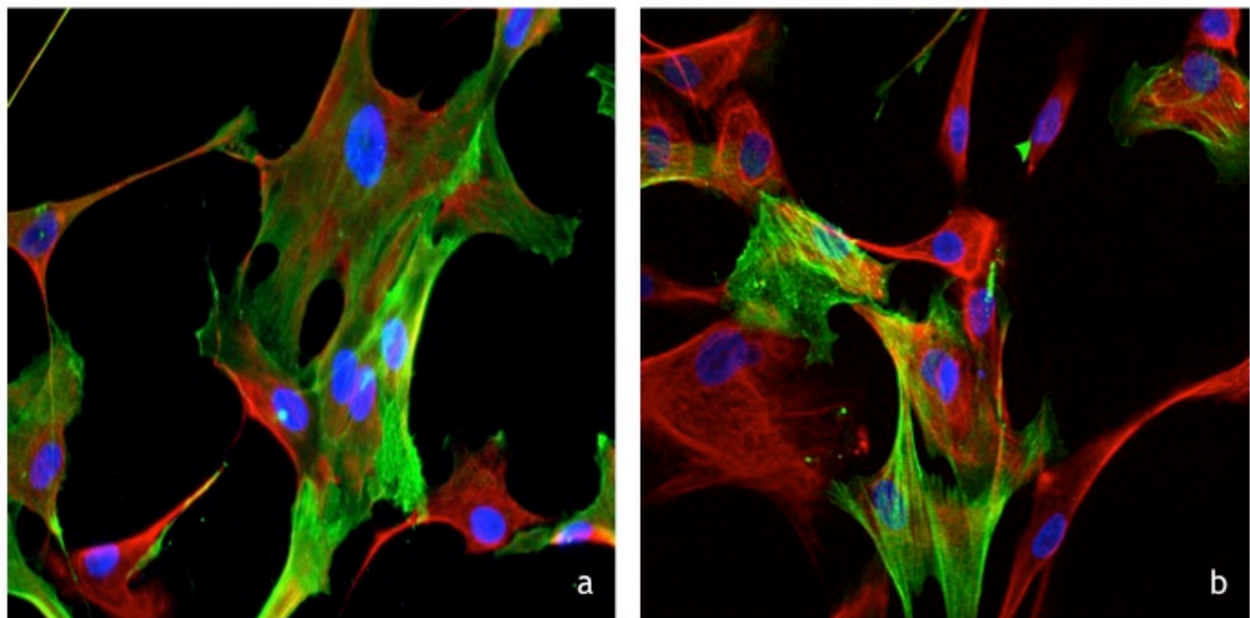


Fig.16 Immunofluorescences staining(40X magnification) anti-a-SMA(green) Vimentin (red) DAPI(blue)
a) NAA cells
b) AA cells

As shown fig 16 alpha smooth muscle actin is present in both L-SMCs and S-SMC and this data identify both populations as vascular smooth muscle cells. However, the two

populations differed, with l-SMC showing higher level of a-SMA than s-SMCs. Vimentin is a marker of mesenchimal origin cells, as SMCs should be. As observed in fig.16 the amount of Vimentin seems to be similar in both types of cells.

Desmin, a 52kD protein that is a subunit of intermediate filaments in skeletal muscle tissue and smooth muscle tissue, was not present either in l-SMCs or s-SMCs(fig.17). This

Data confirm previous findings, obtained by immunohistochemistry on human carotid tissues, showing that SMCs were Desmin negative (Gabbiani et al, 1981).

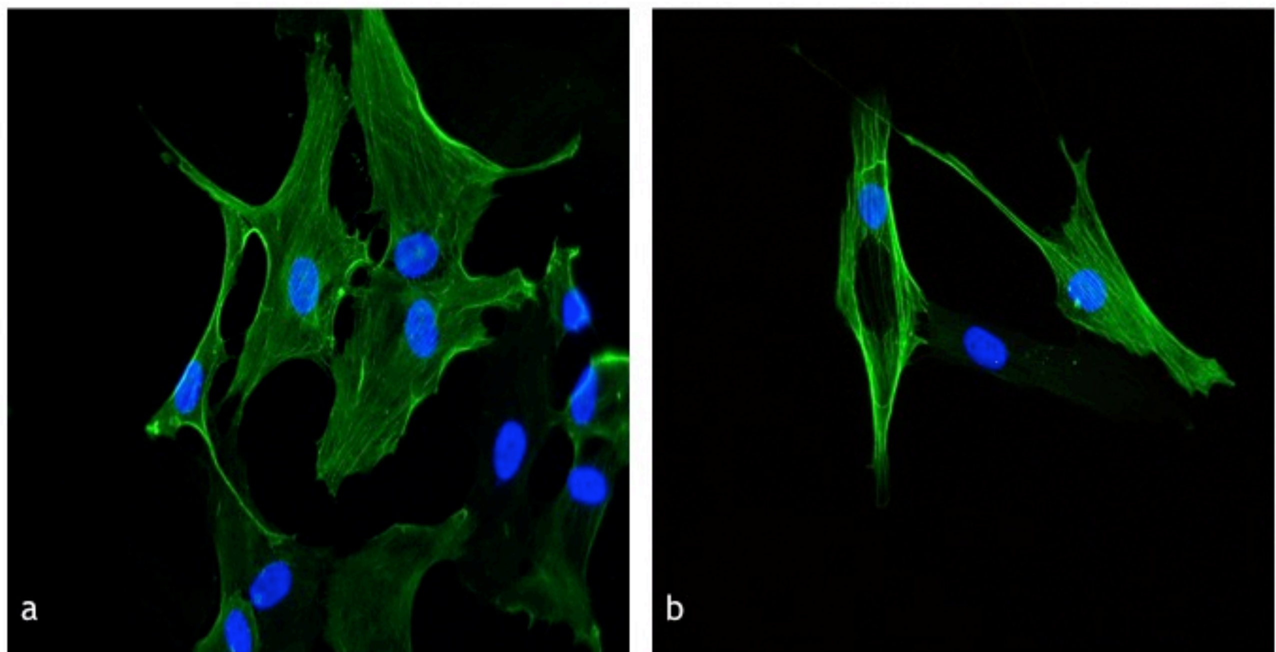


Fig.17
Immunofluorescences staining(40X magnification) with anti-a-SMA (green) Desmin (red) DAPI(blue)
a) l-SMCs
b) s-SMCs

SMMHC, a citoscheletal protein and a marker of contractile phenotype, was present in both cell types, but it was more expressed in NAA cells (fig.18).

