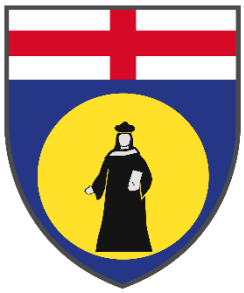

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PhD in Biotechnologies in Translational Medicine

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Identification of biomarkers of plaque vulnerability in carotid artery disease for the prevention of brain ischemic injury

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Summary

Abbreviations list

Artery-Brain Circuit (ABC)
Artificial Intelligence (AI)
Aryl Hydrocarbon Receptor (AhR)
Apolipoprotein E knockout (ApoE-/-)
Ahr Nuclear Translocator (ARNT)
Aryl Hydrocarbon Receptor Nuclear Translocator-Like Protein 1 (ARNTL)
Artery Tertiary Lymphoid Organs (ATLOs)
Basic Helix-Loop-Helix (bHLH)
Basic Helix-Loop-Helix/Per-Arnt-Sim Transcription Factors (bHLH/PAS)
Brain And Muscle ARNT-Like 1 (BMAL1)
Best Medical Therapy (BMT)
Carotid Stenting (CAS)
Cluster Of Differentiation 147 (CD147)
Cluster Of Differentiation 163 (CD163)
Carotid Endarterectomy (CEA)
Celiac Ganglionectomy (CGX)
Carbon Monoxide (CO)
Cardiovascular Disease (CVD)
Computed Tomography (CT)
Extracellular Matrix Metalloproteinase Inducer (EMMPRIN)
Echocolordoppler (ECD)
Extracellular Matrix (ECM)
European Carotid Surgery Trial (ECST)
Extra Domain B (EDB)
Extra Domain A (EDA)
Endothelium-Mesenchymal Transition (EndMT)
Fibronectin (Fn)
EDB-Containing Fibronectin (Fn-EDB)
Hemoglobin-Haptoglobin (Hb-Hp)
High-Density Lipoproteins (HDL)
Hypoxia-Inducible Factor 1α (HIF-1α)
Heme Oxygenase-1 (HO-1)
Intercellular Adhesion Molecule-1 (ICAM-1)
International Atherosclerosis Society (IAS)
Intima-Media Thickness (IMT)
Intraplaque Hemorrhage (IPH)
Low-Density Lipoproteins (LDL)
Matrix Metalloproteinases (MMPs)
Magnetic Resonance Angiography (MRA)
North American Symptomatic Carotid Endarterectomy Trial (NASCET)
Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells (NF-Kb)
200 Kda Heavy Neurofilament (NF200)
Neuroimmune Cardiovascular Interface (NICI)
Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2)
Oxidized LDL (OxLDL)
polycyclic aromatic hydrocarbons (PAHs)
Reactive Oxygen Species (ROS)
soluble receptor CD163 (sCD163)
T Helper (Th)
Tyrosine hydroxylase (TH)
Transient Ischemic Attack (TIA)
Toll-Like Receptors (TLR)
Regulatory T Lymphocytes (Tregs)
Vascular Endothelial Growth Factor A (VEGF-A)
Vascular Cell Adhesion Molecule-1 (VCAM-1)
Vascular Smooth Muscle Cells (VSMCs)
Xenobiotic Response Elements (XREs)

Abstract

Carotid atherosclerosis is a leading cause of cerebrovascular events, yet risk stratification, in severe asymptomatic stenosis, remains limited. Despite advances in imaging and clinical scoring, prediction of prone to rupture plaques remains challenging. This study aimed to identify potential biomarkers of plaque vulnerability by exploring both systemic and local molecular pathways involved in inflammation, oxidative stress, structural remodelling, and neuro-immune interactions. The integration of these markers help to provide a more comprehensive understanding of plaque biology and identifies targets for future diagnostic and therapeutic strategies.

Tissue and peripheral blood mononuclear cells (PBMCs) were obtained from 68 male patients with symptomatic carotid stenosis (n=22) or severe asymptomatic stenosis (n=46) enrolled in the Genoa Vascular Biobank. Biomarkers were analyzed, carefully selected based on previous evidence of involvement in atherosclerosis, grouped as: immuno-inflammatory (CD163), oxidative and stress-response (HO-1, AhR, BMAL1), structural and remodelling (CD147, Fibronectin-EDB), and neuro-immune (Neurofilament 200, Tyrosine Hydroxylase). Gene expression in PBMCs and plaques were analized, and immunohistochemistry evaluated focusing on plaque shoulders prone to instability. Statistical comparisons used the Mann-Whitney test, correlations were assessed by Spearman's rank.

