UNIVERSITA' DEGLI STUDI DELL'INSUBRIA

DOTTORATO DI RICERCA IN SCIENZE DELLA VITA E BIOTECNOLOGIE

XXXIII CICLO

Neo-lymphogenesis in atherosclerotic lesions of carotid bifurcation

Neo-linfogenesi nelle lesioni aterosclerotiche della biforcazione carotidea

Docente guida: Prof. **Matteo Tozzi**
Tutor: Prof. **Matteo Tozzi**

Tesi di dottorato di:
Stefania Ferraro
Matr. 617623

Dip. Biotecnologie e Scienze della Vita - Università degli Studi dell'Insubria

Anno accademico 2019-2020

SOMMARIO

INTRODUCTION

Epidemiology of Ischaemic stroke epidemiology

Cardiovascular diseases, particularly heart diseases and strokes, are the principal death cause for over 17,5 milions of people in the whole world.

The predominance of such cardiovascular diseases increases concerning a patient's age, reaching measures between 4,61 and 7,33 for 100 inhabitants as for individuals older than 65 years old. [1]

87% of most cases, stroke can present an ischaemic nature, whereas the remaining 13% show a haemorrhagic nature (10% intraparenchymal hemorrhage; 3% subarachnoid hemorrhage). [2]

Stroke represents the second most common death cause in cardiovascular diseases with a death rate of 5,8 million dead people per year. Strokes are recognised as the fourth death's life cause before myocardial infarction, tumors and serious respiratory conditions.

The death life risk after an ischemic stroke is equivalent to 7% up to 7 days, 14% up to 30 days, 27% out of a year, and 53% out of 5 years, whereas the chances of experiencing a new cerebral ischemic episode, after a stroke, is of 2% up to 7 days, 4% up to 30 days, 12% up to one year and 29% up to five years. [3]

According to some authors, there has been a reduction of a stroke's incidence and prevalence, particularly as reported by Rothwell et al [4], between 1980 and 2002, the incidence of strokes was reduced by 40% in Great Britain. In a review carried out by Zhang et al [5], they revealed it's impossible to confirm that there has been a reduction in the impact and predominance of cerebral ictus. Nonetheless, they found a decrease in terms of stroke death rates, due to an improvement in the treatment of strokes, both in the United States and in Europe.

In the Western world, ischaemic stroke is the leading cause of disability, causing a heavy impact on the social and economic aspects of people's lives.

Evalueting patients over 65 years, in 50% they have hemiparesis, in 30% they can't walk without support, in 19% they are aphasic and in 26% they live in assistance structure. [6]

Physiopathology of the ischaemic stroke

The risk factors of an ischaemic stroke are the same as those of atherosclerosis. Mainly, such factors can be divided into risk elements that be changeable and unchangeable. [7]

Unchangeable risk factors include:

- Age: the risk of a stroke usually increases with age, particularly after 55 years of age, the risk tends to reduplicate. [8]
- Sex: the risk of a stroke is higher in men than in women, with a man-woman ratio of 1,3:1 [9]
- Genetic factors: patients who show familiarity with myocardial infarction (MI); TIA or stroke, have a risk that usually increases from 1,4 to 3,3 chances of having a stroke. [10]

Changeable risk factors are:

- Arterial hypertension: the risk reduction of a stroke is determined by an appropriate pharmacological antihypertensive therapy (PA <120/80) [11]
- Diabetes mellitus: diabetic patients have a doubled risk of a stroke in comparison to individuals with standard glycaemic numbers [12]
- Hypercholesterolemia: patients with inadequate control of cholesterol values, have a doubled risk of developing a stroke as compared to a subject with standard values. [13]
- Smoking: it increases the risk of brain strokes from 2 to 4 times amongst patients who actively smoke, in relation to non-smoking patients. [13] [14]
- Obesity and lack of physical activity [15] [16] [17]

The INTERSTROKE Study, published in 2010 and performed on 2337 patients from 22 countries of the world, reports that five risk factors, which represent the 80% of the global risk, are represented by arterial hypertension, smoking, obesity, diets and lack of physical activity. [18]
Brain ischemia is caused by a drastic reduction or by an interruption of haematic supply to a cerebral territory that is more or less vast. Atherosclerotic carotid plaques can cause a TIA or a stroke through two pathogenetic mechanisms.

Embolic Mechanism: embolism is nowadays considered the most frequent cause of transitory cerebral ischemia, which interests the vascularisation territory of the internal carotid. Embolism depends on the composition of the atherosclerotic lesion: the lipidic predominance is typical of unstable lesions, with more likelihood of ulceration and embolism.

The *primum movens* of the arterial thromboembolism is the breakage of the overhead intima layer (cap) that determines erosions or deep ulcerations from which atheromasic or thrombotic material can detach in the hematic current, with a consecutive intracranial embolization, or it causes the development of a blood clot obstructing the cavity of the blood vessel.

Hemodynamic mechanism: it is due to a progressive increase of volume in the atheromatous plaque up to the serrated stenosis (with a reduction of the arterial lumen>80%) or the extended thrombosis of a principal truncus arteriosus (ICA or basilar artery) with a resulting reduction of the blood flow, peripheral ischemia, tissue hypoxia and ischemic stroke.
The most frequent pathophysiologic mechanism (80-85% approximately) that pertains to the anterior circulation of carotid is the atheroembolic one.

The Vulnerable Plaque and Atherosclerosis

The term "vulnerable plaque" is used to define an unstable atherosclerotic plaque or a complex plaque. According to the classification of the American Heart Association, atherosclerotic lesions divide into six stages. The first stage is simbolised by isolated foamy cells, (called "lipidic point"). The second stage is characterised by "fatty streaks" and the third stage by intermediate lesions. Whereas in the fourth stage, we can observe the presence of atheroma; and in the fifth, there is fibroatheroma. Complex lesions represent the sixth stage, also including calcifications, breaks with resulting thrombosis, and embolisation of the plaque and intraplaque hemorrhage.

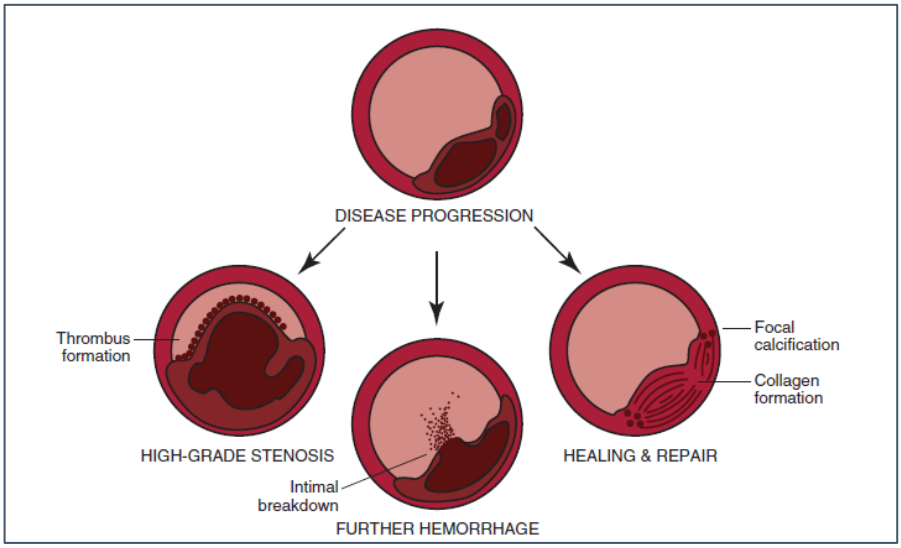


Figure 1 Atherosclerotic plaque

Vulnerable plaques are histologically described by a stringy thin cap and by a voluminous lipidic core. [19] [20]
The identification of vulnerable carotid atherosclerotic plaques can be implemented through first level diagnostics investigations with supra-aortic trunks Eco-Color-Doppler (ECD), and through second level diagnostics (CT or MRI scan). The carotid ECD enables to quantify the stenosis degree and to accurately describe the macroscopic morphological characteristics of the carotid plaque (for example,

the fibrous cap). With the aid of ECD, the plaque's composition can be examined. Hypo-anaecogenic (lipid) and irregular plaques are often associated with cerebrovascular symptomatology as compared to hyperechogenic calcified plaques. [21] [22]

As is common knowledge, since 1970, there has been a connection between histopathological characteristics of the atherosclerotic carotid plaque and the presence of cerebrovascular symptomatology. [23] In 2006, Redgrave et al., published in the Oxford Plaque Study, a histological study regarding atherosclerotic carotid plaques acquired by patients exposed to carotid thromboendoarterectomy (CEA).

Plaques were classified base on the presence of an inflammatory infiltrate and on their instability within the following groups: stable (a plaque that is mainly fibrous with an entire cap), predominantly stable (intraplaque inflammation and entire fibrous cap), unstable with an entire fibrous cap (thin cap and intact with voluminous lipid cap), unstable with a break in the fibrous cap.

In this study, the majority of symptomatic patients' plaques presented a break of the fibrous cap (58,1%), intraplaque hemorrhage (64.4%), and a critical inflammatory infiltrate (66.8%). Even Gao et al. concluded that during their meta-analysis, ulcerated carotid plaques were unstable and significantly linked with neurological events. [20]

The passage from a plaque defined as stable and a vulnerable one depends on a final balance between cellular migration/proliferation, production of the extracellular matrix, inflammatory infiltrate, (predominantly monocyte-macrophage and lymphocyte) and on the development of new vessels.

The break of the fibrous cap is determined by an imbalance between forces that operate on the fibrous cap and on the resistance thereof. It would appear that all of the factors capable of reducing collagen's synthesis from smooth muscle cells (for example, IFN-α) compromise the ability to repair and maintaining the cap intact. Macrophages present in the plaques can produce metalloproteinases (MMP) and elastolytic cathepsins responsible for the break of the collagen and the elastin of the extra-cellular matrix. [24]

The plaque's vulnerability furthermore depends on the presence of new vessels, which appear fragile because they are composed of a single layer of endothelial cells. The new blood vessels can encounter a break with a consecutive intraplaque haemorrhage. Such heavy bleeding in some cases can determine the increase of the plaque's volume and a subsequent rupture of the fibrous cap.

Many studies dedicated to atherosclerotic coronary and carotid plaques have highlighted that the angiogenesis of a plaque can support the progression of plaques. In particular, it encourages the vulnerability and break of the plaque itself. Different factors contribute to angiogenesis, and they are caused by intraplaque inflammatory cells. New blood vessels express cohesion molecules such as E-selectin and VCAM (vascular cell adhesion molecule-1), that summon lymphocyte T cells and

macrophages. Even the inflammatory infiltrate plays a crucial role in the gradual development and variability of the plaque. Taking the example of a study carried out by Moreno et al., they observed that during the break of an atherosclerotic coronary plaque, this fracture linked with the presence of an abundant inflammatory infiltrate. [25]

The affiliation between inflammation and progression of the plaque is supported by inflammatory marker serums, such as the reactive protein C (PCR), serum amyloid A (SAA), IL6, IL18, TNFα, MMP2, and MMP9. [26]

Notwithstanding the numerous investigation lead on atherosclerotic carotid and coronary plaques, the development mechanisms, progression, and break of the plaque remain disjointed.

The development of the atherosclerotic plaque

The accumulation of lipid in the vessel wall is the "primum movens" in the development of the atherosclerotic plaque, followed by the migration of multiple inflammatory cells which determine the formation and the accretion of the atherosclerotic plaque via a release of cytokine, that foster atherosclerosis.

The pathogenetic theory that attempts to explain the atherosclerotic process is a *cross-reaction*, and it considers atherosclerosis as an inflammatory response of the vascular wall due to a damage of the endothelium. The inflammatory course plays a fundamental role in the pathogenesis of atherosclerosis.

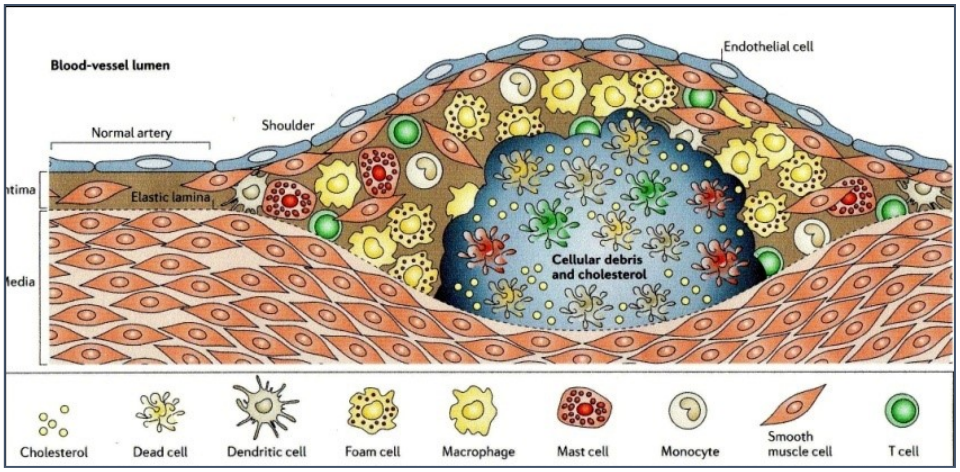


Figure 2 Cells in atherosclerotic plaque

- CRONIC ENDOTHELIAL DAMAGE: inducted by endotoxins, hypoxia, hyperlipidemia, effects derived from smoking cigarettes, homocysteine, and hemodynamic factors. The

endothelial damage appears with an increase of permeability (loss from endothelial cells of intercellular binding and with the underlying connective). Such damage also displays an increment of adhesion molecules (ICAM-1, VICAM-1, E-selectin[1], P-selectin[2]), the activation of endothelial pro-inflammatory and proatherogenic genes, from which derives the creation of cytokines and coagulation proteins.

- EXTRA- CELLULAR LIPIDIC ACCUMULATION:

  Lipo-protein particles accumulate in the tunica intima. These particles tie with proteoglycans of the extra-cellular matrix, and they form "fatty streaks." The lipoproteins tethered to proteoglycans seem susceptible to oxidation and to other biochemical alterations, which facilitate the inflammatory process. In particular, lipoproteins go towards oxidation[3] and a non-enzymatic glycosylation process[4].

  As a result of these processes, it happens that the recruitment and the accumulation of leucocytes, which stick with the endothelium and for diapedesis between endothelial junctions, penetrate the intima where they start to absorb lipids, transforming into "foamy" cell. In such a process, monocytes neutrophils and T lymphocytes are principally involved.

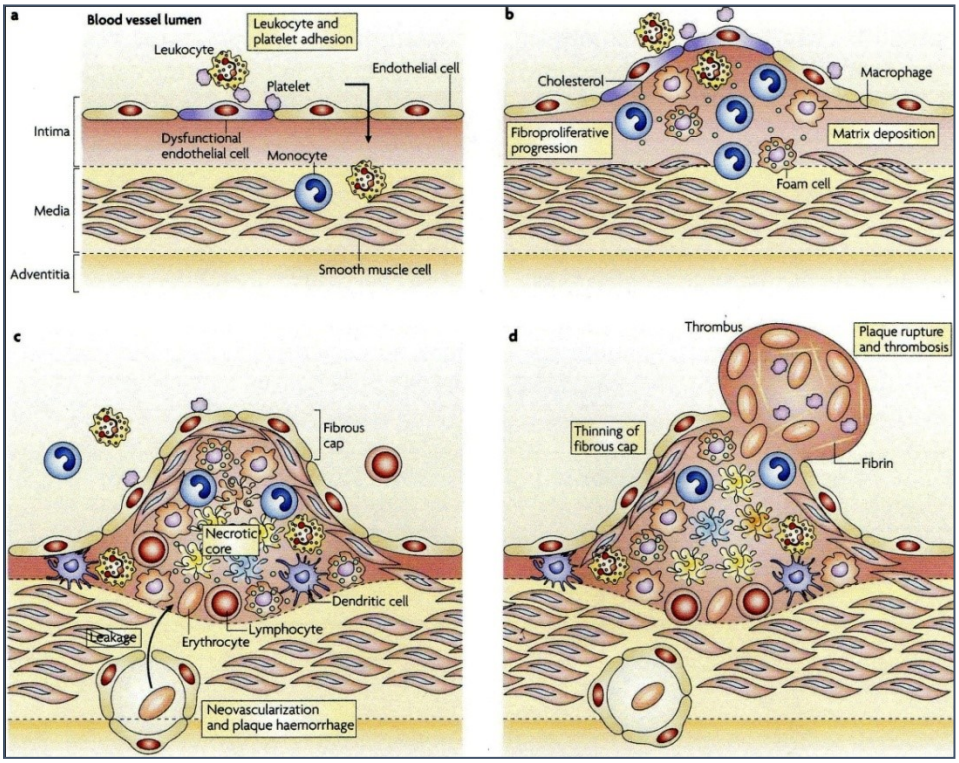


Figure 3 ***the formation of the plaque: a.*** *dysfunction of endothelial cell and their activation with the inflammatory stimulus, leukocyte cohesion and growth of endothelial permeability.* ***b.*** *input of monocytes and transformation into macrophages and progressively into foam cells; sediment of the matrix and recall of smooth muscle cells.* ***c****. development of the necrotic heart and the fibrous cap; neovascularisation.* ***d.*** *erosion of the fibrous cap within unstable plaques and breakage of the plaque.*

Leucocytes penetrate through the arterial wall thanks to chemotactic cytokines, amongst which chemotactic protein-1 for monocytes (MCP-1) produced by the endothelium as a response to oxidized lipoproteins.