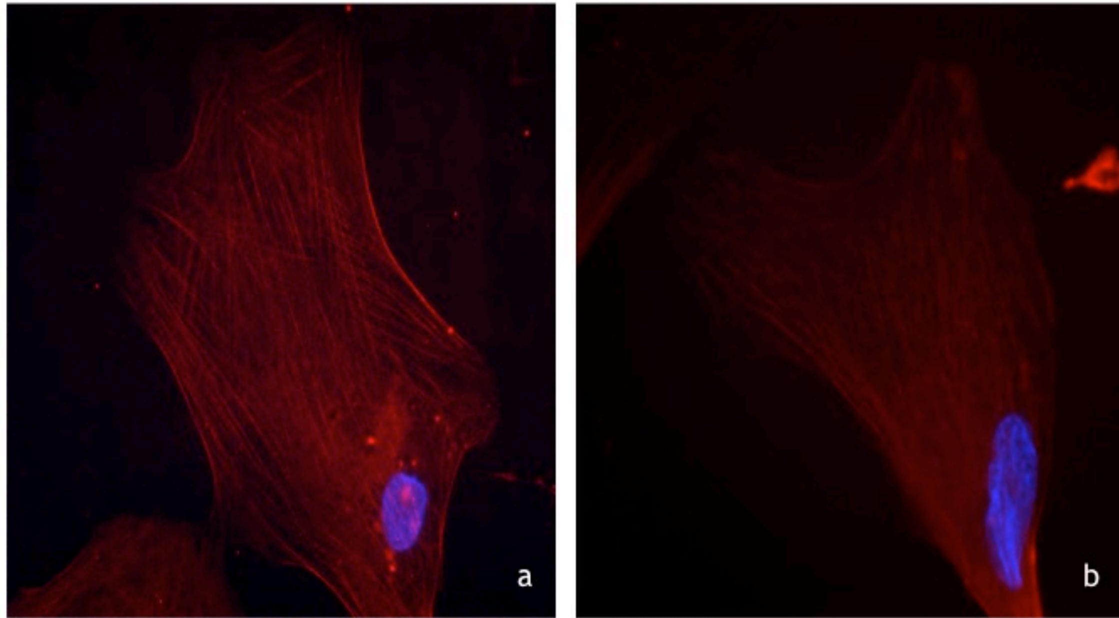


Fig.18
Immunofluorescences staining(40X magnification) with anti-SMMHC (red) DAPI(blue)
a) l-SMCs
b) s-SMCs

S100A4

The protein S100A4 belongs to a large family of low molecular weight calcium binding proteins. In human, S100A4 was barely detectable in coronary artery media and markedly expressed in SMCs of atheromatouse and restenotic coronary artery lesions. S100A4 is a marker of porcine R-SMCs in vitro and of intimal SMCs during intimal thickening development (Brisset et al 2007).

Immunofluorescence staining showed the presence of S100A4 only in s-SMCs (fig.19)

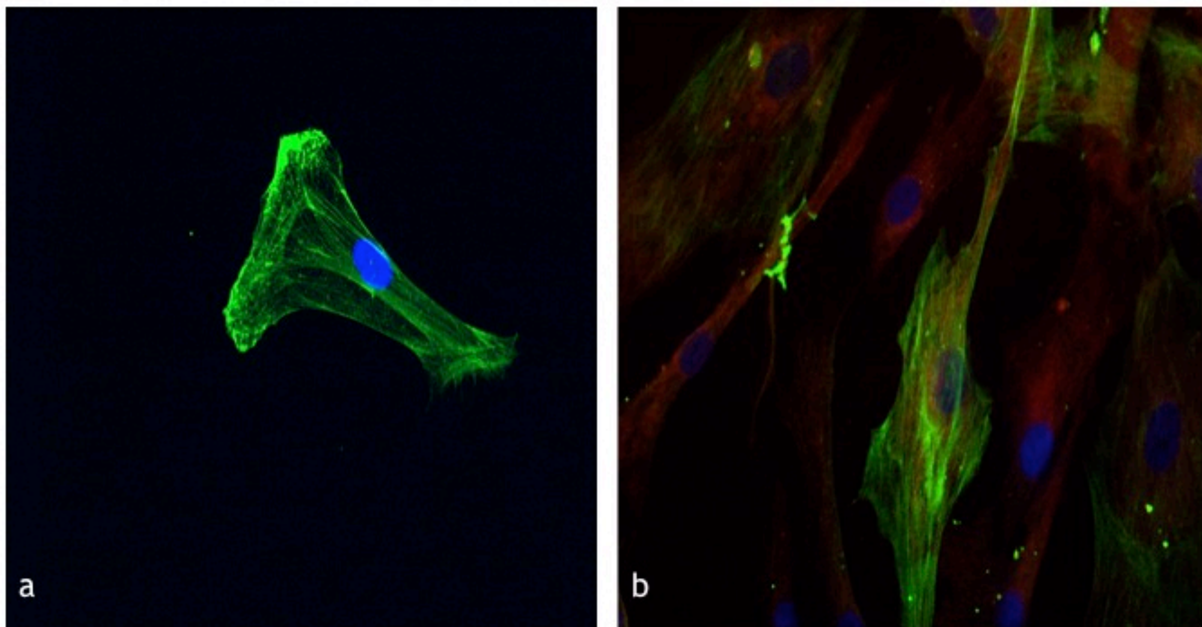


Fig.19

Immunofluorescences staining(40X magnification) anti-S100A4 (red) DAPI(blue) anti-alpha smooth muscle actin (green) a)l-SMC b) s-SMCs

RT PCR

Semiquantitative RT PCR was used to investigate expression of SMC characteristic markers and S100A4 at the RNA level.