Atheromas also express a trio of selective chemokines for lymphocytes: the protein-10 interferon-γ inducible (IP-10), the chemotactic factor α for T cells (I-TAC), and the monokine provoked by the interferon γ (MIG). The interferon γ present in atheromas plaques persuades the genes, which codify for this family of chemotactic cytokines for T cells.

- ACCUMULATION OF LIPIDS AT A MONOCYTE LEVEL: it determines the development of a macrophage full of lipids (foam cells). Such a process is facilitated by a few molecules such as A scavenger receptor, CD36, and macrosialin. After that, foamy cells multiply under the influence of interleukin -3 and the factor, which encourages the growth of macrophages.

- MIGRATION AND PROLIFERATION OF SMOOTH MUSCLE CELLS: these cells derive from tonaca media as a response to a platelet-derived growth factor (PDGF) caused by activated macrophages. Smooth muscle cells synthesize collagen, elastin, and glycoproteins following the stimulus of fibroblast growth factor (FGF) and TGF-β, contained in platelet granules.

- MINERALIZATION: plaques broaden calcification areas during their evolution. A few subsets of smooth muscle cells can foster the calcification through an increase of cytokines' secretion, such as morphogenetic proteins of the bone.

- ANGIOGENESIS: in the plaques, a dense vascular network, often occurs as a response to super angiogenic peptides expressed in the atheromas (VEGF-FGF, oncostatin M).

The description of the presence of new blood vessels in the atherosclerotic carotid plaques was described initially in 1936. It was only at the beginning of the 1980s that scientific projects were conducted to report on new blood vessels within the atherosclerotic carotid plaque.

Lymphatic vessels within the atherosclerotic plaque

Recently, investigations regarding atherosclerosis have pointed out the presence of lymphatic vessels inside the plaque.

Lymphatic vessels are involved in different pathologies such as lymphedema, inflammation, autoimmune diseases, and tumors. Regardless of the presence of a complex physiopathology, literature about lymphatic vessels within atherosclerosis is insufficient. [27]

In normal conditions, lymphatic vessels are made up of a dense network that extends itself inside the tissue, and often, they go with blood vessels, which are mainly venous. [28] Lymphatic vessels are founded on a single layer of endothelial cells, united with each other in a spasmodic way (button-like cell junction) and by unidirectional tubes. [29]

In order to avoid their collapse, they are anchored to the extracellular matrix through strands.

The origin of lymphatic vessels is controversial, taking two potential sources of lymphatic endothelial cells into consideration: blood vessels and mesenchymal cells. [30] During the embryogenesis, the development of lymphatic vessels begins after the stabilization of the advancement of blood vessels; especially, lymphatic vessels appear as if they originate from venous endothelial cells, that resettle in the neighboring tissue. Prox1 stimulates such differentiation and migration. (fig.4) [31] [32] The bond between vascular endothelial growth factor C and D (VEGF-C e VEGF-D), and the receptor R-3 (VEGFR-3) induces lymphogenesis, motivating proliferation, migration, and survival of lymphatic endothelial cells in vitro. [33]

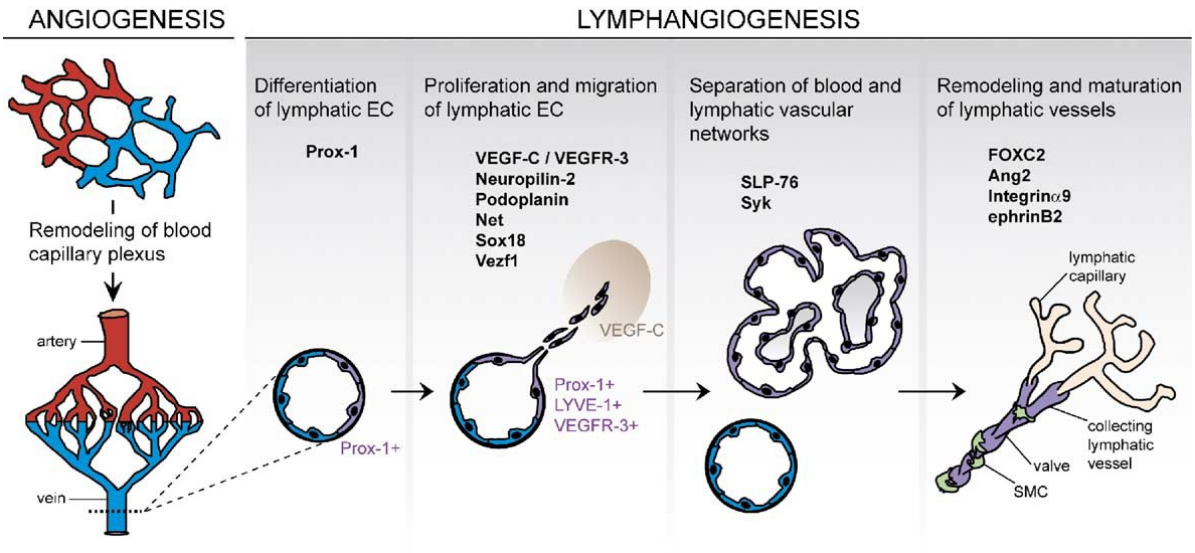


Figure 4 Development of lymphatic vasculature during embryogenesis. After the remodeling of the blood vasculature, lymphatic endothelial cells differentiate and sprout from the major veins to form lymphatic capillary plexus.

Recent studies concerning lymphatic vessels in inflammatory processes show how macrophages are involved in lymphogenesis, producing growth lymphogenic factors (i.e., VEGF-C e D). [34] [35] [36] In conditions of normality, lymphatic vessels of an artery locate primarily in the vicinity of the adventitia (fig.5).

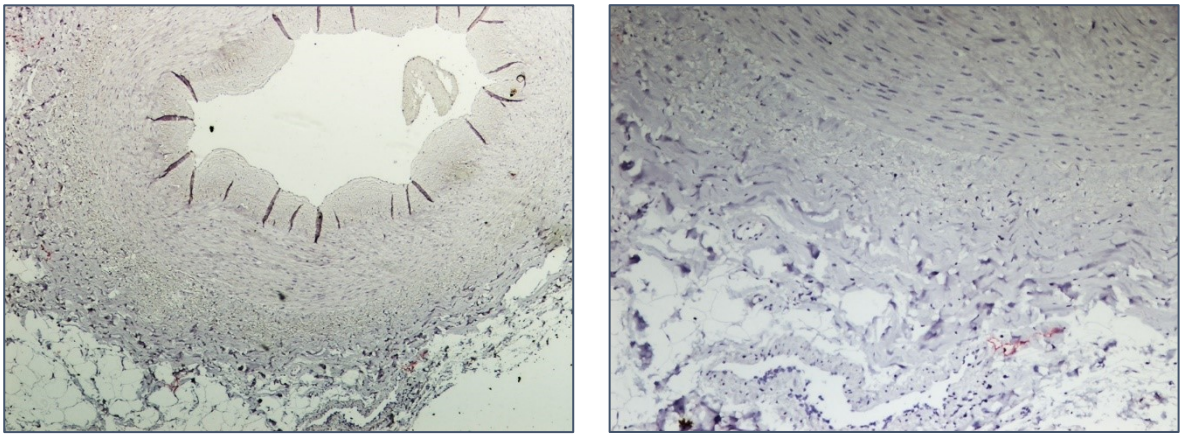


Figure 5 Section of mammary artery, lymphatic vessels (D2-40)

The inflammation rapidly provokes a proliferation of lymphatic endothelial cells. [37]

In literature there are few studies about lymphatic vessels in atherosclerotic carotid plaques.

Nakano et al. researched coronary atherosclerotic arteries, and they observed that lymphatic vessels predominantly locate in the intima layer. [38]

According to Kholova, in atherosclerotic coronary lesions, full of calcium crystals and cholesterol, a relevant lymphogenesis is the intima and media level; instead on an adventitia level, there isn't a proliferation of lymphatic vessels. [39]

Meanwhile, in another investigation lead by Xu and his collaborators, they found that lymphatic vessels are primarily localized at the level of the adventitia. A second study, carried out by Drodtz, progressively confirmed such fact concerning 20 atherosclerotic carotid plaques, where lymphatic vessels, identified at adventitial and pre-adventitial level, were marked with Lyme-1 and with an anti-podoplanin antibody. [40]

Eliska et al., in their analysis regarding healthy coronary and atherosclerotic arteries, concluded that lymphatic vessels were only present in specific fit arteries in the periadventitial region. They also discovered that such vessels, both in healthy coronary and in atherosclerotic ones, did not overstep the adventitial layer, not going through the medium and the intima of the vessel. [41]

Based on the complexity of the atherosclerotic lesion, the number of adventitial lymphatic vessels could seem to expand. Drodtz, in an analysis directed on atherosclerotic plaques of big abdominal

human vessels (aorta and iliac arteries), took in that the total of lymphatic vessels augments with the progression of atherosclerosis as assessed by the intima layer. According to the author, there is a statistically significant ($p<0.05$) correlation between adventitial lymphatics (marked by Lyme-1) and the intima thickness and the patients'age. [42]

The role of lymphatic vessels in atherosclerotic plaques is not yet known. It is thought that the lymphatic system, like in normal conditions, has three functions: the maintenance of the balance of interstitial fluids, transport and cholesterol's and lipid metabolism, and they take part in the immune response.

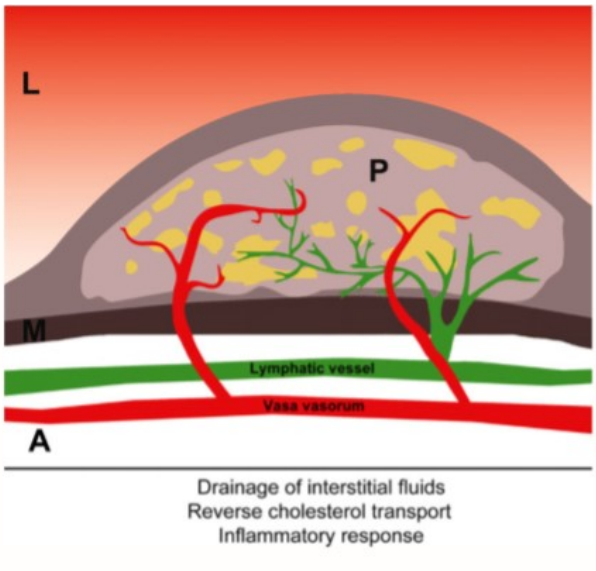


Figure 6 Role of lymphatic vessels

Nakano and his colleagues found that lymphatic vessels played a vital role in the interstitial fluid's outflow from the atherosclerotic carotid plaque. They noticed that the number of lymphatic vessels in atherosclerotic coronary arteries was inferior to the number of adventitial lymphatic vessels within healthy coronary arteries. This fact would determine the growth of interstitial fluid in atherosclerotic plaques, an increase of pressure in the plaque and an alteration in the equilibrium of interstitial fluids.

As a result, this causes an alteration of oxygenation, microcirculation of the plaque, and the following progress of the atherosclerotic lesion and the breakage's risk of the same. [38]

Lymphatic vessels, in particular adventitial ones, could be involved with the transportation of cholesterol in accumulated lipids from the artery wall to the blood.

In a study conducted on hypercholesterolemic apoe-/- mice that cause structural and functional insufficiencies of the vessel's wall, cholesterol is accumulated in the vessels' walls.

Therefore, they reported that the reactivation of the normal vessel's structure and of the liquid's drainage on behalf of lymphatics would improve the clearance of cholesterol and of plaque's lipids. [30]

The clearance's reduction of the cholesterol attributable to an alteration of lymphatic vessels determines the accumulation of cholesterol and lipids in the carotid atherosclerotic plaque.

Lastly, lymphatic vessels actively partake in the transportation of immune cells. The stockpile of lipids and cholesterol can activate pro-inflammatory cytokines (i.e., TNF α) and reactive species of oxygen, which further accelerates the inflammation of the atherosclerotic plaque, recruiting other useful inflammatory cells essential for the progression and variability of the plaque. [43]

Neangiogenesis in atherosclerotic plaque

The presence of new blood vessels in the atherosclerotic plaque and their absence in the intima layer of the vessel in normal conditions, were firstly described by the anatomopathologist Paterson in 1936. [44]

In 1980 researchers started to focus on intraplaque new vessels, and the percentage of angiogenesis's studies increased considerably from 0.2% in 1984 to 2.8% in 2007. [45] [46]

The leading cause which governs neoangiogenesis is hypoxia, as indicated in several of Sluimer's investigations. The shortage of oxygen inside the atherosclerotic plaque determined by the activation of intraplaque inflammatory cells (i.e. monocytes and macrophages).

Hypoxia determines the release, on behalf of inflammatory cells, of hypoxia-inducible factors (HIF) and of metalloproteinases (MMP-2, MMP-9), which participate in the instability of the plaque.

The new blood vessels originate from the migration and proliferation in the vascular areas of endothelial cells coming from adventitial vasa vasorum [47], mainly stimulated by the VEGF-A. VEGF and its principal receptors (VEGFR-1 e VEGFR-2), which nurture the proliferation and formation of tubules throughout the neoangiogenesis.

Angiopoietin 1 and 2 are ligands of the Tie-2 receptor of endothelial cells, and they cover a fundamental role during the final phase of the maturation of the new vessel. HIF and VEGF-A, therefore, induce Ang-2 to destabilize the interaction between pericytes and endothelial cells, allowing the vessel to develop. Ang-1, together with the growth factor deriving from platelets, on the contrary, it covers the role of stabilizing junctures between endothelial cells, encouraging the vessel's stability. [48]

New vessels are fragile, immature, and irregular as compared to vasa vasorum [49], they are characterized by a disjointed basement membrane and by a low number of junctures narrow between endotheliocytes. [50]

Their brittleness causes accretion of the breakage's risk, thus a threat of intraplaque's hemorrhage making the plaque vulnerable and increasing the hazard of the appearance of cardio-cerebrovascular events. As is generally known in the literature, the close relation between new intraplaque vessels and the danger of a plaque's fracture does exist. [51].

Moreno et al. observed that the density of newly formed microvessels was higher than that of complex lipid plaques as compared to calcific plaques. Furthermore, they noticed that new vessels' thickness was superior to that of plaques, where there was an important inflammatory infiltrate. Thus, new vessels facilitate the entry of inflammatory cells in the plaque.

Jeziorska et al. highlighted that within 92% of analyzed plaques, neovascularized areas are located in the vicinity of areas that contain a crucial inflammatory infiltrate, primarily represented by macrophages, T lymphocytes, and mast cells. [52]

New vessels show a rising expression of adhesion molecules, such as E-selectin, CD-40, ICAM-1, VCAM-1. In a study that was conducted by O'Brian, he noted that the manifestation of V-CAM-1 in the coronary artery plaque is rarely represented by endotheliocytes of the lumen (20%). Although, it is mostly displayed in areas intraplaque neovascularisation, rich in the inflammatory infiltrate, proving the pivotal role hematic new vessels in enrolment of inflammatory cells. [53]

Moreover, the transfer of red blood cells through new intraplaque vessels depicts another necessary passage within the creation of the 'vulnerable' plaque. Hemoglobin, thus, provokes oxidative tissue damage, with the formation of oxidizing agents (i.e., cox, INOS).

The removal of hemoglobin, in the form of heme-haptoglobin, takes place through macrophages with a link employing CD163. The production of oxidizing agents negotiated by haemoglobin activates the factor of pro-inflammatory transcription NF-KB that promotes the fabrication of pro-inflammatory cytokines and the cellular recruitment of monocytes/macrophages and T lymphocytes.

AIM OF THE RESEARCH

The primary prevention was implemented through the correction of risk factors, that include: the reduction of arterial pressure values below 140/90 mmHG, LDL cholesterol values under 100 mg/dl and triglycerides beneath 150 mg/dl and an increase of the 'high-density lipoprotein' HDL to superior values of 40 mg/dl. The secondary preclusion was characterized by pharmaceutical therapy (antiplatelets/anticoagulant and statins) and by surgical therapy (carotid thromboendarterectomy and carotid stenting), which are both fundamental in order to forestall the progression of ischemic cerebrovascular events. These prove to be grade A recommendations according to the surgical vascular society [56].

The analysis of macroscopic characteristics concerning the atherosclerotic carotid plaque, the degree of stenosis and the plaque's morphology, carried out via diagnostic radiology investigations (carotid ECD, CT scan and/or MRI) currently represent the factors based on the indication of surgical/pharmaceutical treatment for patients affected by carotid stenosis. Although, the macroscopic study of the carotid plaque doesn't allow us to identify the likelihood of cerebrovascular events and the timeframe of such events from the diagnostic radiology.

With regards to immunohistochemical features of the plaque, with cytokines produced by the inflammatory infiltrate and on the neoangiogenesis, many scientific literature studies do exist, but the real mechanisms at the heart of the growth of the atherosclerotic carotid plaque and its instability are not known yet. We are aware that the evolution and the stability of the atherosclerotic carotid plaque rely on the inflammatory properties of this last one.