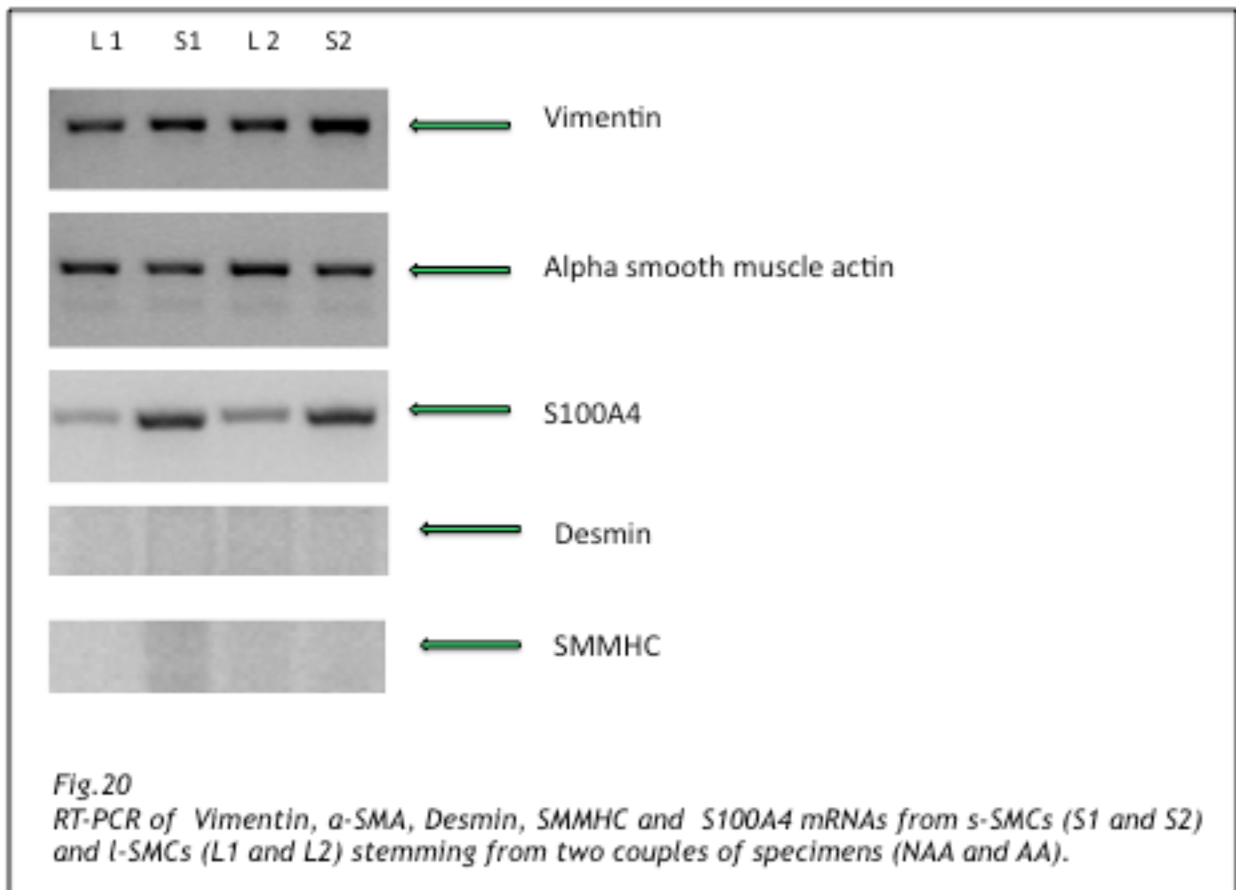


Fig.20
RT-PCR of Vimentin, a-SMA, Desmin, SMMHC and S100A4 mRNAs from s-SMCs (S1 and S2) and l-SMCs (L1 and L2) stemming from two couples of specimens (NAA and AA).

Amplification data indicated that mRNAs of actin and Vimentin were present in both kinds of cells. On the contrary, neither Desmin nor SMMHC were detected.

mRNA for S100A4 was present in both kind of cells, but it was more expressed in AA cells, as shown in fig.20

4.6 Co-colture of NAA and plaque specimens obtained from the same carotid artery

As previously described, plaque is necessary for cellular outgrowth from AA media, suggesting that plaque might release factors able to influences SMC migration.

In order to verify if plaque could influence cellular outgrowth from NAA, we performed tissue explantation in which NAA media was placed in the petri dish alone or with plaque. The two conditions of tissue explantation are schematically reported below:

The monitoring of cellular culture by phase-contrast microscopy showed that:

- NAA cells in the presence of plaque outgrew earlier than cells from NAA alone,
- NAA cells, growth in the two conditions, exhibited different morphological features (fig 21)
- Cells from NAA+ Plaque take less time to raise confluence.

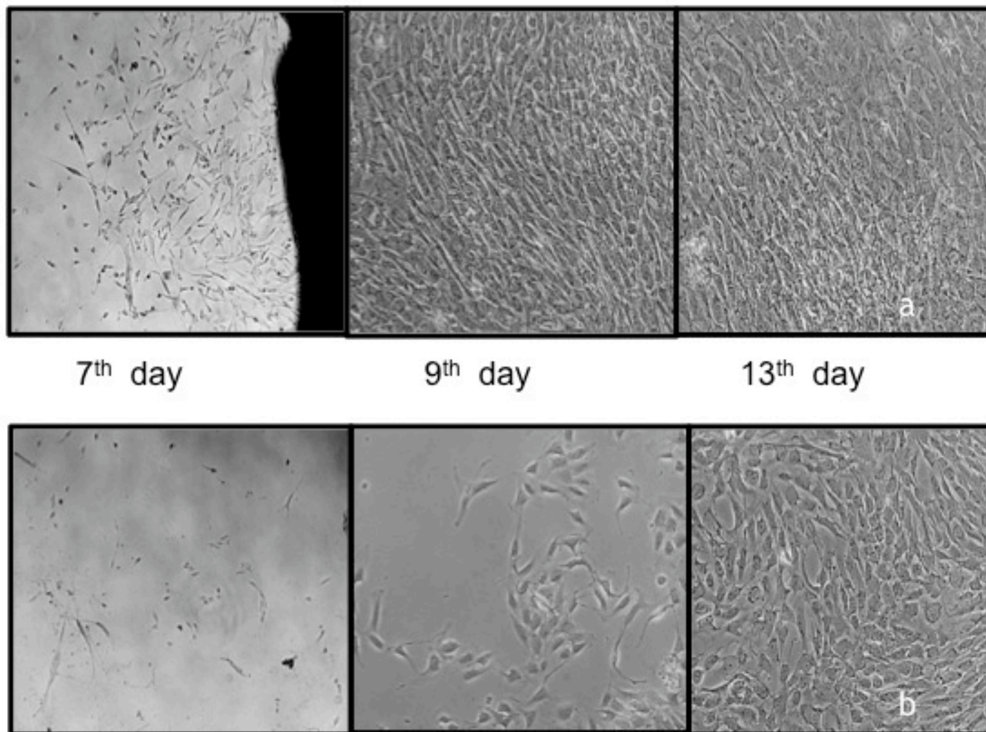


Fig.21
Picture of cellular outgrowth from specimens of NAA a) Cells coming from NAA media with presences of atherosclerotic plaque, b) Cells coming from NAA media alone
7th (4X) 9th and 13th (20X) phase contrast microscopy

NAA cells growth in the presence of the plaque was analyzed by immunofluorescences for a-SMA and S100A4.

By Immunofluorescences staining S100A4 was detected in cells outgrowth from NAA in presences of plaque. This data compare with thee absence of S100A4 protein in NAA cells, suggest that plaque could influences not only time of cellular outgrowth but even molecular features.

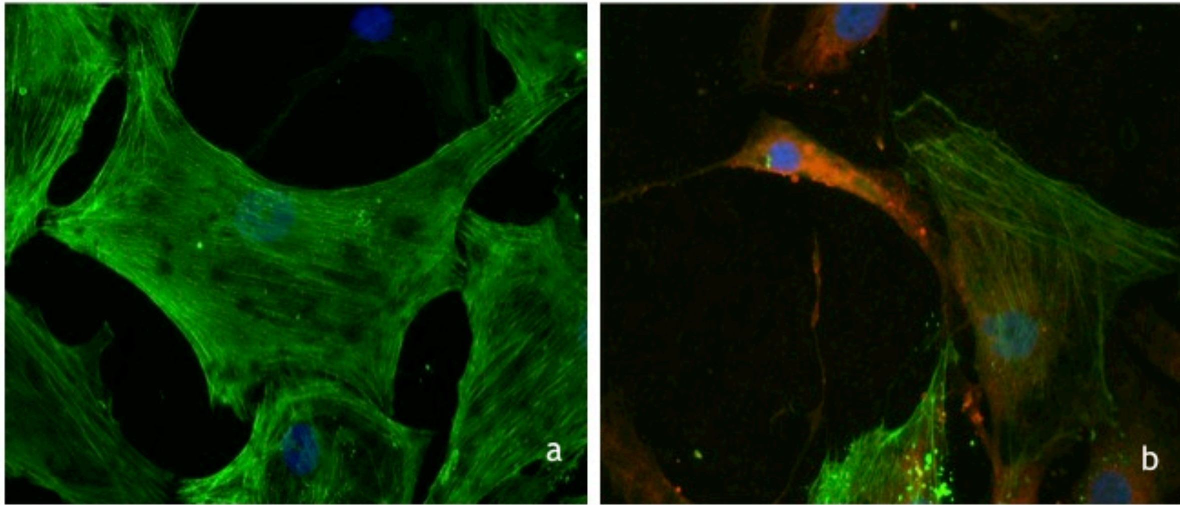


Fig.22
Immunofluorescences staining(40X magnification) anti-S100A4 (red) DAPI(blue) anti-alpha smooth muscle actin (green)
a) l-SMCs
b) s-SMCs

In fig.22 we can observe that NAA with Plaque cells show S100A4 and lower level of a-SMA (compared with cells from NAA alone), in addition they were small and elongate like s-SMCs coming from AA.

These preliminary data suggest that cells from NAA media under the influence of plaque could switch from large to small phenotype.

4.7 RNA profiling

Observation of differences between s-SMCs and l-SMCs was limited by techniques used. Immunofluorescences and RT-PCR permit to analyze just a few numbers of markers at one time. For skip this problem we prepared microarray assay, which provide us general information on genes modulated in these distinct phenotypes.

From 3 male patients between 67-73 years old, specimens both NAA e AA were taken. Specimens obtained were used to prepare tissue explantation. Once cells raised confluence, were cultured until 3rd passage. Then, at subconfluences was collected and RNA was extract using RNA minispin GE health care®. Quality of RNA obtained by cultured SMCs was analyzed using Agilent Bionalazer®. As show in fig.23 in scale from 1(lowest quality) to 10(highest quality) our samples obtained results from 8.80 to 9.80.

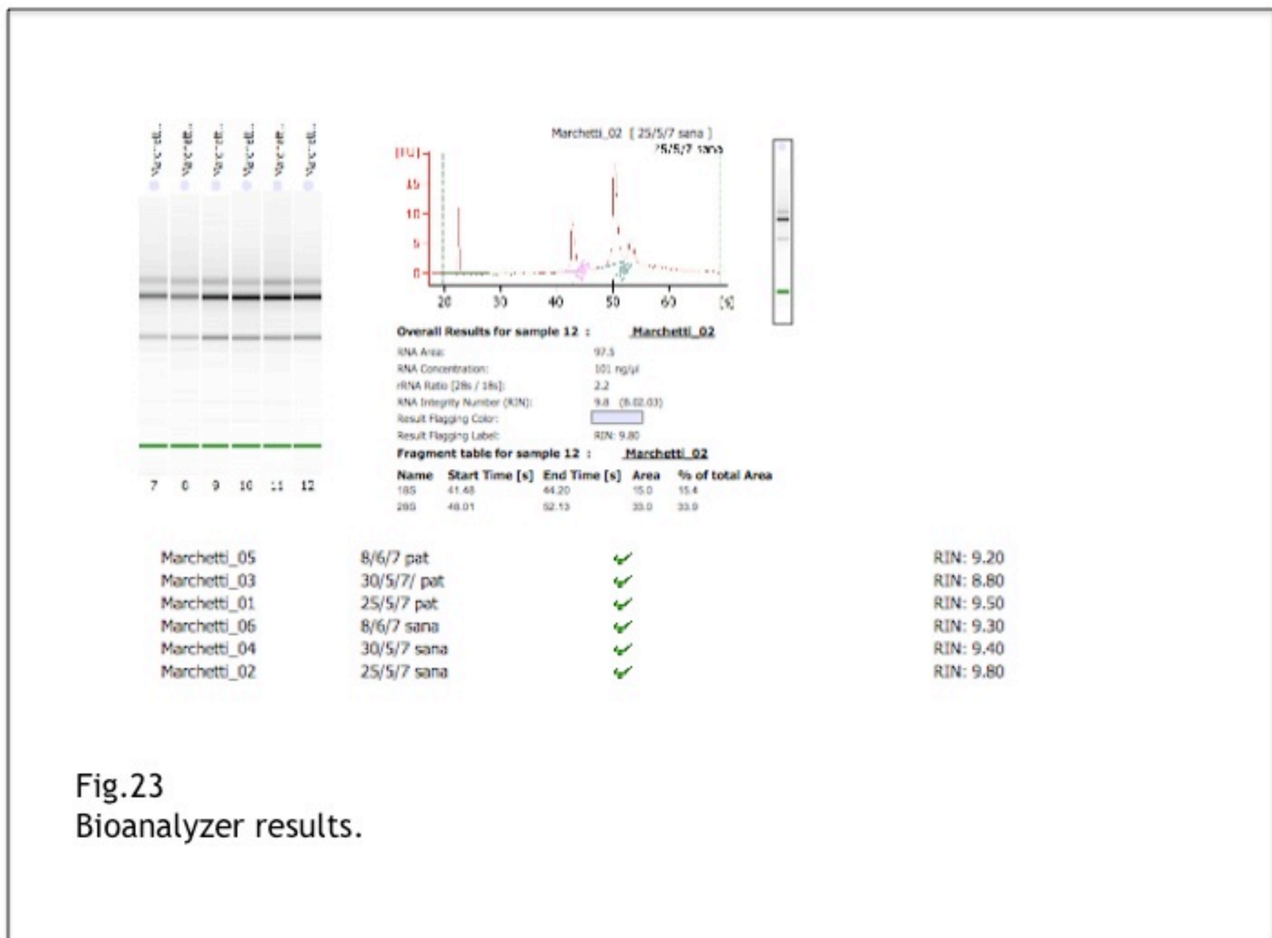


Fig.23
Bioanalyzer results.

These data indicate that RNA samples were suitable for Microarray assay.

Microarray analysis was performed on Agilent platform. Raw data were analyzed using GeneSpring GX software. A Welch t-test was used for a statistical analysis of differentially expressed genes. Only genes with $P<0.05$ and a fold-change >= 2 were retained for further analysis. Because of the small number of samples, these criteria increase the reliability of our microarray analysis allowing us to reduce false-positives.

From this analysis we identified 375 genes differentially expressed in the two populations of SMCs (Fig. 24).