The gradual progression of the atherosclerotic carotid plaque is especially caused by the creation of new vessels, that are responsible for the migration of inflammatory cells and the intraplaque hemorrhage. In parallel with the generation of new blood vessels, we witness the advancement of new lymphatic vessels, which role appears to be still unknown.

During our previous studies of the plaques, the lymphatic vessels in the plaque had been identified involuntarily and surprisingly.

The curiosity about lymphatic vessels in the carotid atherosclerotic plaques led to the birth of this study.

In particular, the aim of this research is the identification and localization of lymphatic vessels in the atherosclerotic carotid plaques and the evaluation of the relationship between neo-lymphatic vessels with new blood ones and inflammatory cells.

In the future the comprehension of the existing connection between neoangiogenesis and neolymphogenesis and inflammatory infiltrate could be the foundation on which to build predictive models of vulnerability identifying premature patients with carotid stenosis at risk of evolving into a TIA or an ischemic stroke.

In this regard, it is essential to deepen the microscopic study of the atherosclerotic carotid plaque in order to pinpoint possible pharmacological treatments to antagonize the atherosclerosis' process or to forbid the development of unstable plaques inhibiting some of the proatherogenic mechanisms.

MATERIALS AND METHODS

Patient's recruitment and clinical characteristics

31 patients with carotid stenosis have been recruited for this study; they have undergone Carotid Throm-boendarterectomy (TEA) surgery. 10 morphologically hard plaques have been used to develop procedures.

Thus, 21 plaques were analyzed in this study. Out of the 21 patients involved in the research, anamnestic-clinical information of interest has been collected. The gathered information includes sex, age, symptomatology, comorbidity, anti-hypertensive, hypoglycaemic, and lipid-lowering.

Clinical characteristics of the cohort			
	total	*soft plaque*	*mix plaques*
Demographic Data			
n	21	13 (62%)	8 (38%)
F/M	7/14	2/11	5/3
Age (years)	73	72	74
Symptomatic/Asymtomatic	9/12	5/8	4/4
Stenosis %	78,33	79,61	77,85
Co-morbidities, (%)			
Smokers	13 (62%)	8 (61%)	5 (39%)
Hypertension	19 (90%)	11 (58%)	8 (42%)
Diabetes	4 (19%)	2 (50%)	2 (50%)
Dyslipidemia	15 (71%)	4 (27%)	11 (73%)
Chronic renal insufficiency	6 (29%)	3 (50%)	3 (50%)
Coronary artery disease	4 (20%)	4 (100%)	0
Chronic obstructive pulmunary disease	3 (14%)	2 (67%)	1 (33%)
ASCVD risk (10 years), %	33,88	30,06	41,54
Pharmacologica treatment			
Antiplatelet/anticoagulant drugs	18 (86%)	12 (67%)	6 (33%)
Statins	17 (81%)	11 (65%)	6 (35%)
β-blockers	7 (33%)	5 (71%)	2 (29%)
Ace inhibitors/AT1R-antagonists	15 (71%)	10 (67%)	5 (33%)
Ca++ channel blockers	6 (29%)	3 (50%)	3 (50%)
Hypoglicemic drugs	4 (19%)	2 (50%)	2 (50%)

Table 1 Anamnestic-clinical information of Patients

Patients affected by autoimmune diseases, neoplasia, or recent infections, have been excluded.
The 21 enrolled patients presented an average age of 73; 14 patients were male (67%), and 7 were female (33%). 9 patients were symptomatic (43%), and 12 were asymptomatic (57%) due to cerebral ischemic events (TIA and or a stroke).
Patients have been considered symptomatic if they could have presented an ischemic transient attack or a stroke in the carotid distribution territory in the previous six months [54].
They were not considered symptomatic, based on OMS's definition, patients with a positive medical history including loss of consciousness, vertigo, global transient amnesia, drop attack, generalized asthenia, and state of confusion.
Of the 9 symptomatic patients (2 females and 7 males), TIA has been diagnosed in 6 cases, and a minor strike established in 3 cases.
90% of patients were affected by arterial hypertension, 71% by dyslipidemia, 29% by mild renal impairment, 19% by diabetes mellitus, 20% by cardiac diseases, 14% by obstructive pulmonary illnesses, and 62% were smokers. Three patients (14%) suffer from glucose intolerance, and they show glycemic values on an empty stomach above 125 mg/dl.

Amongst the primary clinical evaluated outcomes, in addition to TIA/stroke and perioperative death, the atherosclerotic cardiovascular disease risk (ASCVD risk) was also assessed, which is defined as the risk of myocardial infarction and of a stroke in 10 years. These results are based on an equation, which takes into consideration a patient's race and age, the total cholesterol (with levels between 130 mg/dl and 320 mg/dl), HDL cholesterol (20-100 mg/dl), average systolic pressure, presence of pharmacological treatment for arterial hypertension, diabetes mellitus, and smoking.

The ASCVD's average risk in 21 patients is 33,88%; in patients who are carriers of morphologically soft plaques, the percentage is 30,06%, and in mixed plaque-carriers are 41,54%.

Eighteen patients swallowed at least one anti-aggregating/anti-coagulant medicine. (The three patients who did not take such prescriptions manifested a cerebral-vascular ischemic event, and they revealed morphologically soft plaques). 17 of the patients (81%) followed a pharmacological therapy with statin; 12 patients proceeded with atorvastatin with a comprehensive dosage between 20 and 80 mg/daily; 3 patients took simvastatin of 20 mg/daily; 1 patient's therapy requested a dosage of 10 mg/daily and another who had to take 10 mg of ezetimibe jointly with simvastatin 20 mg/daily.

Furthermore, 7 patients (33%) who were treated with β-blockers medications and 15 patients (71%) were cured with ace inhibitors, and 6 patients (29%) took calcium channel blockers. The four patients affected by diabetes mellitus were on a pharmaceutical therapy with oral hypoglycaemic agents, and no one assumed insulin.

All patients underwent a complete clinical and neurological pre-operative evaluation. Thus, during the pre-op phase, the patient was exposed to an instrumental investigation on supra-aortic vessels employing EcoColorDoppler. This medical test ran in order to analyze the morphology and composition of the atherosclerotic plaque (lipidic (soft), mixed or calcified (hard), to consider the plaque's surface (regular, irregular, ulcerated) and its stenosis level. Before proceeding with surgery, every patient was subjected to carotid computed-tomography angiography and intra-cranial circulation to deliver accurate measurements on the stenosis level and to precisely give an estimate of the plaque's composition through the "Hounsfield value". (tab. 2) [55]

Hounsfield Units (HU)	**Composition of the plaque**
20 - 60	**Lipid (soft)**
60 - 130	**Mixed**
≥ 130	**Calcific (hard)**

Table 2 Hounsfield Units range

All symptomatic patients went through a CT brain and a magnetic resonance imaging (MRI) of brain diffusion-perfusion for the analysis of ischemic wound's outcome. Moreover, all patients had to go through medical investigations before the operation in order to obtain a pre-operation analysis: ECG, complete hematic routine, and a thorax's X-ray.

The enrolled patients experienced a carotid revascularisation's surgery through carotid – thromboendarterectomy (TEA) by semi-eversion at the Unit of Vascular Surgery of University Hospital Macchi, ASST Sette Laghi, in Varese, Italy.

Carotid TEA was implemented according to the European Society for Vascular Surgery's (ESVS) guidelines under which symptomatic patients must be treated if they show carotid plaques super by 50% (NASCET) with a risk of a stroke or of a perioperative mortality 6%.

With regards to asymptomatic patients, they are treated when they present carotid stenosis included between 70 and 90% with a post-surgery risk of stroke and minor death of 3%.
Twenty-one patients presented medium stenosis of 78,33%, measured according to Nascet's criteria: 13 (62%) carotid plaques were morphologically soft, and 8 (38%) were morphologically mixed. In patients with soft-plaque the medium stenosis is 79,61%, and in the mixed ones, it is of 77,85%.

The operations executed on 21 patients were carried out via under-local regional anaesthesia and they ran clinical monitoring. Afterward they performed Transcranial Doppler (TCD) for the analysis of possible embolic manifestations during the cross-clamping and declamping maneuvers and for the evaluation of compensatory effect determined by Willis polygon. It was not necessary to position a shunt. The medium clamping time was 22 minutes. There was no sign of TIA, stroke or perioperative mortality

The atherosclerotic carotid plaque resulted from the carotid TEA was initially placed in physiological solution at 37° to remove blood residues from the surface, and it was successively positioned in paraformaldehyde at 4% and sent for microscopic immunohistochemical analysis.

Immunohistochemical analysis: preparation *and sample's processing*

The atherosclerotic plaques collected after the TEA carotid surgery, have been put in paraformaldehyde at 4% phosphate swab for at least 48 hours and sent to MIA's Consortium laboratory of Milano-Bicocca for immunohistochemical analysis. At the laboratory, as fixation occurred, the plaque was included in paraffin using automatic processors based on a standard method adopted by all laboratories of Pathological Anatomy. Processing roughly lasts 18 hours.

Reagent	Time min	Temperature °C
Ethanol 70%	60	22°
Ethanol 95%	90	22°
Ethanol 95%	120	22°
Ethanol 100%	60	22°
Ethanol 100%	90	22°
Ethanol 100%	120	22°
Xylol	120	22°
Xylol	120	22°
Paraffin	90	60°
Paraffin	90	60°
Paraffin	120	60°

Table 3 Reagenta, timing and temperature of samples

Once paraffin is soaked in, the pieces are included in biology pathology boxes, and from the embedded tissues, slices of 3-10 μm are obtained, and they are assembled on the slide compartment, using a steel blade microtome.

For an initial histochemical analysis, the obtained samples are dyed in hematoxylin-eosin. Hematoxylin-eosin is a two-toned coloring based on the different pH measure of the various tissues and organelles components of the cell. The nucleus and the cytoplasm's acidic elements (ribosomes, acid secretion) are colored in purple from hematoxylin, which is a basic colorant. Meanwhile, the cytoplasm and the different kinds of basic tissues (muscular, connective, and bone) are dyed in pink, more or less intense, from an eosin's acid mixture.

Other slide sections are used to identify immunohistochemical investigations through the use of specific antibodies that allow characterizing cellular and tissue components.

Numerous passages executed in different reagents:

- Himmuno-phosphatase on paraffin
- Xylol: 2 passages, 30 minutes long
- Alcohol 100°: 2 passages, 3 minutes long
- Alcohol 96°: 1 passage, 3minutes long
- Distilled water: 2 swift passages

At this stage it is possible to carry out the antigen exposure (if required).

For identification, slides are placed in EDTA 0,1mM or on a 5mM citrate swab in the designated containers. Such containers are placed inside a pressure cooker containing 700 ml of distilled water. The distilled water is brought to boil. After that, the lid is closed for about 5 minutes. After going through this phase, the pot is removed from the grill and brought back to ambient pressure by opening the valve.

This operation is repeated for a total of three passages. Subsequently, repositories are removed out of the pressure cooker, and after 5 minutes, lids are opened.

Slides are washed 3 times, for 5 minutes, with TBS (tris swab 0,5M in buffered saline solution and Triton)

It is necessary to proceed by drying the slides and processing them as it follows:

- PBS+BSA (phosphate buffer solution 0,05M in bovine albumin in saline solution at 1%).
- Primary antibody 1h RT (alternatively ON at 4°) 1 passage, 10 minutes long
- Rinse slide with TBS+T one by one

- TBS+T 3 washes, 5 minutes long
- Biotinylated secondary antibodies (AB2 Dako REAL K5005): 1 passage, 15 minutes long
- TBS+T: 3 washes, 5 minutes long
- Streptavidin conjugated with alkaline phosphatase (AP Dako REAL K5005): 1 passage, 15 minutes long
- TBS+T: 3 washes, 5 minutes long

Progression in chromogen prepared according to the advisable procedure in the kit (RED Dako REAL K 5005): after 20-30 minutes check on the microscope:

- Counterstain with Carazzi's hematoxylin
- Rinse with springwater
- Dry the slides

Soft and mixed plaques have been examined, and for each plaque, each marker has been taken into account:

- CD3 antibody used for T lymphocyte identification
- CD 4 antibody used for T lymphocyte (helper) identification
- CD8 antibody used to identify cytotoxic T lymphocytes
- CD11b antibody used to recognize cells of the monocyte-macrophage line, especially taking action on the admission and phagocytosis performed by macrophages.
- CD14 antibody used for the recognition of monocytes during their distinction
- CD34 antibody used to determine progenitor cells of the hemopoietic line and endothelial cells.
- CD66b antibody used to label triggered neutrophils
- CD68 used to identify macrophages (lysosomes, phagocytosis capability)
- CD163 antibody used for the identification of monocytes/macrophages
- CD208 antibody used to put a name on dendritic cells
- CD 209 antibody used to identify dendritic cells
- IL-8 chemokine is pro-inflammatory and pro-angiogenic. Produced by endothelial cells, macrophages, and muscular cells.
- TGF-β growth-factor beta that monitors the cellular proliferation
- TRYPTASE antibody used to identify mast cells
- INOS (inducible nitric oxide synthase) enzyme that produces nitric oxide
- ULEX marker used to determine endothelial cells of blood vessels of different sizes

- HLA-DR transmembrane molecule expressed by T lymphocytes, activated lymphocytes, macrophages, and monocytes.
- MMP-2, MMP-9 metalloproteinases able to downgrade various proteins of the extracellular matrix
- Neutrophil elastase: protease produced during inflammatory processes able to degrade the extracellular matrix
- Arginase 1 enzyme, which hydrolyzes with an inflammatory effect
- D2-40 (podoplanin) marker used to identify lymphatic endothelial cells
- Ki67 useful for the assessment of the cellular proliferation from G1phase

Immunohistochemical coloring has been scanned using Aperio Technologies®, Vista, California, U.S., and quantified using *the MATLAB*®portal.
Certain carotid atherosclerotic plaque sections have been analyzed via confocal microscope.

Automatic analysis' software

A designated software has been invented in collaboration with department of the University of Studies of Milan, in order to carry out a quantitative and reproducible assessment of the images obtained from immunohistochemical analysis, that will allow not to lose sight of the entirety of the plaque, where markers do not appear evenly spread throughout.

This software authorizes automatic analysis, which is repeatable and quantitative of the images that came out of the immunohistochemical examination of the carotid atherosclerotic plaques.

In order to re-elaborate them, immunolabelled sections have been scanned, producing hundreds of digital colour images (RGB) of large size/resolution (often >4Gb). This software uses Matlab's language for automatic tracking (segmentation) of markers, from digital colour images of sections of the immunolabelled plaque with alkaline phosphatize.

After segmenting markers, the software must quantify:

- The consistency of distribution of markers in every section (section's density) and in all divisions of one same patient (a parient's solidity);
- Measures that contrast both the density of different portions, and the consistency of diverse patients;

- An image (map) of localization for each segmented marker and an image (map) which shows tracking of all different markers segmented by standard sections, which show the same plaque's area;
- Measurements which enable the comparison between two maps, or among one map and morphological structures, identified by expert users, who describe the macroscopic shape of the section.

In this regard, algorithms have been realized to segment the section's interest area (ROI), excluding it from the "background" of bright colour. Therefore, in the ROI algorithms of segmentation of markers have been applied, namely, the algorithms that look for pixel's areas of various shapes, characterized by an 'averagely' red colour.

Segmented areas have been quantified by measuring density per section and patient, and they have been visually located through the creation of maps to whom should be applied suitable transformations of distance for the mutual comparison. These transformed must resume both comparative relations among maps and the contrast between a map and the morphological structures of a plaque.

There are some problems linked with the software, and they are characterized primarily by substantial sizes and resolutions of each image, as high computational costs occur in terms of space and time. To resolve this set of problems, experts have developed fast and straightforward computational algorithms, which are independent of global image features (i.e., medium luminance, medium contrast) to apply to image's subregions and to progressively put together the achieved results.

Another set of problems is represented by the presence of pigments with a structure and colouring similar to that of markers, to segment with the possibility of achieving an inaccurate segmentation of unspecified structures with similar colour shades (false positives). For this reason, in order to obtain sensible segmentation results (low or invalid marker loss, or somewhat elevated percentages of true positives) and specific results (low or invalid percentage of false positives), they have designed software capable of learning examples semi-automatically given by expert users. Values of pixels establish the examples in the images of sections and of their label (ground truth) of marker pixels (pixels that belong to markers) and no-marker pixels.

Thanks to this software, all sections received by each plaque and patient have been thoroughly examined.

RESULTS

Immunohistochemical analysis was executed on 10 atherosclerotic carotid plaques hard in the first part of the investigation.

In spite of the trouble had with the preparation of slides, in relation to the difficulties of cutting the same ones for the presence of intraplaque calcium, from the analysed data it has been observed that there is an absence of inflammatory cell population, a lack of inflammatory cytokines and molecules and lymphatic and arterial vessels in hard atherosclerotic plaques.

Therefore, our study went on by enlisting patients who were bearers of, morphologically defined, soft and mixed plaques, after a radiologic evaluation via ECD and successively, by means of angio-CT TSA scan.