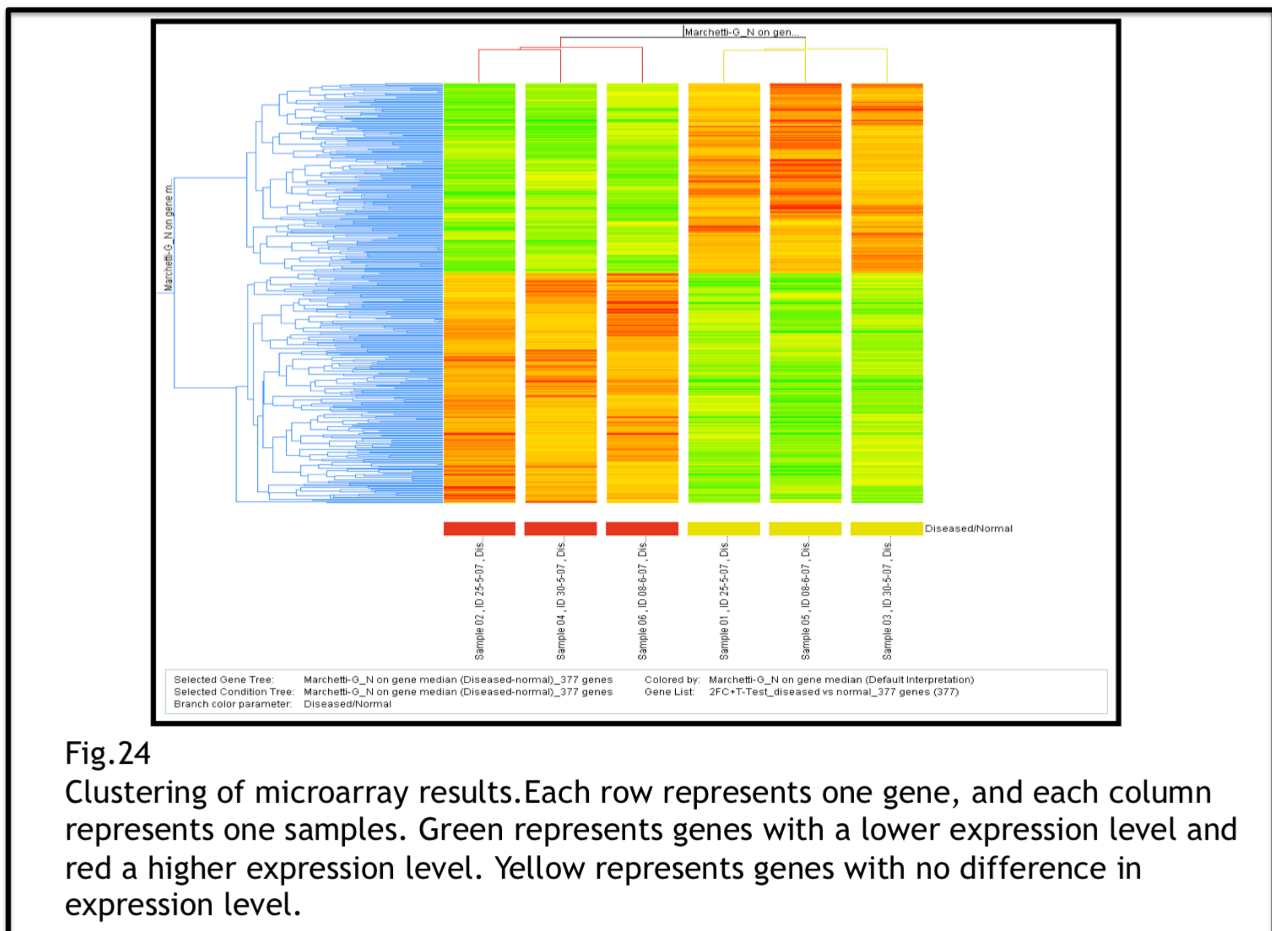


Fig.24
Clustering of microarray results.Each row represents one gene, and each column represents one samples. Green represents genes with a lower expression level and red a higher expression level. Yellow represents genes with no difference in expression level.

Gene ontology (GO) analysis was used to assign the regulated genes into functional categories. A classification for biological processes was available for 225 genes (125 up-regulated in disease, 100 up-regulated in normal). Given the two lists of differentially

expressed genes (125 and 100 genes), we scored each GO category by evaluating, with the Fisher's exact test, the enrichment of each class in the list of interest if compared to the list of all genes spotted on the microarray. comparing the number of genes in the list annotated by this term to the expected number of such genes based on the total number of genes annotated by this term in the whole array

The most representative categories in the two lists are schematically reported in figs 25 and 26 respectively.

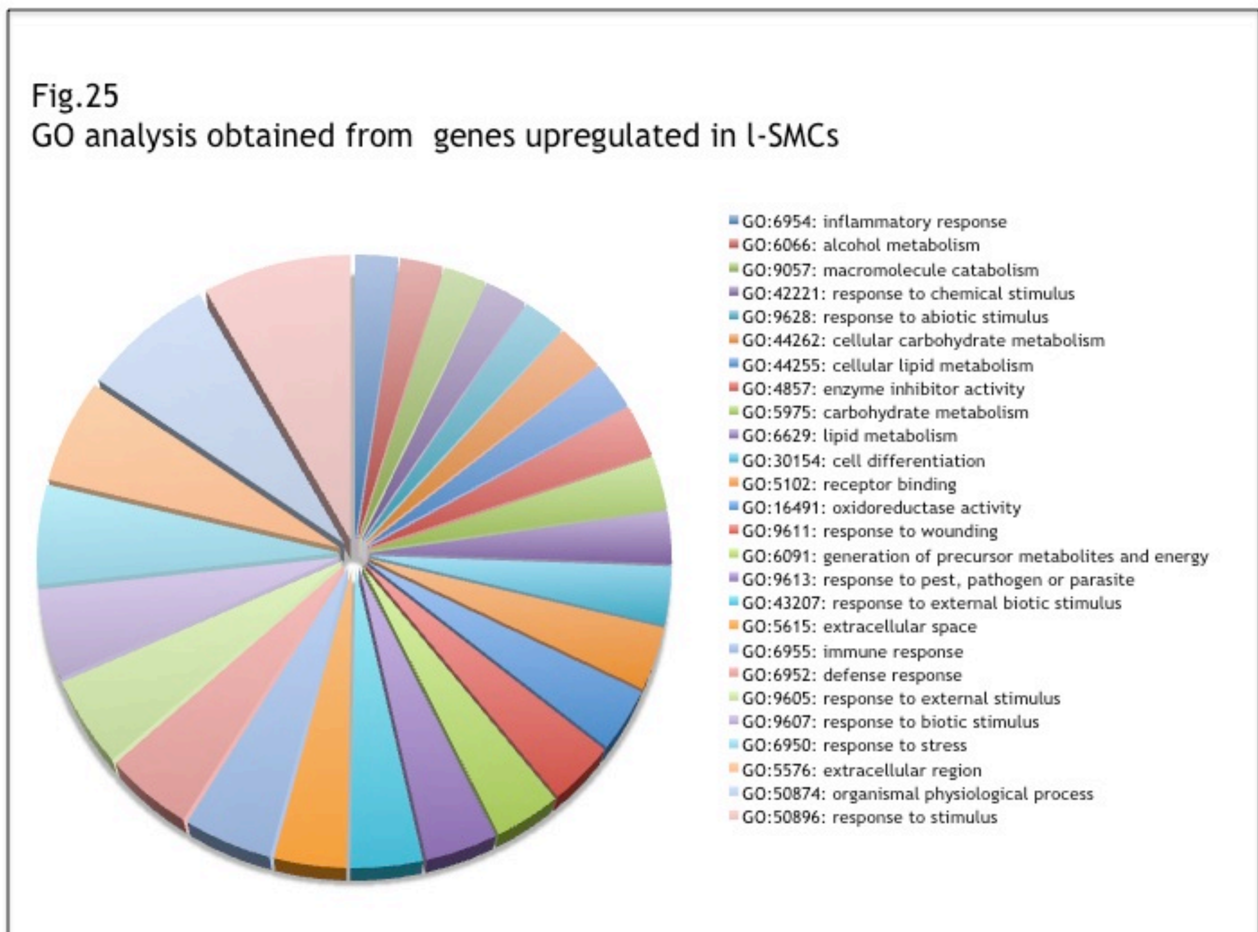
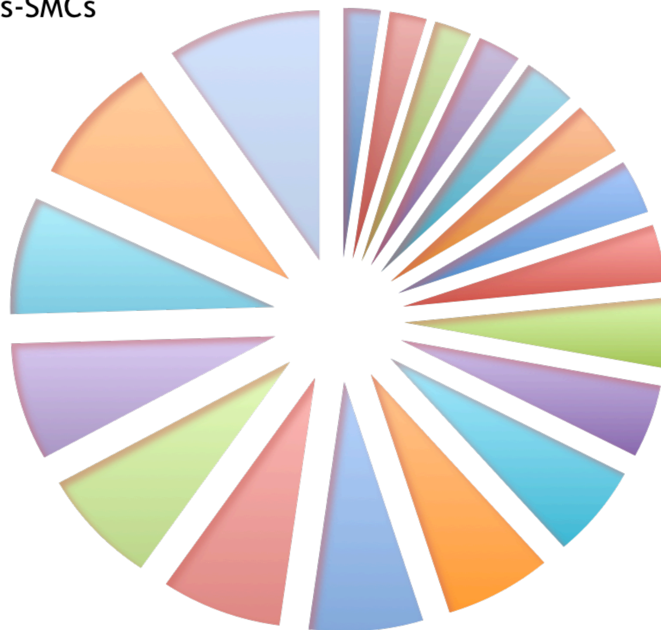


Fig.25
GO analysis obtained from genes upregulated in l-SMCs

Fig.26
GO analysis obtained from genes upregulated in s-SMCs



Validation of microarray data at the mRNA level through semi-quantitative RT-PCR

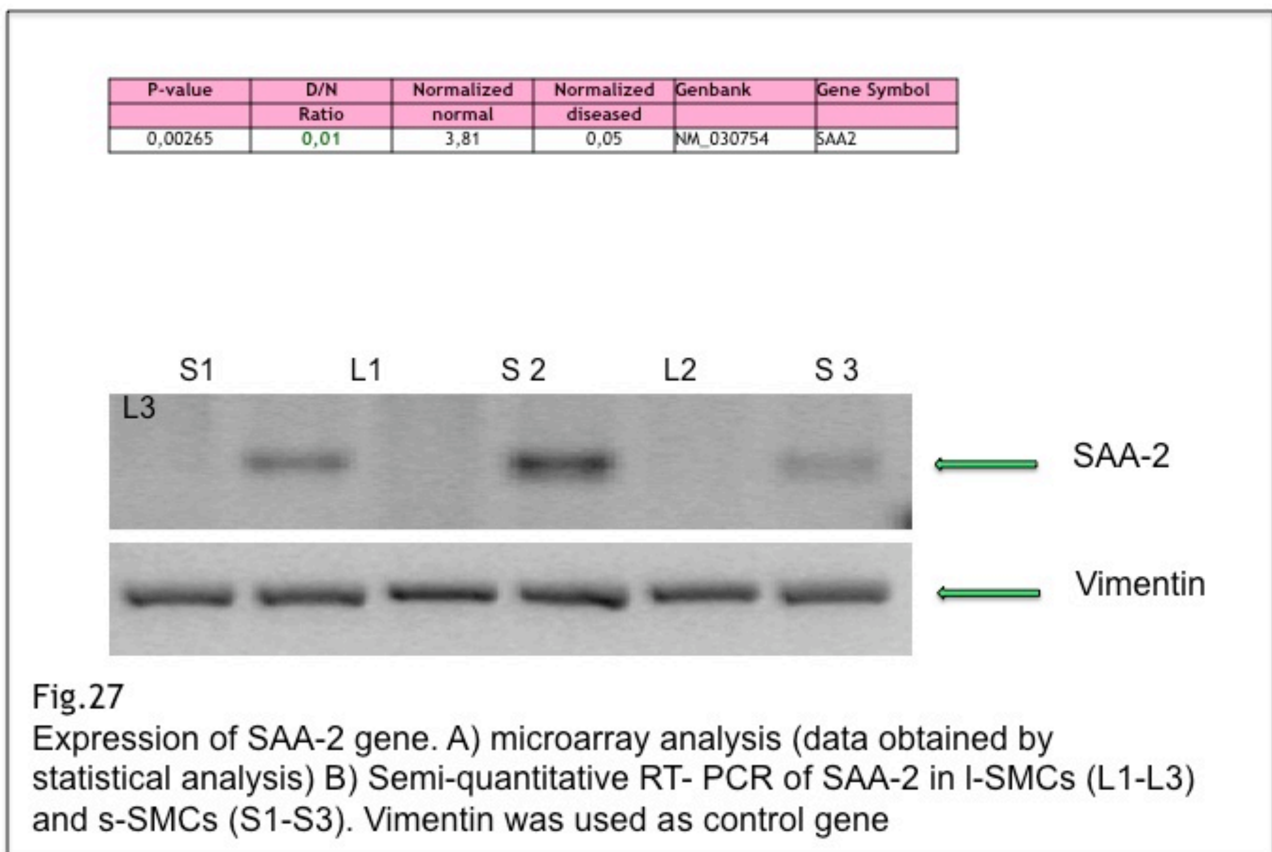
In order to validate microarray data we used semi-quantitative RT-PCR analysis for selected genes. Particularly, among inflammation response genes, two were analysed, serum amyloid A2 (SAA-2), and interleukin 6 (IL-6).

SAA-2, an acute-phase serum

adipokine, was chosen because of its highly significant difference in expression between NAA and AA cells (100:1, P=0,00265) and IL-6, because its correlation with SAA-2.

and involvement in atherosclerosis pathogenesis (1227 PubMed citations).

In semi-quantitative RT-PCR SAA-2 mRNA was found only in l-SMCs, thus confirming microarray data (Fig.27)

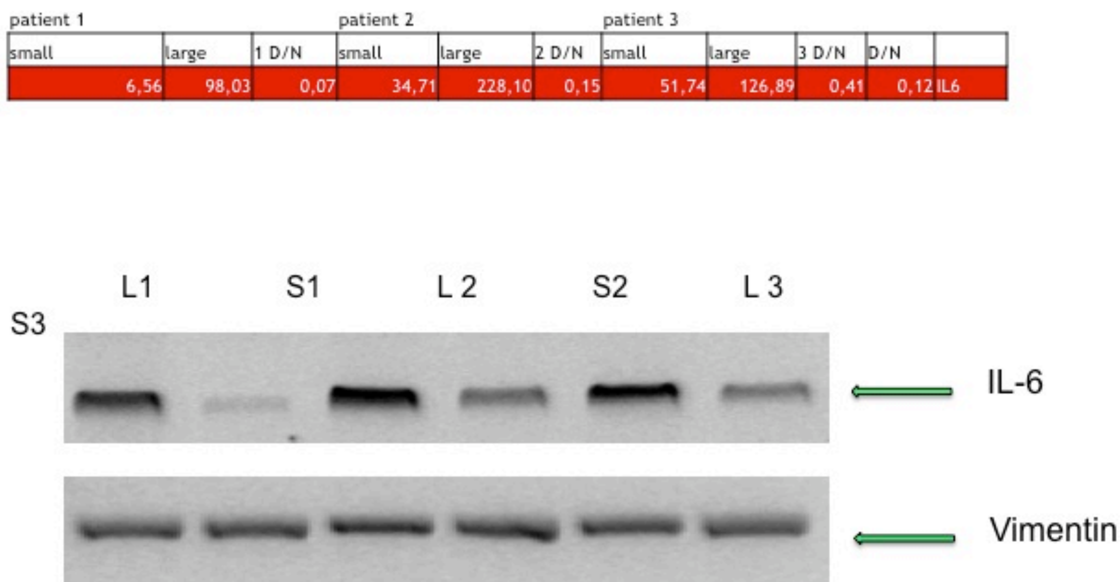


P-value	D/N Ratio	Normalized normal	Normalized diseased	Genbank	Gene Symbol
0,00265	0,01	3,81	0,05	NM_030754	SAA2