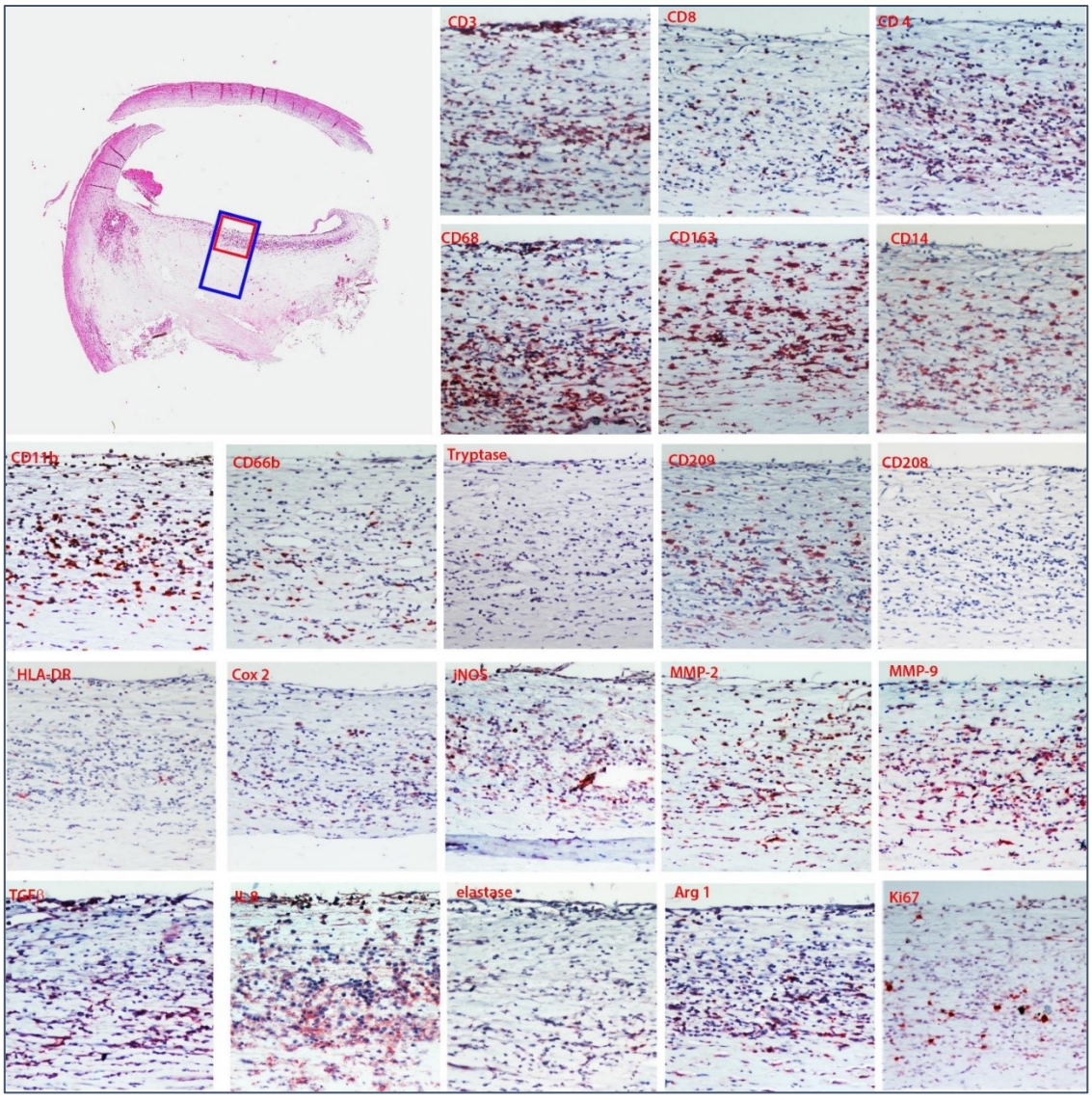


Figure 7 Immunohistochemical analysis of soft atherosclerotic carotid plaque

Immunohistochemical analysis of atherosclerotic carotid plaques, using different markers, which identified cells and molecules that were involved with inflammatory and neovascularisation, has shown an extreme variability of expression amongst the several inspected plaques, notwithstanding that areas neighboring the lipidic and the fibrous cap have been evaluated.

Making a comparison with immunohistochemical images obtained after the analysis of two plaques, one lipidic and one mixed, it is observed that the markers' display is very variable (fig 7-8).

In both illustrations, the first image presents a whole plaque's section examined with hematoxylin-eosin.

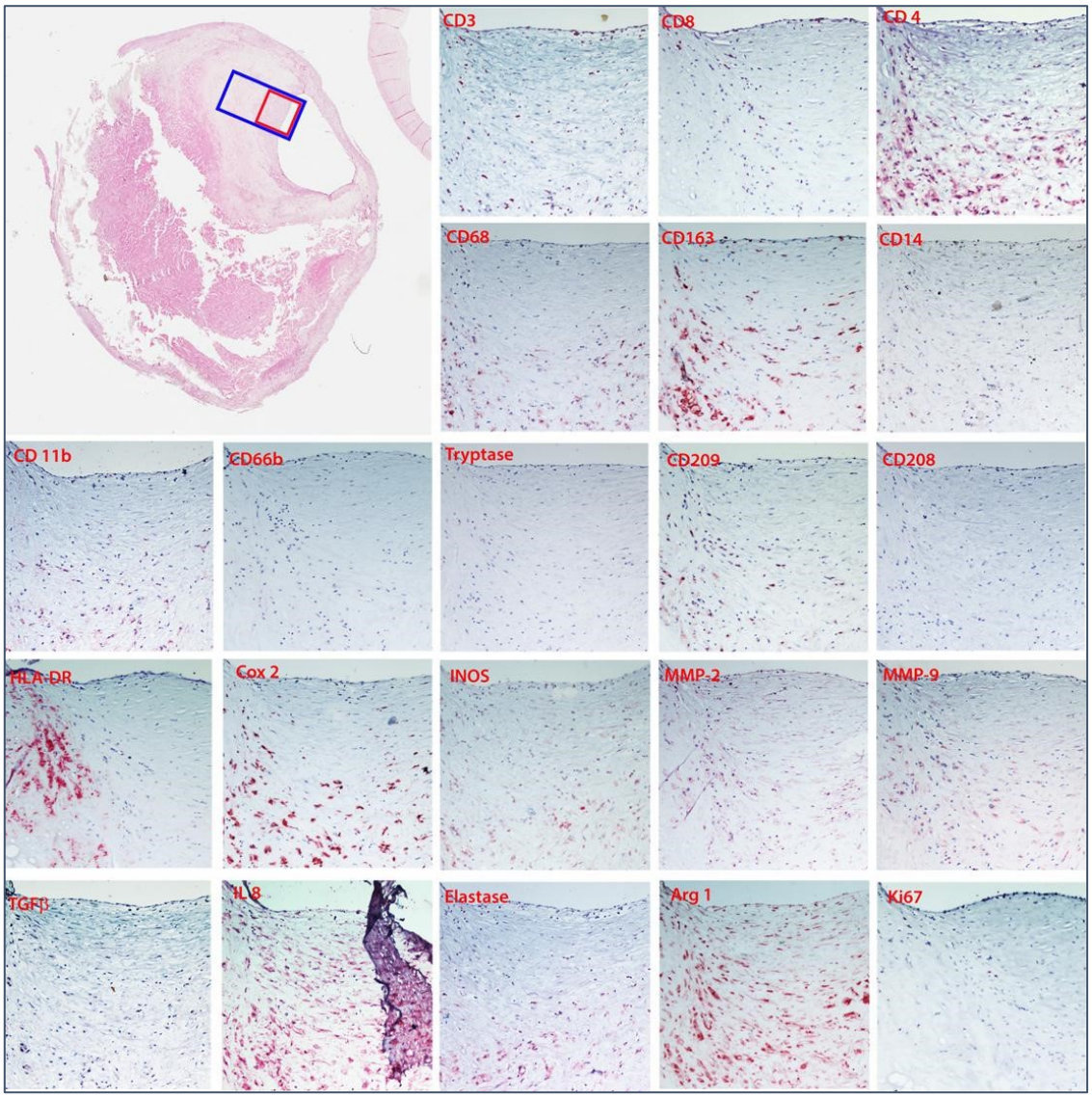


Figure 8 Immunohistochemical analysis of mixed atherosclerotic carotid plaque

In the soft inspected atherosclerotic carotid plaque's portion (fig. 7), especially in the first section coloured with hematoxylin-eosin, it is possible to observe the presence of an important inflammatory

infiltrated. It is mainly represented by cells of the monocyte-macrophage line (CD-11b, CD-14, CD-68, CD-163), by T lymphocytes (CD-3, CD-4, CD-8), by dendritic cells (CD-208, CD-209), by (Tryptase) mast cells and also by the existence of molecules implied with cellular proliferation (Ki67) and with inflammatory-atherosclerotic process: metalloproteases, Elastase, arginase 1, TGF-β, Cox-2, IL-8 ed Inos.

Such markers result to be less expressed in the mixed atherosclerotic carotid plaque examined in picture 8. In such plaque, we can observe the presence of an inflammatory lymphocyte T infiltrate weakly expressed in CD4 and the presence of a few cells of the monocyte-macrophage line, predominantly CD-163. Even the molecules' expression implied in the inflammatory-atherosclerotic is weak.

Taking into consideration the extreme display variability of the markers inside the atherosclerotic carotid plaques and the extremely changeable nature of plaques amongst different patients, the focus was on new hematic and lymphatic vessels, and macrophage and lymphocytic.

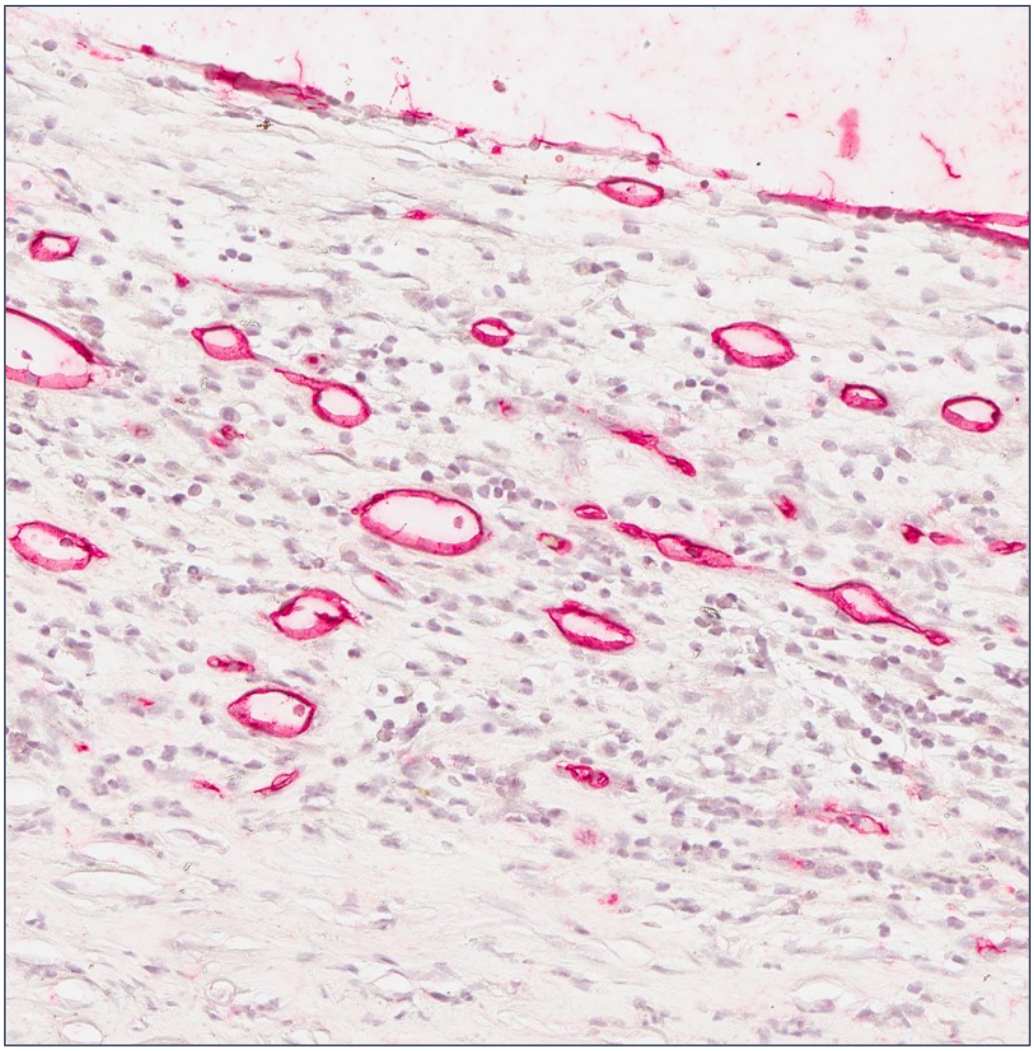


Figure 9 Blood vessels (marked with Ulex) in atherosclerotic plaque

Regarding new hematic vessels, inside the atherosclerotic carotid plaque numerous studies in literature exist and their immunohistochemistry identification results simple, appearing as they composed by a single layer of mature endothelial cells. Furthermore, they do not result flatted and they present tubular structures. To recognise blood endothelial vessels markers are multiple, and we have made the decision to use ULEX e CD-34 (Fig. 9).

On the other hand, to date, investigations on lymphatic vessels are less copious and at times their identification appears demanding because they look like flatted structures constituted by a single-layer of lymphatic endothelial cells (Fig. 10). The most utilized markers to classify the lymphatic endothelium, are characterized by the podoplanin (D2-40) and by the VEGFR3.

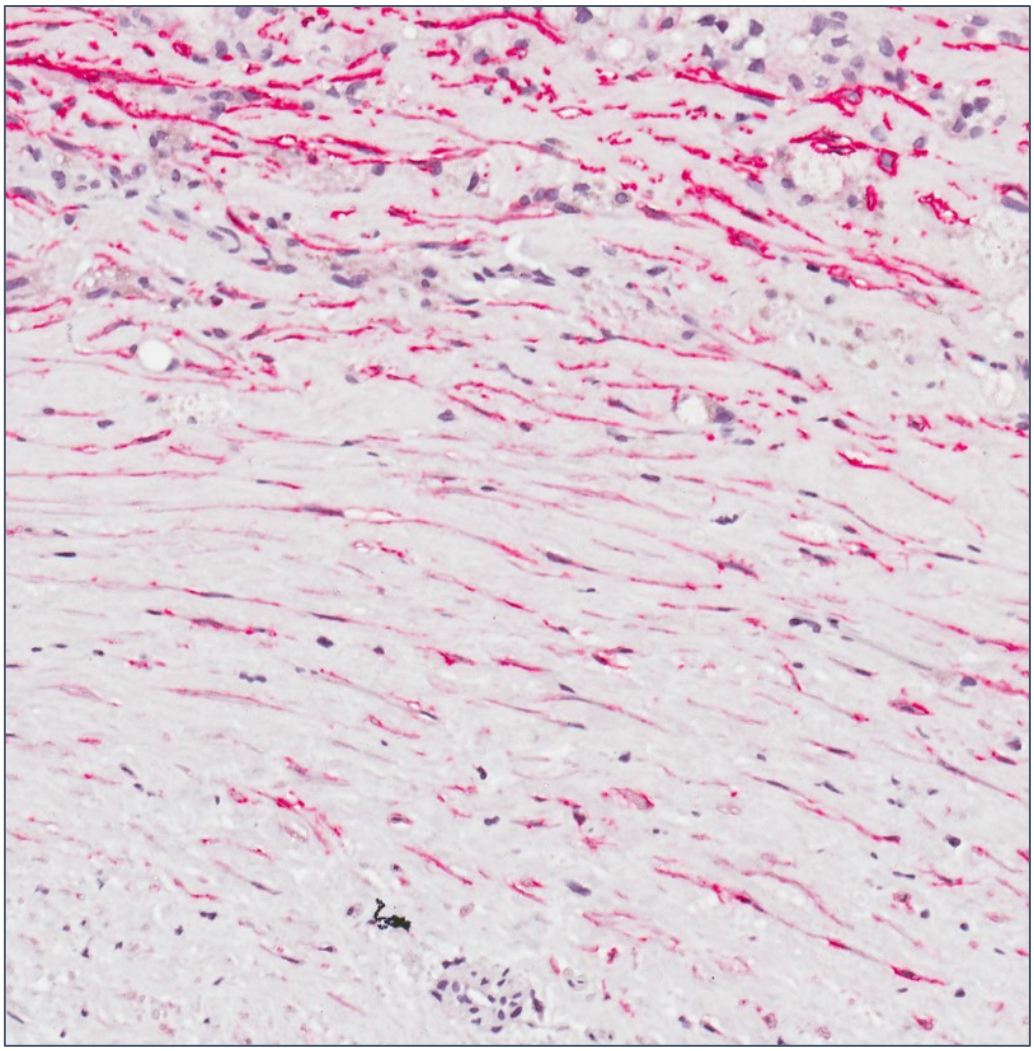


Figure 10 lymphatic vessels (marked with D2-40) in atherosclerotic plaque

Considering the radical heterogeneity of the sample and the difficulty to recognise lymphatic endothelial cells, with a view to have confirmation that the pinpointed vessel were actually new lymphatic vessels, a double marking was done with D2-40 3 VEGFR3 on atherosclerotic carotid plaques using a confocal microscope. Thus, going through an analysis on the confocal microscope, in picture 11a two vessels made of lymphatic endothelial cells in red are observed.