Fig.27
Expression of SAA-2 gene. A) microarray analysis (data obtained by statistical analysis) B) Semi-quantitative RT- PCR of SAA-2 in l-SMCs (L1-L3) and s-SMCs (S1-S3). Vimentin was used as control gene

IL-6 mRNA was detected in both l-SMCs and s-SMCs, and it was

differentially expressed (higher level in l-SMCs than in s-SMCs), with the same direction of change as measured by the expression array (fig.28).



patient 1			patient 2			patient 3				
small	large	1 D/N	small	large	2 D/N	small	large	3 D/N	D/N	
6,56	98,03	0,07	34,71	228,10	0,15	51,74	126,89	0,41	0,12	IL6

Fig.28
Expression of SAA-2 gene. A) microarray analysis (row data and s-SMCs/l-SMCs expression ratio of each sample was reported. B) Semi-quantitative RT- PCR of IL-6 in l-SMCs (L1-L3) and s-SMCs (S1-S3). Vimentin was used as housekeeping genes.

4.8 Tissue factor (TF) pathway inhibitor (TFPI) in SMCs

A principal focus of our laboratory is Haemostasis. For this reason, in microarray data analysis, a particular attention was reserved to genes involved in coagulation and its regulation.

Among the genes listed in the coagulation cascade (KEGG database), TFPI expression was modulated in all three couples of RNA samples. Expression of TFPI was 1.3 – 5 fold lower in s-SMCs than l-SMCs.

TFPI is the major downregulator of the procoagulant activity of the TF–factor VIIa complex (TF - FVIIa). Beside a regulation activity of the coagulation cascade, TFPI exhibits additional biological functions (Fig.29).

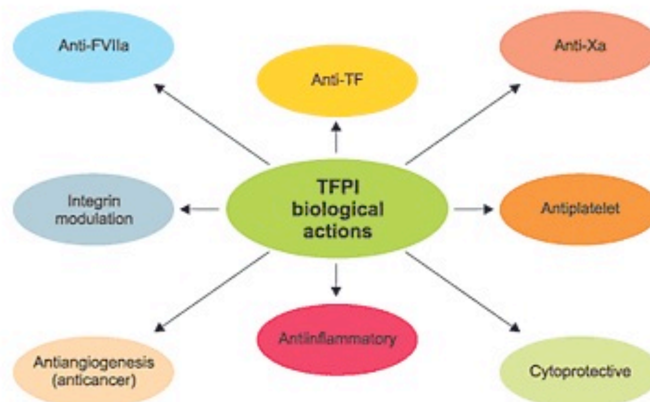


Fig.29
Biological function of TFPI

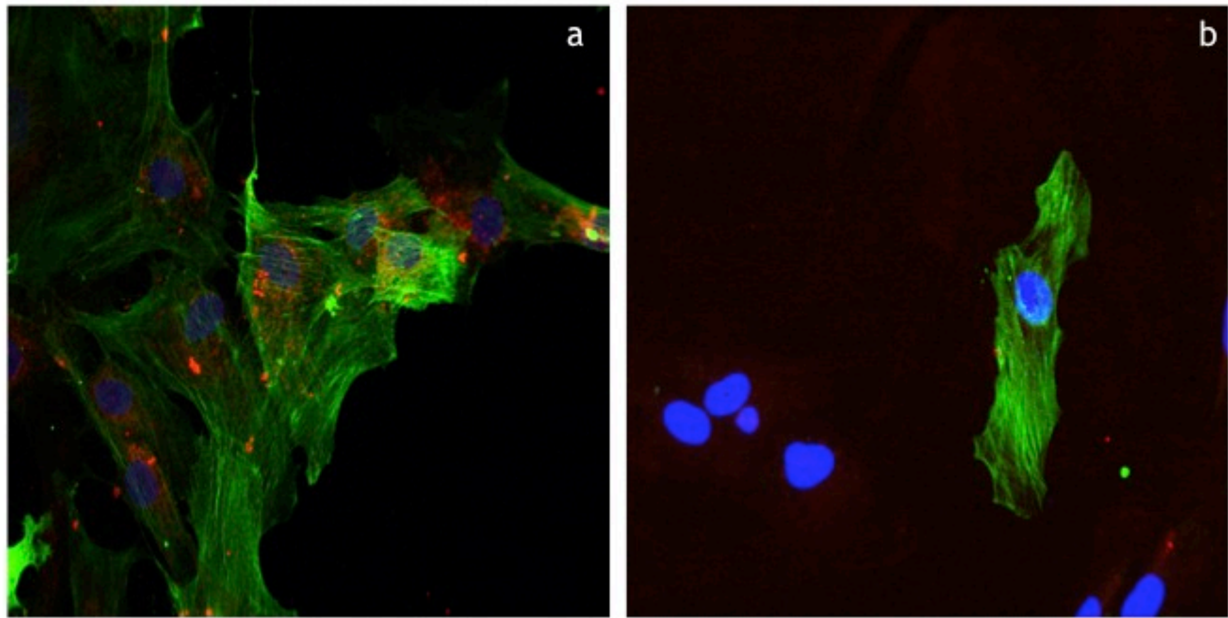


Fig.30
Immunofluorescences staining(40X magnification) anti-TFPI (red) DAPI(blue) anti-a-SMA a) l-SMCs b) s-SMCs

5. Discussion and Conclusion

5.1 Primary culture of SMCs

The involvement of selected cellular components in intimal hyperplasia and atherosclerosis progression has been inferred from the ability of only some cells to respond to stimuli in vitro, their accumulation in injury-induced intima, and the differences in gene expression between neointimal and medial SMCs (Zalewski et al, 2002)

Several animals have been used for the study of atherosclerosis, the nonhuman primates, swine, mice, guinea pigs and hamsters. The disadvantages of animal models are that there is no possibility to reproduce the same history of pathogenesis in animals as that in man. There is no perfect animal model that completely replicates all stages of human atherosclerosis.

The availability of normal human arterial samples for experimental studies is limited. Primary cultures of vascular SMCs can be difficult to obtain and grow, and commercially available cells are expensive. Vascular tissues can be obtained from various organs during organ transplant procedures, but access to these tissues is limited. Furthermore, the age of the donor is often advanced and health problems or death of the donor may have altered the phenotype of the cells. There is always a question as to whether donor cells are truly normal (Leik et al, 2004).

A readily available source of normal human arterial smooth muscle cells for in vitro studies would be useful.

In this thesis we developed an experimental protocol for the isolation and culture of SMCs from human samples of atherosclerotic and no-atherosclerotic carotid artery. This protocol provides a standard method for primary culture by tissue explantation. Cells stably maintained their features until tenth passage, thus permitting to perform experimental investigations for a relative long time.

Nevertheless, drawbacks in setting up this method were manifold.

First of all, tissue explantation needed to be performed as quickly as possible to avoid sample damage. For this reason within an hour from surgery, the specimens were taken to the laboratory.

Although the number of samples was large enough, because CEA is one of the most performed surgical interventions in vascular surgery, not all CEA specimens were usable for tissue explantation. Particularly, using a surgical blade could not cut lesions presenting extended calcifications.

In addition, as all samples of no-atherosclerotic artery were very small for understandable reason, the recovery of SMCs from atherosclerotic and no-atherosclerotic portions of carotid artery from the same patient was difficult.

Several time-consuming experiments were preliminarily performed, before understanding that, to obtain cellular outgrowth, the side of the specimen (luminal or abluminal) to be placed in contact with petri dish surface was the abluminal one.

5.2 Distinct SMC Phenotypes

Several groups have obtained and characterized phenotypically different SMC populations from normal and pathological arteries, mainly in animals such as rats and

pigs (Walker et al, 1986; Bochaton-Piallat et al, 1996). However, human SMC heterogeneity has only been sporadically described. Through tissue explantation we were able to isolate two distinct SMC populations from human carotid endarterectomy specimens. L-SMCs from NAA were large and grew in a monolayer, whereas s-SMCs from AA were small and elongated, growing multilayered with a typical pattern of hills and valley. Moreover, proliferation rate differentiated l-SMCs from s-SMCs.

The presence of S100A4 was an additional feature that distinguished l-SMCs and s-SMCs. S100A4 was detecting only in s-SMCs outgrowing from AA media, and in SMCs from NAA media in the presence of plaque. This protein has been detected in human IT SMCs (Brisset et al, 2007) and in atheroma prone SMCs in porcine coronary culture. Accordingly with this previous study, our finding might suggest that S100A4 could be also a marker of human atheroma prone cells.

5.3 Plaque influence on cellular outgrowth and proliferation

From our results we demonstrated that plaque plays a fundamental role in cellular outgrowth and proliferation from both NAA and AA media.

The co-culture experiments were useful for the identification of media as the proliferative compartment. Moreover by this approach we demonstrated that presence of plaque was necessary for AA media cellular outgrowth. Interestingly, cells coming from NAA in the presence of plaque showed morphological and molecular features similar to s-SMCs outgrown from AA tissue. This indicates that plaque could influence SMC phenotype independently of the starting tissue. Plaque component/s, able to influence cellular outgrowth, proliferation and phenotype, should be soluble in culture medium, because the holes of the transwell membrane do not allow cells to move from a compartment to other. These component/s could be present as molecule/s in the plaque or secreted by cells. One hypothesis is that macrophages, which are the most abundant cell type in plaque after SMCs, could release grow factors affecting SMC proliferation. The observation that macrophages outgrowth from plaque before SMCs reinforces this hypothesis.

5.4 5.3.RNA profiling

Despite compelling evidence that phenotypic modulation of SMCs plays a key role in the development and/or progression of atherosclerosis and in vascular injury repair, the molecular mechanisms and their regulation leading to the phenotypic switching are still largely unknown (Kawai-Kowas & Owens, 2007). Particularly, very few information are available for SMCs and for their expression patterns in normal and atherosclerotic vessels.

The comparison between SMCs from atherosclerotic and non atherosclerotic carotid artery through microarray assay, is an original approach to identify molecular signatures of the atherosclerotic process. Microarray data analysis indicated that 375 genes were differentially expressed, and among annotated genes 125 were up regulated in s-SMCs and 100 up regulated in l-SMCs.

In particular, our attention was focused on IL-6 and SAA-2, both involved not only in inflammation but also in the atherosclerosis progression.

IL-6

IL-6 is an important stimulator of the acute phase reaction, acting on both liver and hypothalamus (Fattori, 1994) leading to release of Serum amyloid A (SAA), and fibrinogen, all know to have proinflammatory role. IL-6 deficiency was reported to result in an enhanced formation of atherosclerotic lesion, reduced collagen metabolism and

elevated level of serum cholesterol (Schieffer et al,2004). However, IL-6 is a pleyotropic cytokine and its role in the modulation of inflammation-related process, particularly cytokine response and tissue inflammatory cells infiltration, remains equivocal (*Madan et al,* 2007). One function of IL-6 is to suppress the level of proinflammatory cytokines without compromising the level of antinflammatory cytokines. Therefore the absence of IL-6 results in more pronounced response of proinflammatory cytokines. (VB)

The evidence of a lower level of IL-6 mRNA in s-SMCs, supports data obtain by microarray assay and semiquantitative RT-PCR, in which L-SMCs showed higher level of IL-6 mRNA, compared with s-SMCs.

SAA2

Serum amyloid A (SAA) proteins are a family of apolipoproteins associated with high-density lipoprotein (HDL) in plasma. SAA2 belong to SAA protein family subclassified as acute phase SAA (SAA1; SAA2) and constitutive SAA. Although most SAA is produced by the liver, SAA1, SAA2, and SAA4 also can be produced by extra-hepatic sources. During inflammation, SAA1 and SAA2 are principally expressed and induced in the liver, while SAA3 is induced in many distinct tissues. SAA1 and SAA2 genes are regulated in liver cells by the proinflammatory cytokines IL-1, IL-6, and TNF-α. It is of interest that mRNA for SAA has been detected in all of the major cell types present in atherosclerotic, which contain both acute-phase and constitutive forms of SAA protein. It was demonstrated that SAA2 might serve as autocrine factor to influence SMC migration of aortic tissue injury and inflammation (*Kumon et al*, 2002).

Cultured SMCs expressed SAA2 (Meek et al, 1993). Previous studies demonstrated an association between increased levels of serum amyloid A (SAA), and increased cardiovascular risk (Johnson, et al 2004).

Differently from literature, in which SAA2 is described as a pro-atherosclerotic marker, both in microarray analysis and in semiquantitative RT-PCR, it was more expressed in l-SMCs outgrown from NAA samples. This finding requires further investigation.

TFPI

Several evidences support the strong relationships between coagulation and atherosclerosis. Acute thrombosis after atherosclerotic plaque disruption is a major complication of primary atherosclerosis, leading to acute ischemic syndromes and atherosclerosis progression. Several studies have identified TF in the intima of human atherosclerotic plaque and have suggested that it is an important determinant of thrombogenicity after plaque rupture. Vascular smooth muscle cells were identified as a significant source of TFPI synthesis in culture and in normal coronary arteries (*Caplice N et al,* 1997). Biologically active TFPI is present within human atherosclerotic plaque and is associated with attenuated TF activity (*Noel M et al*, 1998). TFPI is currently under clinical investigation as an anticoagulant and its effects on intimal hyperplasia in animal models have been studied (*Taubman MB et al,* 1997).

TFPI downregulation in s-SMC from atherosclerotic carotid, observed both in microarray and immunofluorescence analysis is consistent with previous studies.

Althought microarray analysis is a powerful approach to identify molecular signature, we are aware of the limitations of RNA profiling of cultured cells.

A disadvantage is that the in vitro culture may cause a shift in the transcriptome (Bijnens et al, 2005). However, in our study all cells were maintained in similar experimental conditions, which should favour the detection of differences related to the vessel conditions (presence/absence of atherosclerosis).

One of the most challenging aspects of gene expression analysis is the selection, among the vast quantities of data, those genes that could have a real causal role. Indeed, the change in the transcriptional level of a gene is not correlated with the causal role of that gene.

Sometimes small changes in a key gene can produce a large biological effect. In addition, changes in gene expression are not invariably associated with changes in protein synthesis. Thus the microarray techniques may be viewed as guiding tools to point the study in the right direction, with more conclusive functional information collected in subsequent complementary studies.

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