Thereupon, a confocal estimate was carried out on a section adjacent to the first, of labeling with VEGFR3 (VEGF's receptor typical of lymphatic endothelial cells), identified in the colour blue (fig. 11b). Lastly, another section adjacent to the first ones, with double marking, was studied closely on the confocal microscope (D2-40 e VEGFR3). This confirms that immunolabelling structures show both colourings simultaneously (fig. 11c).

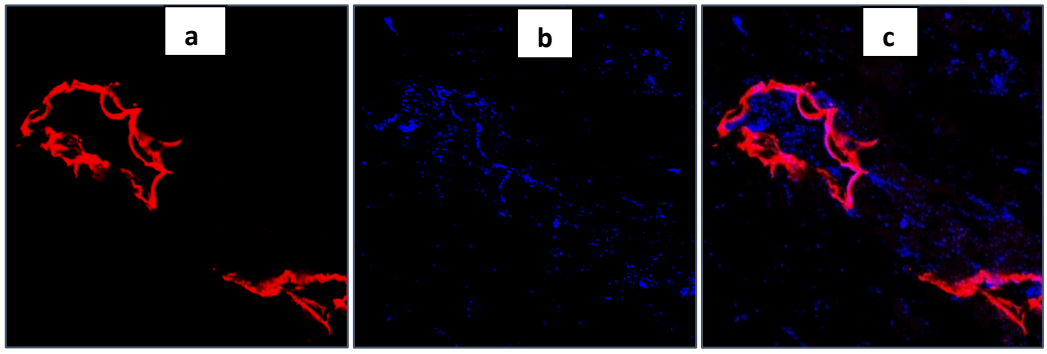


Figure 11 Lymphatic vessels marked with D2-40 (red, a) and VEGFR-3 (blue, b) by confocal microscope, double marking (c).

Thence, new hematic and lymphatic cells, present in morphologically soft atherosclerotic plaques and in mixed ones, were evaluated.

The first section of a soft plaque (Fig. 12a) has been marked by hematoxolyn-eosin and shows the presence of an important inflammatory infiltrate scattered in the whole section.

In picture 12b-c the new hematic vessels have been marked with CD-34 and Ulex, they characterized by a single-layer of endothelial cells. On the contrary, in picture 12d the plaque's section has been marked with D2-40 locating in a different place compared to that of hematic vessels, away from the stringy cap, the new lymphatic flatted vessels.

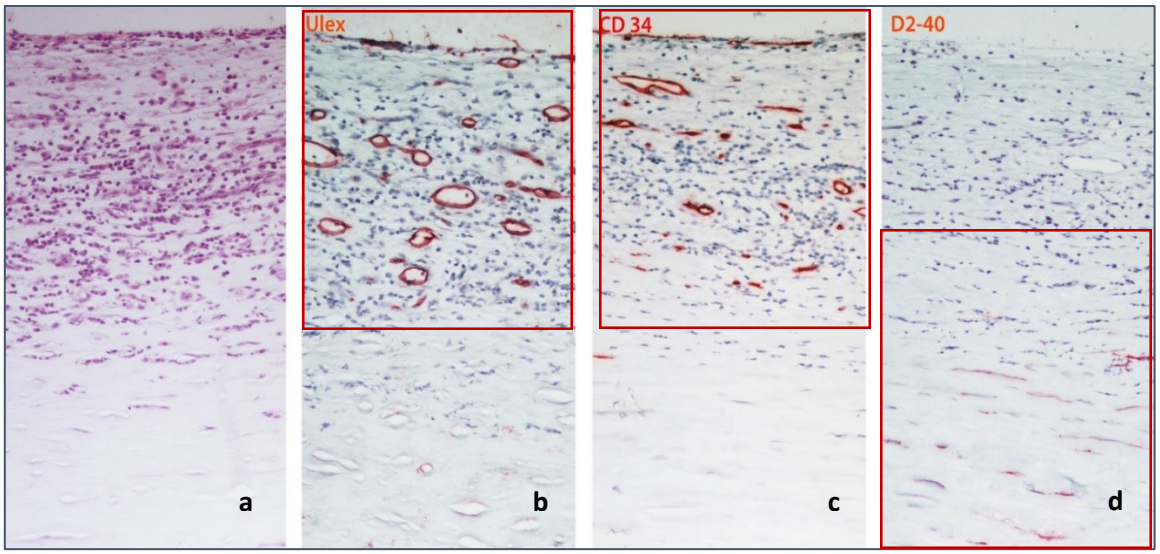


Figure 12 Immunohistochemestry analysis of soft carotid plaque: a-hematoxolyn-eosin, b-c-blood vessels (Ulex, CD34); d- lymphatic vessels (D2-40)

In the atherosclerotic carotid mixed plaque analyzed using hematoxylin-eosin (fig. 13a), it is observed that the inflammatory infiltrate is less represented compared to soft plaques.

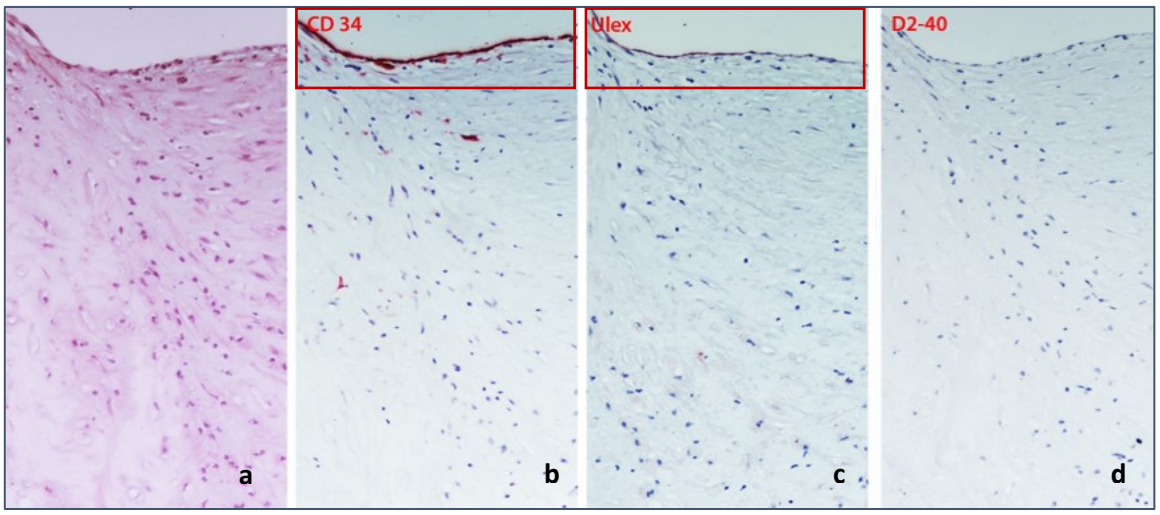


Figure 13 Immunohistochemestry analysis of mixed carotid plaque: a-hematoxolyn-eosin, b-c-blood vessels (Ulex, CD34); d- lymphatic vessels (D2-40)

Furthermore, after the section's colouring with Ulex and CD34 (fig.13 b-c) new vessels aren't identified. It is observed that there's a colouring on the stringy cap's surface symbolised by intimal endothelial cells. In figure 13d the presence of endothelial lymphatic cells is not seen.

The immunohistochemical assessment allows us to evaluate only small plaque areas. This can lead to a loss of an overview of the atherosclerotic carotid plaque, as inside the plaque markers have an extreme variability of display and they don't result as evenly distributed.

For this reason, immunohistochemical images of the atherosclerotic carotid plaque have been judged using a specifically developed software, it allows to examine the entire plaque, speeding analysis' duration of all plaques and granting a comparison amongst them. The given results, (concerning predominantly consistency and distance between lipidic/necrosis core and dissection plan), are comparable amongst them, reproducible as it regards an automatic analysis of all immunolabeled sections of every single plaque.

As previously seen in figure 13, D2-40's display, lymphatic vessels' marker, in a morphologically soft plaque (fig.14b, on the left) as compared to a mixed plaque (fig.14b, on the left), it is substantially represented.

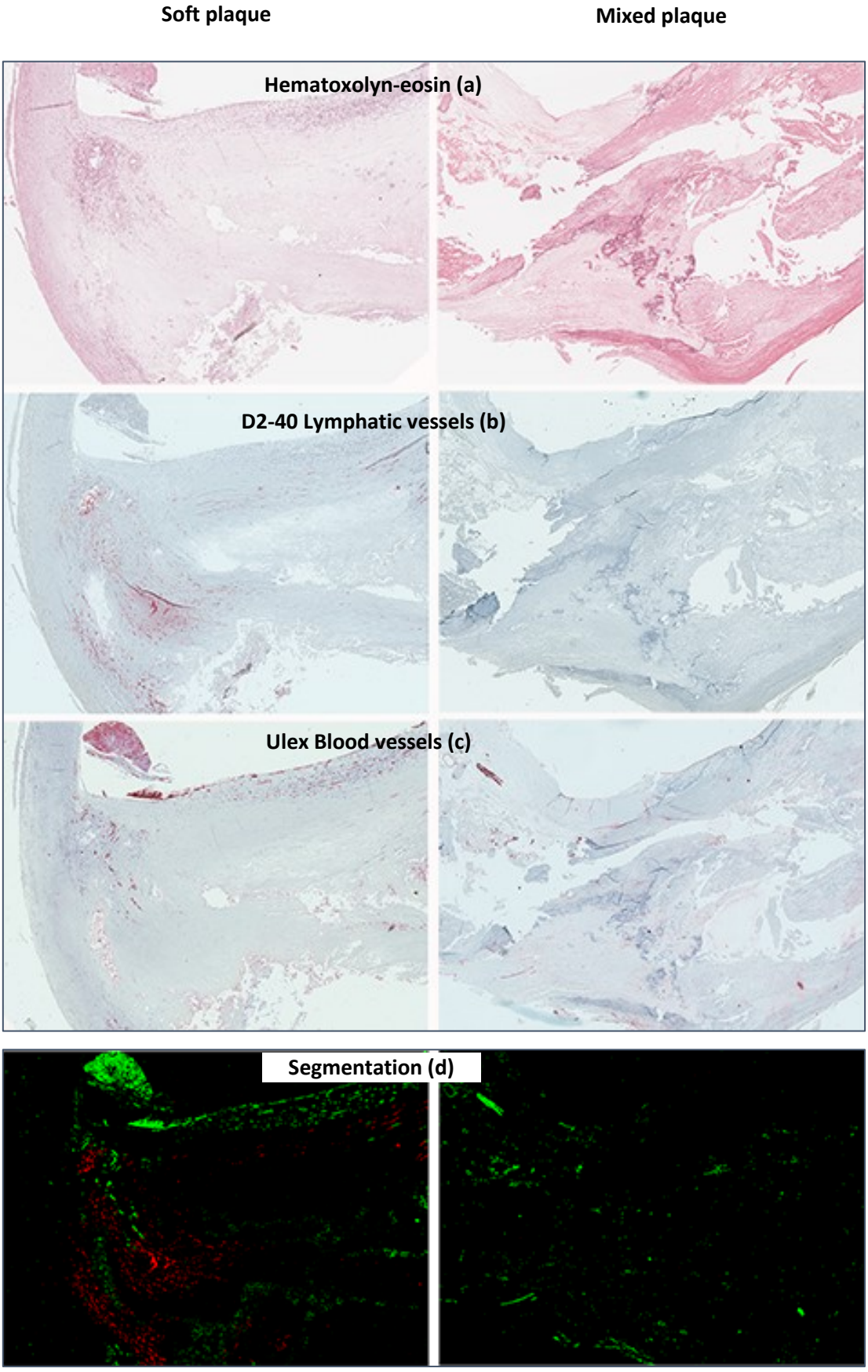


Figure 14 Soft plaque (left) and mixed one (right): hematoxolyn-eosin coloring (A); D2-40 (B), Ulex (C). Automatic segmentation by software (D)

In the soft plaque, the greatest of D2-40 is visible near to the lipidic core. With regards to blood vessels (fig.14c), they are present in both analysed plaques, mostly in the vicinity of the surgical margin and showing no relation with the lipidic core.

The last two sections (fig. 14d) depict a segmentation example (a process of automatic analysis from the software) of the plaque. The lymphatic vessels are represented with a red colour, whereas hematic vessels are depicted with a green colour.

The automatic software analysis obtained a localization map of two types of new vessels and it is observed that the red colouring is missing in the mixed plaque, whereas it is well depicted in the lipidic plaque near to the lipidic core. In both plaques, the hematic vessels, in green, have a casual arrangement, primarily close to the stringy cap and the surgical margin.

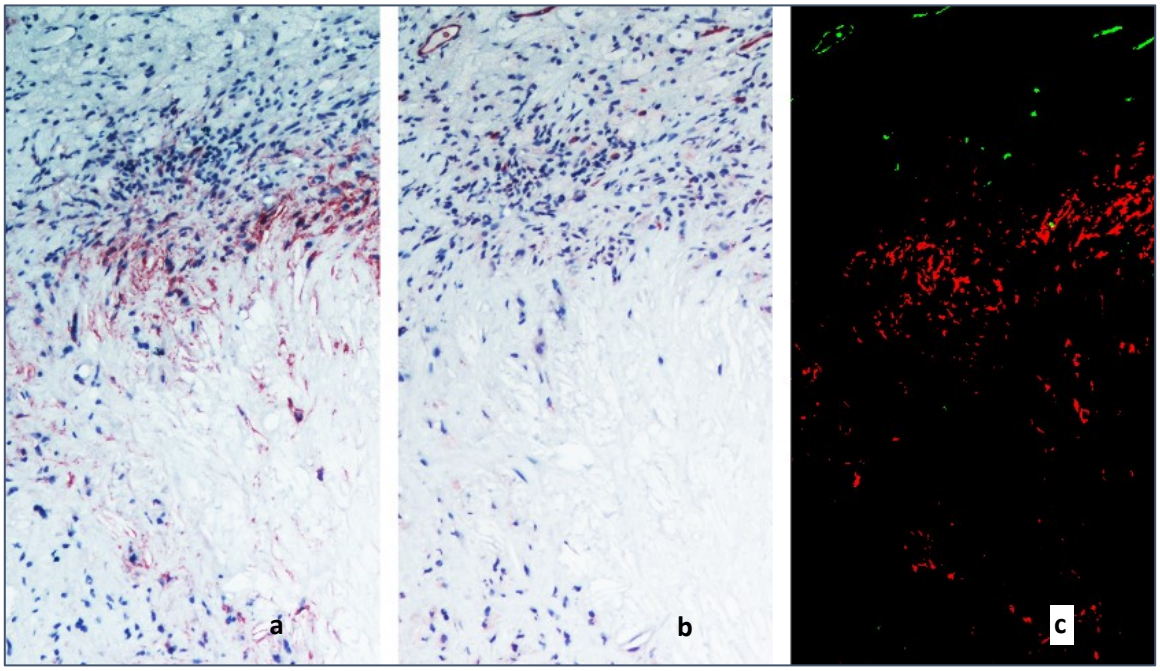


Figure 15 Soft plaque: colouring with D2-40 (a), Ulex (b), Automatic segmentation by software (c)

In picture 15 it is noted that there's an expansion of one section of the atherosclerotic carotid lipidic plaque. The lipidic core and the position of the lymphatic vessels around it are well noticeable (fig.15a), whereas blood vessels are located far away from the lipidic core near the cap (fig.15b). Conclusively, an automatic segmentation was also performed on this section using the software of the analysed plaque, which has allowed us to deeply monitor the position of the lipidic vessels around the lipidic core.

By using the software all structures of mixed and lipidic plaques have been examined, and D2-40's density has been evaluated through the software, in each section and all sections of one same patient,

gaining a numerical value that is repeatable and comparable. Different sections were standardized automatically by the software for a correct comparison between different plaques.

	Patients	Density D2-40
Mixed plaques	a05	0.00001
	b03	0.00001
	a14	0.00010
	b10	0.00011
	b14	0.00012
	a03	0.00017
	a02	0.00053
	a01	0.00131
Soft plaques	a15	0.00247
	a16	0.00380
	a10	0.00361
	b01	0.00424
	b02	0.00489
	a08	0.00557
	a07	0.00632
	a12	0.01025
	a09	0.01184
	a18	0.01818
	a17	0.01873
	a11	0.03707
	b05	0.03983

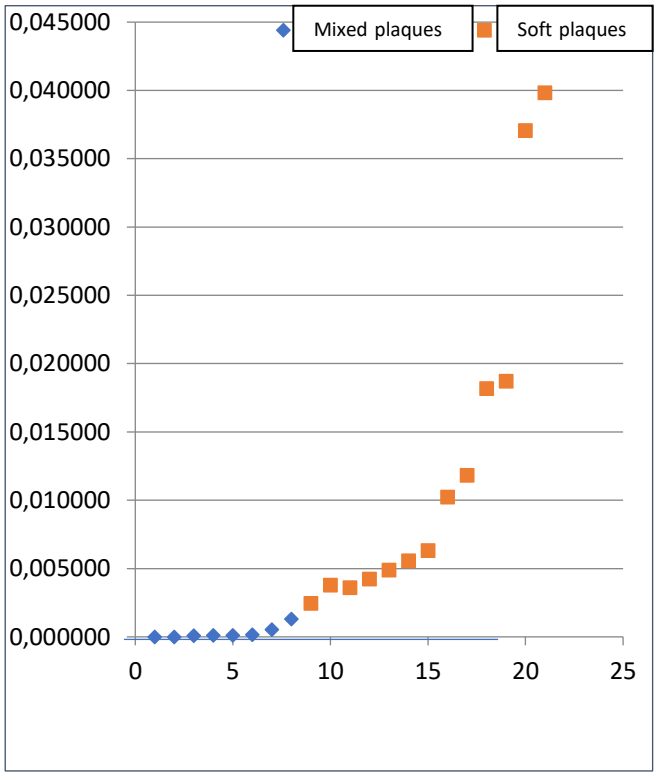


Table 4 D2-40 density in soft and mixed carotid plaques and in the graph: distribution of lymphatic vessels density in the mixed plaques (blue) and soft ones (orange).

From the automatic analysis using the software, density values of mixed and soft lymphatic vessels were extracted (table.4). As also noted on the histogram, the density of lymphatic vessels in the atherosclerotic lipidic plaque (medium value 0,0128) is greater than mixed plaque (medium value 0,000295), and such dissimilarity is statistically significant with $p<0,05$.

For a better understanding of the origin and function of lymphatic and hematic vessels inside atherosclerotic carotid plaques, some components of the inflammatory infiltrate were examined, particularly, T lymphocytes (CD-3) and two different macrophage populations (CD-68, CD-163) were analyzed. In addition to this, their position was also evaluated against lymphatic and hematic vessels.

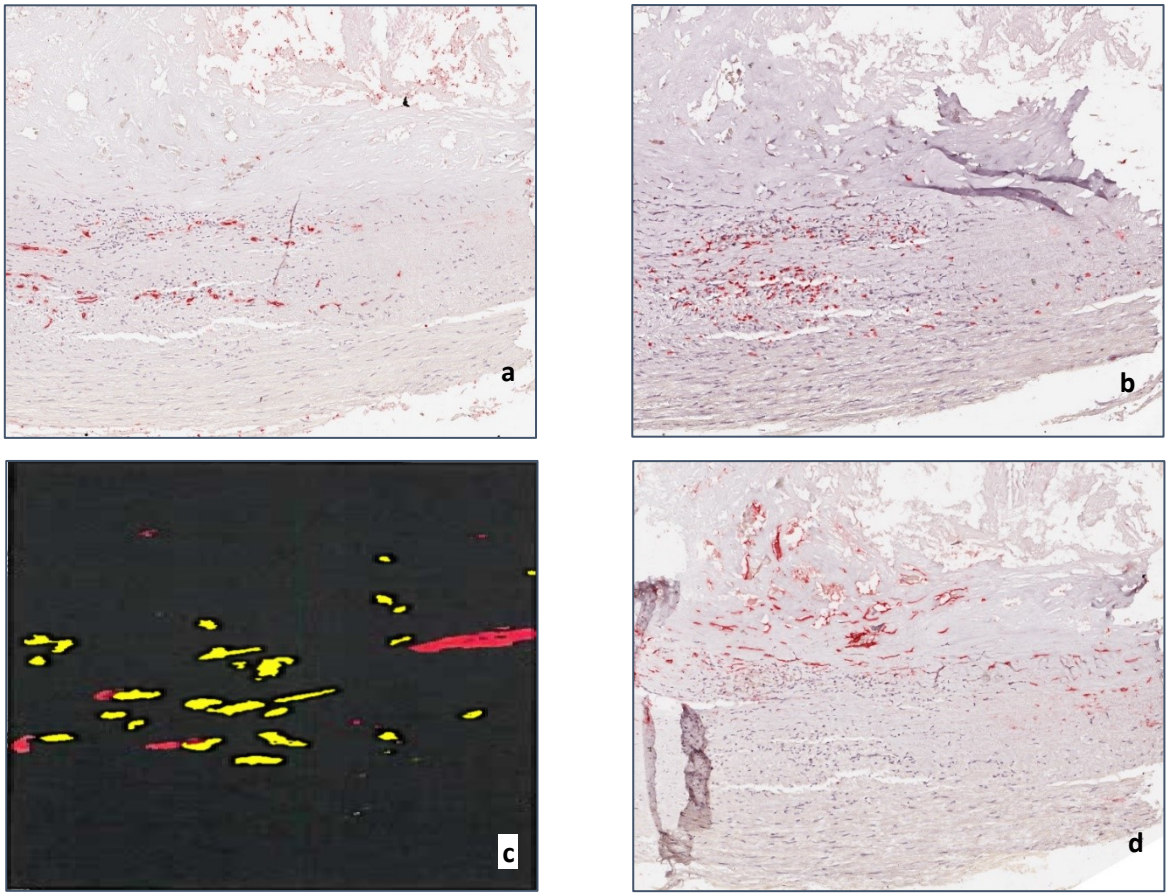


Figure 16 Immunohistochemistry analysis of carotid plaque marked with Ulex (a), CD-3 (b), double marking on the confocal microscope (ulex red, CD-3 yellow) and D2-40 marked (d).

Figure 16a represents an atherosclerotic carotid plaque's section marked with Ulex to identify endothelial hematic cells. Picture 16b shows a colouring with CD-3 and it enables the identification of T lymphocytes. As observed in these pictures, hematic vessels tend to co-locate with T lymphocytes. Such information is confirmed by the double-marking on the confocal microscope (fig.16c), where the lymphocytes CD-3 coloured in yellow are near endothelial hematic cells marked with Ulex in red colour.

T lymphocytes and hematic vessels are not localized in the same territory that is occupied by lymphatic vessels marked with D2-40 (fig.16d).

With regards to the inflammatory infiltrate of macrographic origin (CD-68, CD-163) it is mainly displayed near the lipidic core and close to the areas where an elevated expression of D2-40 is observed.

Two sections of atherosclerotic carotid marked plaques with D2-40 have been shown in figure 17a, to identify lymphatic endothelial cells. They arrange themselves around the lipidic core, together with macrophage cells CD-68 (fig.17b). The co-location of lymphatic endothelial cells and macrophage cells was proved by a double-marking on the confocal microscope (fig.17c).

In figure 17c lymphatic vessels are seen in red, macrophage cells are observed in green. The blue colouring is defined by nuclear staining, which is typical of the methodology.

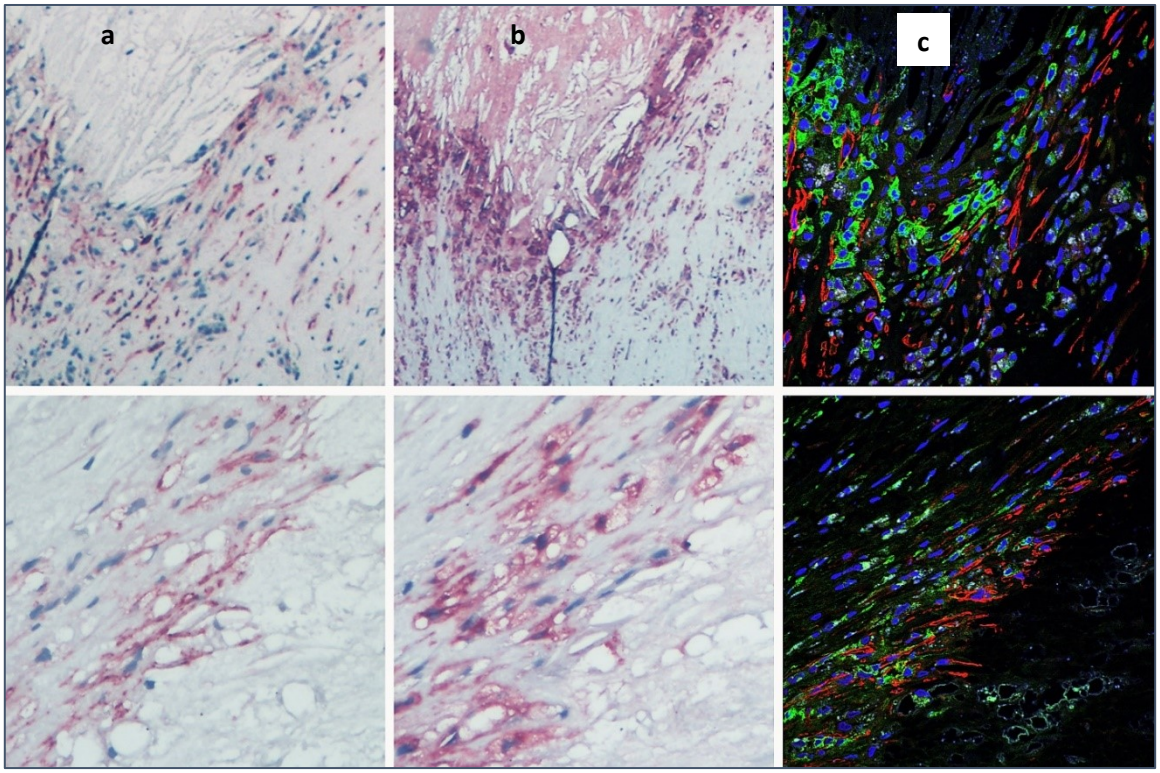


Figure 17 Immunohistochemistry analysis with D2-40 (a), CD-68 (b), and double marked by confocal microscope.

The co-location analysis of lymphatic vessels' and macrophage cells was further scrutinized by way of immunofluorescence in confocal microscopy. Taking advantage of the resolution power of confocal microscopy, it observed that only rarely we can find tubular open structures attributable to one or two collapsed lymphatic vessels (fig.18a, red). This is frequently seen in structures that could also be isolated cells (fig.18b, red) near macrophage cells CD68, coloured green. In some cases, cells seem that they express both markers (fig.18c).

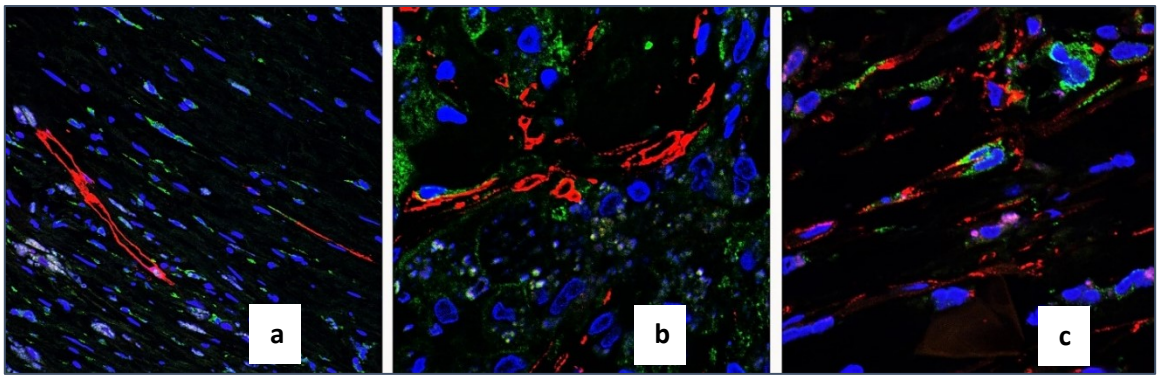


Figure 18 confocal microscope: lymphatic vessels (red), macrophage cells CD68+ (green)

To characterise macrophage cells and to better understand how they could influence new lymph genesis, an analysis of macrophage cells CD-68 was done on the confocal microscope with double-markings.

As seen in picture 19, the first section of the plaque was marked with CD-68 (fig.19a, green) and it shows the presence of a macrophage cell. The following section, forthwith adjacent to the first, was marked with HLA-DR (fig.19b, red), showing cells that present HLA-DR topside. Conclusively, double-marking was implemented with CD-68 and HLA-DR (fig.19c), which has highlighted that a few macrophages show positive labeling for CD-68, although they do not show topside HLA-DR.

Such features are typical of myeloid-derived suppressor cells (MDSC) of the monocyte-macrophage line.

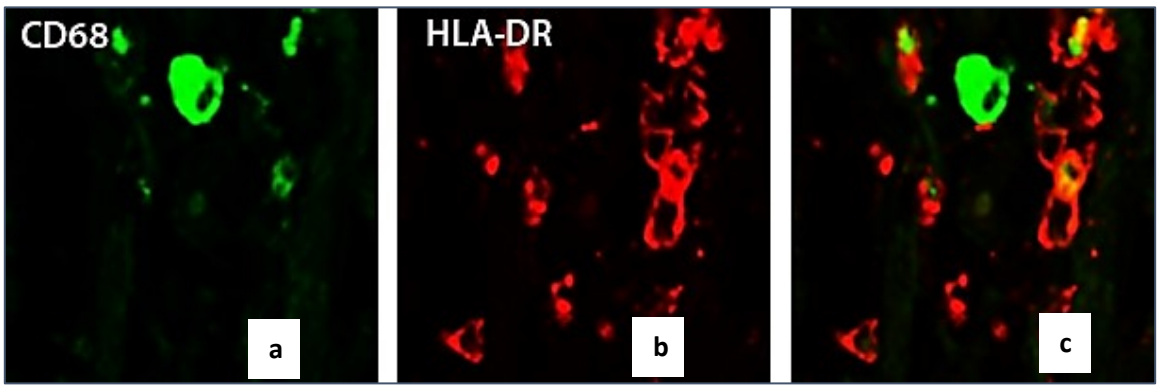


Figure 19 Confocal microscope analysis: CD-68 marked (green) and HLA-DR (red)

To achieve comparable and reproducible data, all soft plaque sections have been examined automatically, by the software, to obtain a localization image for each segmented marker (map), which shows the locations of all markers inside the atherosclerotic carotid plaque. Furthermore, the software has created maps, which have been compared by the software itself, attaining with precision

the distance of different markers from the lipidic/necrotic core and the surgical dissection plan (in pixels).

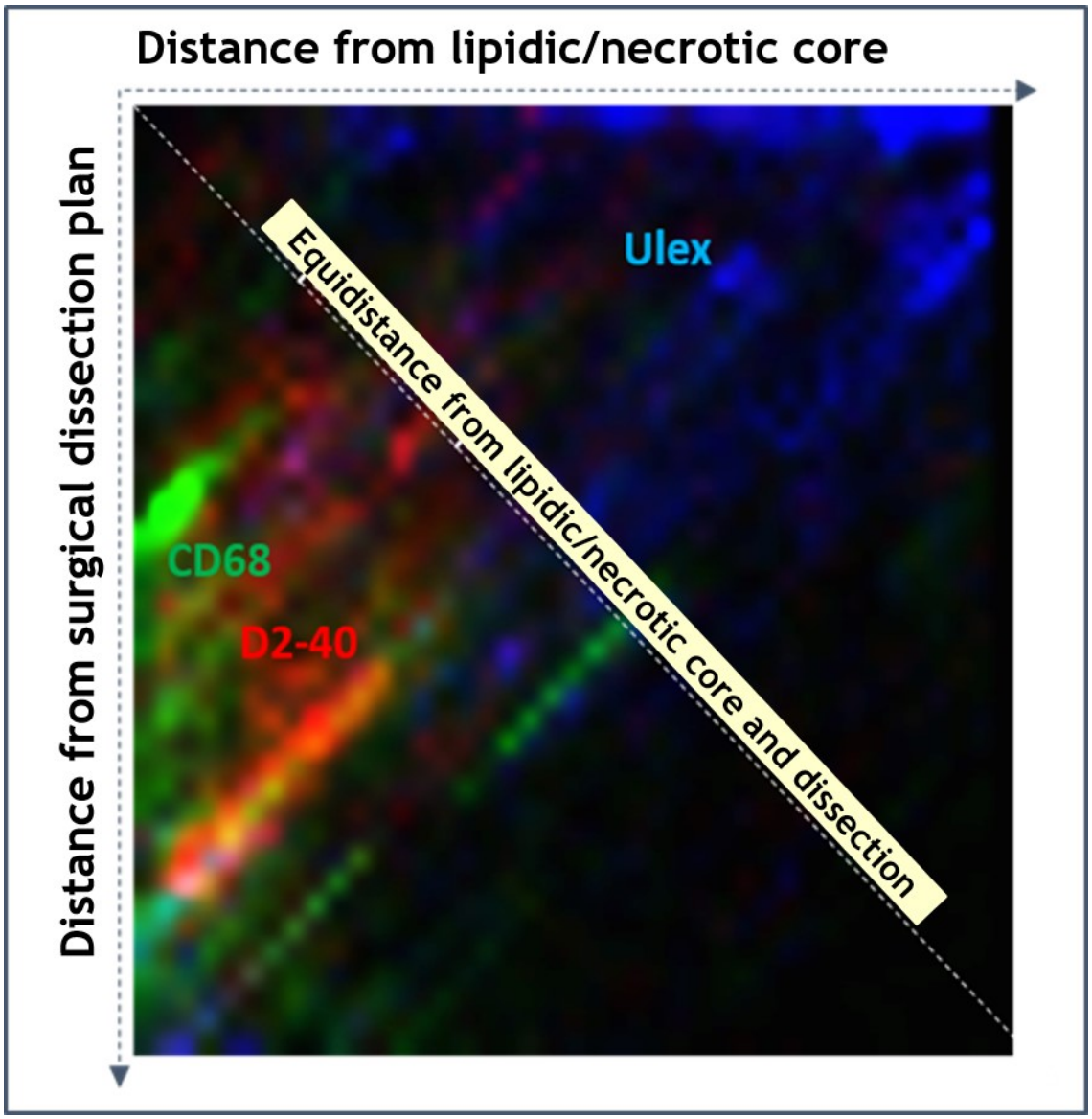


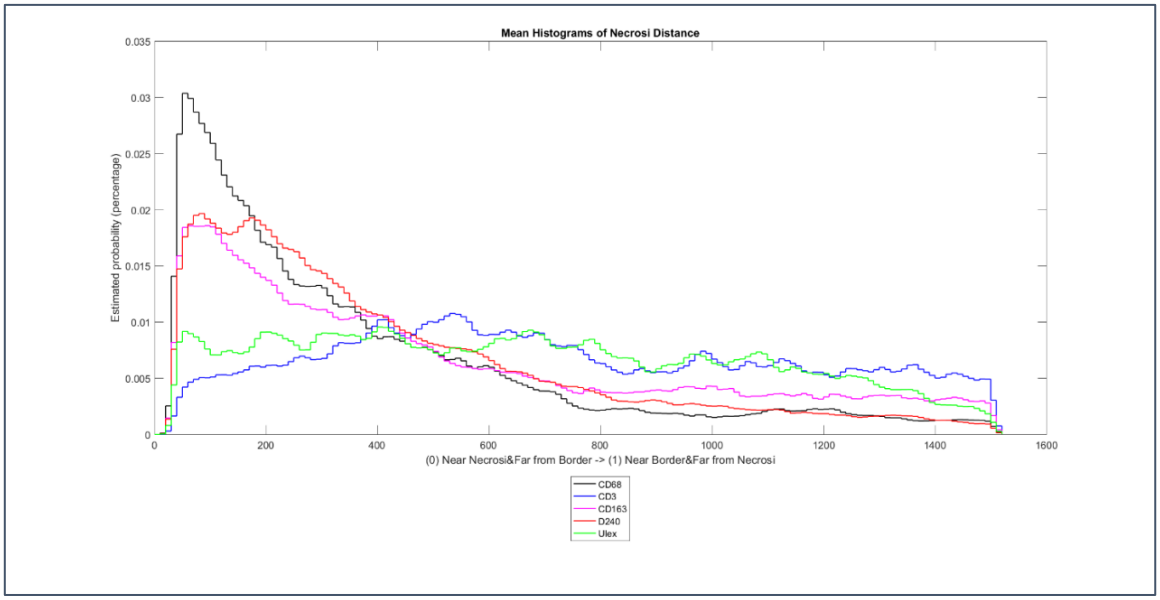
Figure 20 map of localization of lymphatic vessels (D2-40, red), ematic ones (Ulex, blue), macrophages cells (CD-68, green)

Figure 20 represents an example obtained from an automatic analysis of all sections of all atherosclerotic carotid soft plaques. The software has segmented markers D2-40, Ulex, CD-68 of all plaques and putting all the data together, it has given information regarding the distance between such markers from the lipidic core and the dissection surgical plan.

This analysis, defined automatically by the software, has confirmed that inside lipidic plaques lymphatic vessels are located near the lipidic core, which co-locates with macrophage cells CD-68.

Futhermore the software has confirmed that blood vessels are located far off lipid core/necrosis but near the surgical dissection surface.

Connecting the data obtained from automatic analysis of all immunoistochemical sections of all lipidic plaques, it is created an histogram. This one creates the connection between lymphatic vessels, blood vessels and inflammatory macrophage cells (CD-68 and CD-163) and lymphocytes T (CD-3)



Graph 1 Histogram of lymphatic and blood vessels distribution with macrophage cells (CD-68, CD-163) and lymphocytes (CD-3)

From Graph 4 it is plained that the expression of D2-40 (red line) is near the lipid core with its maximum peak between 0 to 400 pixels from lipid core. Macrophage cells CD-163 have a peak between 0 to 400 pixels (pink line) from lipid core and therefore macrophages are located near the lymphatic vessels and lipid core.

Near lipid core and near macrophage cells CD-163 there is a peak of CD-68 cells, this peak is between 0-200 pixels (black line).

Blood vessels, in all lipid plaques, are homogeneously distributed between lipid core and surgical plan (green), they haven't peak. Moreover lymphocites T CD-3 (blue line) follow the distribution of blood vessels and they don't show peak.

DISCUSSION

The atherosclerotic carotid plaque's inflammatory process plays a main role in the instability of the plaque and its fracture.

The extreme variability of the inflammatory cells of cytokines produced by the cells and new vessels present in atherosclerotic carotid plaques have made the investigation very complicated, with considerable evaluation difficulties amongst the analyzed plaques, particularly for the extreme irregularity of distribution of the different considered markers.

The analysis appears to show a higher number of inflammatory cells, cytokines, pro-inflammatory, and new vessels inside soft plaques compared to mixed and hard plaques. Such information can be explained by the fact that soft plaques show a vast lipidic core, and they do not contain calcification areas occupying space. It is then clear that the atherosclerotic carotid plaque is not a static structure. However, it is a dynamic structure inside of which inflammatory cells play a fundamental role in the stability-instability process of the plaque itself.

Numerous and still unknown are the signs and the balance between pro-inflammatory and anti-inflammatory stimuli useful for the development of a neovascularization and plaque instability. Several pieces of research, with the example of Van der Wal et al. [59] and L. H. Arroyo et al. [60], have concluded that the presence of leucocytes and especially T lymphocytes, neutrophils, macrophages and inflammatory cytokines produced by them, may reduce plaque's stability.

These cells can generate cytokines and proteolytic enzymes (i.e., urokinase, plasmin, metalloproteinases MMPs) in the extracellular matrix. For example, the production of IFNγ on behalf of T lymphocytes reduces the proliferation of smooth muscle cells and the creation of collagen, making the plaque more vulnerable to breakage. At the same time, MMPs possess the capacity to decompose the extracellular matrix, making the plaque unstable.

In this study we observed that is very important to evaluate lymphatic vessels and their correlation with blood ones because they have different position in the plaques and different relationship with inflammatory cells.

In this study, we have observed the presence of lymphatic and hematic new vessels inside atherosclerotic carotid plaques. These new vessels tend to not position amongst each other. Blood

vessels expand themselves primarily away from the lipidic core, in the proximity of the fibrous cap, while lymphatic vessels situate near the lipidic core.

There are several studies in which they mention that the stability of the atherosclerotic carotid plaque might depend on, as well as for the presence of a significant inflammatory infiltrate, it might also be determined by the presence of new blood vessels.

According to Sluimer, there is a close relationship between the density of intraplaque new vessels, the growth and risk of fracture of the plaque. [36]

In our project, there is not a significant difference between the density of new lymphatic and blood vessels and the development of cerebrovascular symptoms. As commented in Moreno's investigation, neoangiogenesis demonstrates a higher expression in lipidic plaques than in mixed and calcified plaques; it is also superior in plaques where there is a significant inflammatory infiltrate.

It is believed that blood vessels play a crucial role in the entrance and exiting of inflammatory cells. More importantly, we have noticed that newly formed blood vessels co-localize with inflammatory cells, especially with lymphocytes CD-3. T lymphocytes determine the progression of a lesion and support apoptosis, producing cytokines that nurture the endothelial proliferation and advancement of new vessels. It would also seem that they would lower the proliferation of macrophages, causing a higher instability of the plaque. [61]

Lymphatic vessels in all analyzed plaques have a superior density in lipidic plaques as compared to calcified or mixed plaques.

For those investigations which are part of literature, the lymphatic vessels' boundary inside atherosclerotic arteries is not univocal. According to Xu [62] and his associates, lymphatic vessels situate on the adventitia's level, as in healthy vessels, which are positioned on the intima's level, as stated by Nakano [50] These tend to increase density with a growth of the intimal area, as declared by Kholova [51], ultimately at an intimal level and on media layer.

Drozdz and his colleagues [54] [52] found a substantial correlation between lymphatic vessels pinpointed at an adventitial layer within atherosclerotic carotid arteries and the intimal area; therefore, the more significant that intimal area was, the more prominent was the LYME-1 's (lymphatic vessels' marker) expression at the level of the adventitia.

In our study, we have observed that lymphatic vessels situate on media layer, and, in all plaques, they arrange themselves around the lipidic core, on the intimal area; we have not spotted any lymphatic vessels, but only blood vessels. While, we have not considered the adventitial layer, as the cleavage plan of the carotid plaque was implemented on media layer between the internal and external elastic lamina.

Lymphatic vessels have three fundamental functions: maintain the balance between interstitial fluids, transporting inflammatory cells, and, finally, regulating the metabolism and the absorption of lipids. In normal conditions, they adjust the drainage and the stability of interstitial fluids in atherosclerotic plaques. As specified by Nakano, lymphatic vessels have little representation, mainly arranged at an intimal layer, thence they determine an increase of liquids and interstitial pressure, causing hypoxia, a progression of atherosclerotic lesions and breakage of the plaque.

The arrangement of the lymphatic vessels around the lipidic core, inside the atherosclerotic carotid plaque, it allows us to hypothesize that the stimulus of the neo-lymphogenesis might be the attempt to absorb and metabolize lipids. Lymphatic vessels play a crucial role in the opposite transportation of cholesterol, mobilizing cholesterol's macromolecules, and lipids from the peripheral site to blood. [63]

According to Coso [64] in cardiovascular diseases and atherosclerosis, there is a dysfunction of lymphatic vessels and their functions. In a research study conducted on hypercholesterolaemic mice, the excessive accumulation of cholesterol and lipids in tissues determines structural and functional defects of lymphatic vessels. [65]

In fact, in the same model of hypercholesterolaemic mice, the reinstatement of the lymphatic drainage has established a better clearance of the cholesterol and lipids from the tissue [66] . This altered function of the lymphatic vessels sets a stockpile of lipids and cholesterol inside atherosclerotic plaques.

It is, however, inexplicable how cholesterol HDL leaves the plaque. Martel and his collaborators have created a few models to explain such a mechanism. As specified by these authors, cholesterol pours out of the plaque following the interstitial fluid's direction, and it merges inside the adventitial lymphatic vessels. They have increased the cholesterol in particular (using an isotope of the cholesterol) in atherosclerotic plaques of the murine aorta, and they have restricted the development of lymphatic vessels on the plaque's level through the inhibition of VEGFR-3.

As a result, they have monitored that cholesterol's transportation blocked outside of the atherosclerotic plaque. [67]

Lymphatic vessels often located in the proximity of the lipidic core and in areas where the inflammatory infiltrate results very well represented. New lymphatic vessels seem to perform an activity of the transportation of inflammatory cells inside and outside of the atherosclerotic plaque. [68]

The lipids' stack can define the launch of inflammatory cells, which produce pro-inflammatory cytokines, such as tumor necrosis factor (TNF- α) and reactive oxygen molecules, that raise the local

inflammation. For this reason, anomalies at the expense of the lymphatic system set out the stash of inflammatory cells, toxic metabolite, and pro-inflammatory cytokines which facilitate atherosclerosis progression. [69]

Also, in our investigation, we have scrutinized a colocalization amongst lymphatic vessels and macrophage cells, CD-68 and CD-163 in particular.

Macrophages in the atherosclerotic plaque play a crucial role in the phagocytosis' process of lipids and cholesterol carrying the scavenger's role out.

From a phenotypic point of view, macrophages can divide into two groups: M1 macrophage, which is activated and stimulated by molecules, as, for example, IFN-γ, TNF-α. They secrete nitric oxide and pro-inflammatory cytokines (IL-1, IL-6, IL-12, IL-23) and TNF-α. Secondly, there are M2 macrophages that have a potent anti-inflammatory activity ("Deactivated" macrophages) and incited by cytokines, such as IL-4, IL-10, and IL-12. Furthermore, M2 macrophages defined as regulators.

Amongst the analyzed atherosclerotic plaques, we have identified macrophage cells CD-68 positive, which include all types of macrophages and macrophage cells CD-163 positive, that in most cases are M2 macrophages.

Macrophages situate in proximity with the lipidic core s their fundamental role is to phagocytize lipids and cholesterol. Besides, they foster the removal of HDL cholesterol on behalf of lymphatic vessels even if only a few macrophages seem to come out of the atherosclerotic plaque through lymphatic vessels. [70] [71]

Moreover, macrophages localize in the proximity of the lymphatic vessels and co-localize with themselves, probably because they represent inflammatory cells mainly involved in the lymphogenesis' process. They nurture lymphogenesis via two methods: the production of paracrine signals represents the first one on behalf of macrophages; the second one is symbolized by the differentiation of macrophages in lymphatic endothelial cells.

The first mechanism of macrophages that supports lymphogenesis, is determined by the up-regulation of VEGF-C during the inflammatory process inside the atherosclerotic plaque. In fact, macrophages produce TNF-α that activates the TNFR1 receptor facilitating the creating of the VEGF-C, which activates VEGFR-3 promoting the development and the activation of lymphatic endothelial cells. [72]

In addition to the paracrine mechanism, macrophages contribute to lymphogenesis using transdifferentiation in lymphatic endothelial cells. In our study, we have monitored the confocal microscope, the presence of lymphatic endothelial cells (marked with D2-40), that expressed on their surface CD-68, macrophages' marker. In literature, such data is confirmed thanks to investigations run on vitro and animals. For example, Hall, in vitro, has found on the level of the lymphatic endothelium macrophagic cells that expressed markers of lymphatic endothelial cells. [73].

The macrophagic cells' in-depth research has allowed us to identify inside the atherosclerotic carotid plaque, the presence of CD-68 positive cells and HLA-DR negative cells. These are myeloid-derived suppressor cells (MDSC) of monocyte-macrophage origin.

Myeloid-derived suppressor cells were described for the first time in tumors in 1970. [74] In spite of the numerous investigations carried out inside neoplasias, their phenotypical and functional characteristics are not entirely defined.

These cells in neoplasias inhibit the immune response mediated by T lymphocytes, both in vitro and in vivo. [75] [76] The inhibition happens to regulate the immune response and to prevent excessive activation of T lymphocytes, which can develop an auto-reactivity, damaging healthy tissues. MDSCs facilitate tumor growth, releasing reactive species of oxygen, nitrogen, and nitric oxide and intensifying the inflammatory process. [77] [78] [79]

Moreover, myeloid-derived suppressor cells are involved in angiogenesis and lymphogenesis' processes [80]. In literature, there are not projects in which myeloid-derived suppressor cells have been analyzed inside atherosclerotic carotid plaques. Wang and his colleagues have evaluated the presence of myeloid-derived suppressor cells in the blood of patients affected by acute coronary syndrome, stable angina, and healthy individuals. Wang et al. monitored a more significant suppression (higher MDSC expression, arg-1, IL-1β, IL-6, TNF-α) of T lymphocytes in patients affected by acute coronary syndrome as compared to patients with stable angina and healthy controls. [81]

In our investigation, there are relatively small numbers of myeloid-derived suppressor cells and predominately arranged in the proximity of blood and lymphatic vessels; for this reason, it is thought that they might intervene in the stimulation processes of neoangiogenesis and neolymphogenesis.

CONCLUSIONS

The inflammatory process represents the cornerstone in the development of the atherosclerotic carotid plaque, but the literature's facts are still lacking a definitive vision.

The analyzed plaques in this study have allowed achieving preliminary and interesting results that show the presence of a new lymphatic and blood neovascularisation and to determine existing relations between such vessels and the inflammatory infiltrate, with a particular focus on macrophages and T lymphocytes.

Through the use of the software, purposely created, it has been possible to examine plaques automatically and to obtain data that could be compared amongst each other, also amongst different

patients, regardless of the variability of markers' expression, between the analyzed areas of the same plaque and amongst different plaques.

The distribution of blood vessels is variable, but they generally place in areas where the inflammatory infiltrate is abundant. Such information suggests that these vessels may regulate the entrance and exit of inflammatory cells inside the plaque.

As illustrated by literature, blood vessels seem to make the plaque vulnerable and unstable in the first place because they appear composed of a single layer of endothelial cells; therefore, they are fragile, and can encounter a lesion; secondly, because they nurture the entrance of inflammatory cells inside the plaque.

A new piece of information, not previously identified in the literature, is the localization of lymphatic vessels near the lipidic core. Undoubtedly lymphatic vessels inside atherosclerotic carotid plaques represent a new "actor" in the atherosclerotic process, although their role is still unknown. One of the keystone functions of lymphatic vessels is represented by lipids' metabolism and cholesterol's opposite transport, but it is not yet known if this function could make the atherosclerotic carotid plaque vulnerable, or if it could have a protective and stabilizing effect on the same.

With such utter clarity, it is even more evident that in atherosclerotic plaques, there is a 'reparative' tendency of our organism, that starts in the 'fatty streak' deriving from the attempt made by macrophages to metabolize oxidized LDLs. It symbolizes the first evolution phase of the atherosclerotic plaque, which has a new phase made up of the creation of new lymphatic vessels.

Lymphatic vessels could highlight the drainage attempt of the lipidic core. These phases depict different moments in the evolution of the atherosclerotic plaque, but they allow us to recognize how

the reparative tendency of the individual, mediated by immunity, may intervene in the physiopathology of the atherosclerotic lesion.

For that reason, it will be necessary to increase the numerousness of the sample and considering new markers, which can allow us to define cholesterol metabolism, and lipids' transport mechanisms with more precision, and so the function of lymphatic vessels and cytokines involved in such mechanisms.

Another important, revealed by the study, is the co-localization of lymphatic vessels with macrophage cells. Macrophages are the principal cells involved in the lymphogenesis, via VEGF-C's secretion. By increasing the study sample, we will be able to identify other cells implicated in such a mechanism, and some cytokines that might support the development of lymphatic vessels. Furthermore, it will be useful finding ways to pinpoint all macrophage subpopulations, both M1 and M2, with the intent of understanding how these populations may act on neo-lymphogenesis, on the atherosclerotic plaque's progression and the stability-instability of the plaque.

Through this investigation, we have also recognized the presence of myeloid-derived suppressor cells of the monocyte-macrophage line inside the atherosclerotic carotid plaque. These cells inside neoplasms have a dual function: trying to inhibit T lymphocytes activity from avoiding self-inflicted damage, although, at the same time, they facilitate neo-angiogenesis, and thus tumor's growth and its dissemination.

In atherosclerotic carotid plaques, these cells will probably inhibit T cells' activity, as they do not localize near such cells, and they foster lymphogenesis more extensively that angiogenesis, given their localization close to lymphatic vessels and not near blood vessels.

It will be beneficial to continue the study trying to identify the functions of myeloid-derived suppressor cells in particular, by pinpointing the cytokines that they produce, to understand that type of activity they possess inside the atherosclerotic plaque.

Therefore, this investigation, regardless of the riveting and not yet identified data in the literature, it represents the preliminary phase of other possible examinations of atherosclerotic carotid plaques.

The inspection of new plaques and additional markers will allow us to acquire significant results for an overall assessment of the plaque, and hopefully, to the identification of areas at a higher atheroembolic risk inside the atherosclerotic carotid plaque.

REFERENCES

[1] D. Mukherjee, «Epidemiology and the global burden of stroke,» *World Neurosurg.,* vol. 76, pp. S85-90, 2011 Dec.

[2] V. Roger, «Heart disease and stroke statistics - 2012 update: a report from the American Heart Association,» *Circulation,* vol. 125, pp. 2-220, 2012.

[3] CDC, «Prevalence and most common causes of disability among adults - United States,» *MMWR,* vol. 58, pp. 421-426, 2009.

[4] P. Rothwell, «Change in stroke incidence, mortality, casefatality, severity and risck factors in Oxfordshire, Uk from 1981 to 2004,» *Lancet,* vol. 363, pp. 1925-1933, 2004.

[5] Y. Zhang, «The incidence, prevalence and mortalityof stroke in France, Germany, Italy, Spain, UK, and the US: a literature review,» *Stroke Res Treat,* p. 436125, 2012.

[6] M. Kelly-Hayes, «The influence of gender and age on disability following ischemic stroke: the Framingham study,» *J. Stroke Cerebrovasc Disease,* vol. 12, pp. 119-126, 2003.

[7] R. Grysiewicz, «Epidemiology of ischemic and hemorrhagic stroke: incidence, prevalence, mortality and risk factors,» *Neurol Clin,* vol. 26, pp. 871-895, 2008.

[8] P. Wolf, «Secular trends in stroke incidence and mortality,» *Stroke,* vol. 23, pp. 1551-1955, 1992.

[9] R. Brown, «Stroke incidence, prevalence and survival: secular trends in Rochester, Minnesota, through 1989,» *Stroke,* vol. 27, pp. 373-380, 1996.

[10] D. Kiely, «Familial aggregation of stroke. The Framingham Study,» *Stroke,* vol. 24, pp. 1366-1371, 1993.

[11] W. Cushman, «Effects of intensive blood-pressure control in type 2 diabetes mellitus,» *N. Engl J Med,* vol. 362, pp. 1575-1585, 2010.

[12] S. Vermeer, «Impaired glucose tolerance increases stroke risk in nondiabetic patients with transient ischemic attack or minor ischemic stroke,» *Stroke,* vol. 37, pp. 1413-1417, 2006.

[13] L. Goldstein, «Guidelines for the primary prevention of stroke: a guideline for healthcare professionals from the American Heart Association,» *Stroke,* vol. 42, pp. 517-584, 2011.

[14] R. Shah, «Smoking and stroke: the more you smoke the more you stroke,» *Expert Rev Cardiovasc Ther,* vol. 8, pp. 917-932, 2010.

[15] T. Kurth, «Body mass index and the risk of stroke in men,» *Arch Intern Med,* vol. 162, pp. 2557-2562, 2002.

[16] K. Rexrode, «A prospective study of body mass index, weight chance and risk of stroke in women,» *JAMA,* vol. 277, pp. 1539-1545, 1997.

[17] G. Hu, «Body mass index, waist circunference and waist-hip ratio on the risk of total and type-specific stroke,» *Arch Intern Med,* vol. 2007, pp. 1420-1427, 2007.

[18] M. O'Donnel, «Risk factors for ischaemic and intracerebral haemorrhagic stroke in 22 countries: a case control study,» *Lancet,* vol. 376, pp. 112-123, 2010.

[19] J. Redgrave, «Histological assessment of 526 symtomatic carotid plaques in relation to the nature and timing of ischemic symptoms: the Oxford plaque study,» *Circulation,* vol. 113, pp. 2320-2328, 2006.

[20] P. Gao, «The correlation of carotid plaque pathohistologic features and neurological symptoms: a meta-analysis of observational studies,» *Neurol India,* vol. 55, pp. 122-129, 2007.

[21] A. Nicolaides, «Asymptomatic internal carotid artery stenosis and cerebrovascular risk stratification,» *J Vasc surg,* vol. 52, pp. 1486-1496, 2010.

[22] S. Jmor, «Grading internal carotid artery stenosis using B-mode ultrasound,» *Eur J Vasc Endovasc Surg,* vol. 18, pp. 315-322, 1999.

[23] A. Imparato, «The carotid bifurcation plaque: phatologic findings associated with cerebral ischemia,» *Stroke,* vol. 10, pp. 238-245, 1979.

[24] P. Libby, «patogenesi, prevenzione e trattamento dell'aterosclerosi,» *Principi di medicina interna,* pp. 1460-1467, 2008.

[25] P. Moreno, «Macrophage infiltration in acute coronary syndromes. Implications for plaque rupture.,» *Circulation,* vol. 1994, pp. 775-778, 1994.

[26] M. Schillinger, «Inflammation and Carotid Artery-Risk for atherosclerosis study (ICARAS),» *Circulation,* pp. 2203-2209, 2005.

[27] K. Alitalo, «Molecular mechanism of lymphangiogenesis in health and disease,» *Cancer Cell,* vol. 1, pp. 219-27, 2002.

[28] I. Martinez-Corral, «Regulation of lymphatic vascolatur morphologenesis: implication for pathological (tumor) lymphangiogenesis,» *Experimental Cell Research,* pp. 213-226, 2013.

[29] P. Baluk, «Functionally specializedjunctions between endothelial cells of lymphatic vessels,» *J Exp Med,* vol. 204, pp. 2349-2362, 2007.

[30] S. Coso, «Pressing the right buttons: signalining in lymphangiogenesis,» *Blood,* vol. 123, pp. 2614-2642, 2014.

[31] T. Karpanem, «Regulation of lymphangiogenesis-from cell fate determination to vessel remodeling,» *Experimental cell research,* vol. 312, pp. 575-583, 2006.

[32] G. Oliver, «The rediscovery of the lymphatic system: old and new insights into the development and biological function of the lymphatic vasculature,» *Genes Dev,* vol. 16, pp. 773-783, 2002.

[33] T. Petrova, «Lymphatic endothelial reprogramming of vascular endothelial cells by prox-1 homeobox trascription factor,» *Embo J,* vol. 21, pp. 4593-4599, 2002.

[34] S. Schoppmann, «Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis,» *Am J Pathol,* vol. 161, pp. 947-956, 2002.

[35] D. Kerjaschki, «The crucial role of macrophages in lymphangiogenesis,» *J Clin Invest,* vol. 115, pp. 2316-2319, 2005.

[36] K. Maruyama, «Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages,» *J Clin Invest,* vol. 115, pp. 2363-2372, 2005.

[37] *IMMUNE NETWORK ,* vol. 4, pp. 182-186, 2014.

[38] T. Nakano, «Angiogenesis and lymphangiogenesis and expression of lymphangiogenetic factors in the atherosclerotic intima of human coronary arteries,» *Human Pathology,* vol. 36, pp. 330-340, 2005.

[39] I. Kholova, «Lymphatic vasculature is increased in heart valves, ischaemic and inflamed hearts and in cholesterol rich and calcified atherosclerotic lesions,» *Eur J Clin,* vol. 41, pp. 487-497, 2011.

[40] K. Drozdtz, «Adventitial lymphatics of internal carotid artery in healthy and atherosclerotic vessels,» *Folia Histochem Cytobiol,* vol. 46, pp. 443-436, 2008.

[41] O. Eliska, «The absence of lymphatics in normal and atherosclerotic coronary arteries in man: a morphologic study,» *Lymphology,* vol. 39, pp. 76-83, 2006.

[42] K. Drozdz, «Adventitial lymphatics and atherosclerosis,» *Lymphology,* vol. 45, pp. 26-33, 2012.

[43] T. Vuorio, «Lymphatic vessel insufficiency in Hypercholesterolemic mice alters lipoprotein Levels and promotes atherogenesis,» *Arterioscler Thromb Vasc Biol,* vol. 34, pp. 1162-1170, 2014.

[44] J. Paterson, «Vascularization and hemorrhage of the intima of atherosclerotic coronary arteries,» *Arch path,* vol. 22, p. 313, 1936.

[45] S. Schwartz, «Cellular proliferation in atherosclerosis and hypertension,» *Proc Soc Exp Biol Med,* vol. 173, pp. 1-13, 1983.

[46] S. Grundy, «Atherosclerosis: pathology, pathogenesis and role of risk factors,» *Dis Mon,* vol. 29, pp. 1-58, 1983.

[47] P. Carmeliet, «Melocular mechanisms and clinical applications of angiogenesis,» *Nature,* vol. 473, pp. 298-307, 2011.

[48] S. Goel, «Vascular normalization as a therapeutic strategy for malignant and nonmalignant disease,» *Cold spring harb perspect med,* vol. 2, 2012.

[49] J. Xu, «Vasa vasorum in atherosclerosis and clinical significance,» *Int J Mol Sci,* vol. 16, pp. 11574-11608, 2015.

[50] J. Sluimer , «Thin walled microvessels in human coronary atherosclerotic plaques show incomplete endothelial junctions relevance of compromised structural integrity for intraplaque microvasculature leakage,» *J Am Coll Cardiol,* vol. 53, pp. 1517-1527, 2009.

[51] J. Sluimer, «Novel concepts in atherogenesis: angiogenesis and hypoxia in atherosclerosis,» *J Pathol,* vol. 218, pp. 7-29, 2009.

[52] A. Simonini, «IL-8 is an angiogenic factor in human coronary atherectomy tissue,» *Circulation,* vol. 101, pp. 1519-1526, 2000.

[53] M. McCormick, «S100A8 and S100A9 in human arterial wall. Implications for atherogenesis,» *J Biol Chem. ,* vol. 16, pp. 41521-9, 2005.

[54] A. Vega, «Angiotensin II induces CD62L shedding in human neutrophils,» *Atherosclerosis,* vol. 209, pp. 344-351, 2010.

[55] L. Saba, «Carotid artery plaque classification: does contrast enhancement play a significant role?,» *J Neuroradiol,* vol. 33, pp. 1814-1817, 2012.

[56] X. Zhang, «Cholesterol, coronary heart disease and stroke in the Asia Pacific region,» *Int J Epidemiol,* vol. 32, pp. 563-572, 2003.

[57] M. Fleiner, «Arterial neovascularization and inflammation in vulnerable patients early and late signs of symtomatic atherosclerosis,» *Circulation,* vol. 110, pp. 2843-2850, 2004.

[58] P. Gao, «The correlation of carotid plaque pathohistologic festures and neurological symptoms: a meta-analysis of observational studies,» *Neurol India,* vol. 55, pp. 122-129, 2007.

[59] Van der Wal, «Atherosclerotic plaque rupture phatologic basis of plaque stability and intsbility,» *Cardiovasc Res,* vol. 41, pp. 334-344, 1999.

[60] Arroyo, «Mechanisms of plaque rupture: mechanical and biologic interactions,» *Cardiovasc Res,* vol. 41, pp. 369-75, 1999.

[61] S. Lhotak, «Characterization of proliferating lesion-resident cell during all stages of atherosclerotic growth,» *J Am Heart Assoc,* vol. 5, pp. 1-14, 2016.

[62] T. Vuorio, «lymphatic vessel insufficiency in hypercolesterolemic mice alters lipoprotein levels and promote atherogenesis,» *Atheroscl Thromb Vasc Biol,* vol. 34, pp. 1162-1170, 2014.

[63] S. Coso, «pressing the right buttons: signaling in lymphangiogenesis,» *Blood,* vol. 123, pp. 2614-2624, 2014.

[64] H. Lim, «Hypercolesterolemic mice exhibit lymphatic vessels dysfuction and degeneration,» *Am J Pathol,* vol. 175, pp. 1328-1337, 2009.

[65] H. Lim, «Lymphatic vessels are essential for the removel of cholesterol from peripheral tissues by SR-BI-mediated transport of HDL,» *Cell Metab,* vol. 17, pp. 671-684, 2013.

[66] C. Martel, «Lymphatic vasculature mediates machrophage reverse cholesterol transport in mice,» *J Clin Invest,* vol. 123, pp. 1571-1579, 2013.

[67] D. Jones, «An overview of lymphatic vessels and their emerging role in cardiovascular disease,» *J CArdiovasc Dis Res,* vol. 2, pp. 141-152, 2011.

[68] D. Aebischer, «The inflammatory response of lymphatic endothelium,» *Angiogenesis,* vol. 17, pp. 383-393, 2014.

[69] S. Potteaux, «Suppressed monocyte recruitment drives macrophage removal from atherosclerotic plaques of apoe-/- mice during disease regression,» *J Clin Invest,* pp. 2025-2036, 2011.

[70] G. Randolph, «Mechanism that regulate macrophage burden in atherosclerosis,» *Circ Res,* vol. 114, pp. 1757-1771, 2014.

[71] Y. Suzuki, «Transforming growth factor-beta induces vascular endothelial growth factor-C expression leading to lymphangiogenesis in rat unilateral ureteral obstruction,» *Kydney Int,* vol. 81, pp. 865-879, 2012.

[72] K. Hall, «New model of macrophage acquisition of the lymphatic endothelial phenotype,» *Plos one,* p. 31794, 2012.

[73] M. Young , «Hematopoiesis and suppressor bone marrow cells in mice bearing large metastatic lewis lung carcinoma tumors,» *Cancer Res,* vol. 47, pp. 100-105, 1987.

[74] Gabrilovich, «Mechanisms and fuctional significance of tumor-induced dendritic cell defects,» *Nat Rev Immunol,* vol. 4, pp. 941-952, 2004.

[75] L. Dolcetti, «MDSC role in tumor related inflammation,» *Cancer Lett,* vol. 267, pp. 216-25, 2008.

[76] J. Youn, «The biology of mieloid-derived suppressor cells: the blessing and curse of morphological and functional heterogeneity,» *Eur J Immunol,* vol. 40, pp. 2969-2975, 2010.

[77] M. Srivastava, «Myeloid-derived suppressor cells inhibit t-cell activation by depleting cystine and cysteine,» *Cancer Res,* vol. 70, pp. 68-70, 2010.

[78] C. Corzo, «Mechanism regulating reactive oxygen species in tumor-induced myeloid-derived suppressor cells,» *J Immunol,* vol. 182, pp. 5693-5701, 2009.

[79] W. Shojaei, «Bv8 regulates myeloid cell dependent tumour angiogenesis,» *Nat Biothecnol,* vol. 25, pp. 911-920, 2007.

[80] Y. Wang, «Expansion of myeloid-derived suppressor cells in patients with acute coronary syndrome,» *Cell Physiol Biochem,* vol. 35, pp. 292-304, 2015.

[81] Ricotta, «Updated society for vascular surgery guidelines for management of extracranial carotid disease,» *J Vasc Surg,* vol. 54, pp. 1-31, 2011.

[82] X. Xu, «adventitial lymphatic vessels an important role in atherosclerosis,» *Medical Hypotesis,* vol. 69, pp. 1238-1241, 2007.

[83] E. M. H. C. A. M. Z. J. S. R. O. A. A. I. Rodriguez PC.

[84] E. B. R. C. P. V. I. M. J. A. I. i. C. s. i. Vega A.

[85] «Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults,» *JAMA 2001; 285:2486-2497.*