Symptomatic patients exhibited a higher CD163 and AhR expression in PBMCs, correlates with HO-1 and BMAL1, indicating systemic activation of inflammatory and antioxidant pathways. These findings suggest that circulating immune cells reflect the ongoing vascular stress. Gene expression in plaques showed that AhR strongly correlated with CD147 and Fibronectin-EDB, particularly in asymptomatic patients, suggesting local remodelling and early destabilization. AhR immunopositivity was higher in symptomatic lesions (whole lesion $p=0.064$), mainly localized in neovasa and inflammatory infiltrates of the media and plaque shoulder, the most active and vulnerable sites of carotid stenosis. CD147 immunopositivity inversely associated with circulating stress markers in symptomatic patients. Preliminary results of immunohistochemistry with neuro, immune and cardiovascular biomarkers revealed localization of $CD3^+$ T cells, $CD163^+$ macrophages, and nerve fibers expressing NF200 and Tyrosine Hydroxylase in AhR-rich regions of the plaque shoulder, indicating a complex neuro-immune interface contributing to local inflammation and tissue remodelling.

Taken together these results show that systemic CD163 and AhR, linked to BMAL-1 and HO-1, associate with plaque vulnerability, while AhR, CD147, Fn-EDB correlations, especially in asymptomatic patients, suggest early process of plaque vulnerability. Circulating AhR, CD163, and HO-1 inversely correlate with local CD147, highlighting biomarker potential. AhR expression by neuronal and immune cells supports a neuro-immune interface in plaque inflammation.

Overall, these findings represent a snapshot of a stress-related molecular axis in severe carotid stenosis, which may be proposed as a biomarker panel for vulnerable plaques. Larger multicentric studies including women and healthy controls are needed to validate these results.

1. Introduction

1.1 Atherosclerosis

1.1.1 Definition

Atherosclerosis is a chronic and inflammatory disease that mainly affects large-caliber arteries, including the aorta, carotid arteries, iliac arteries, coronary arteries, and popliteal arteries. The terms "atheroma" (from the Greek "athero", meaning "gruel") and "sclerosis" (from the Greek "sklerosis" meaning "hardening") refer specifically to the stiffening of the vessel wall. The atherosclerotic process develops over time and is characterized by the accumulation of lipids, inflammatory cells, cell degradation products, extracellular matrix (ECM) deposition and calcification within the intima layer of the arteries, leading to narrowing of the vessel lumen and loss of elasticity (Lusis AJ., 2000; Holm Nielsen S. et al., 2020).

The initial mechanism is endothelial dysfunction, often triggered by both modifiable and non-modifiable cardiovascular risk factors such as hypercholesterolemia, diabetes, smoking, and hypertension. This event facilitates the penetration and accumulation of low-density lipoproteins (LDL) in the vascular wall, where lipoproteins undergo oxidative changes. Oxidized LDL (oxLDL) stimulates the recruitment of monocytes and their differentiation into macrophages, which, when engulfed by LDL, transform into foam cells, a distinctive feature of early lesions (Gimbrone MA Jr. et al., 2016; Kong P. et al., 2022).

The review by Roy and colleagues highlights the crucial role of the interaction between lipid metabolism and immunity in determining the evolution of atherosclerotic plaques. The lipoproteins that infiltrate the arterial wall, and their subsequent oxidation, modify the microenvironment, which activates monocytes into macrophages (Roy P. et al., 2022).

During lesion progression, ECM deposition is mediated by vascular smooth muscle cells (VSMCs), which transition from a contractile to a synthetic and migratory phenotype, and then move to the intima, where they synthesize collagen and proteoglycans. However, these cells can acquire pro-inflammatory phenotypes and contribute to the degradation of the fibrous cap via the secretion of matrix metalloproteinases (MMPs), making the plaque prone to rupture (Holm Nielsen S. et al., 2020).

In this context, dendritic cells also play an important role; they are stimulated by antigens derived from modified LDL and altered endogenous proteins. These cells, depending on the

context and the maturation signals received, can induce pro-inflammatory responses or promote immune tolerance through the expansion of regulatory T cells. Other components of innate immunity, such as mast cells, neutrophils, natural killer cells, and innate lymphoid cells, also contribute to the atherosclerotic microenvironment, influencing the balance between inflammation and repair (Roy P. et al., 2022).

Adaptive immunity exerts an equally decisive influence. CD4+ T cells represent the main protagonists: T helper (Th) 1 cells, producers of IFN-γ, are strongly pro-atherogenic, promoting plaque instability and leukocyte infiltration; Th 2 cells have a more ambiguous role, sometimes protective thanks to cytokines such as IL-5 and IL-13; Th 17 cells, on the other hand, show variable functions depending on the inflammatory context. Regulatory T cells (Tregs) exert a stabilizing effect, promoting immunotolerance and limiting chronic inflammation. Another subgroup, the T follicular helpers, is mainly pro-atherogenic, as it stimulates the antibody response and the formation of autoantibodies (Roy P. et al., 2022; Mallat Z. et al., 2022).

B cells, by producing antibodies, contribute to modulate the disease in a biphasic way: natural IgM antibodies directed against oxidized epitopes exert protection by favoring the clearance of modified LDL and apoptosis; while IgG antibodies promote inflammation and progression of the lesion (Roy P. et al., 2022; Mallat Z. et al., 2022).

Atherosclerotic plaques are not static entities but dynamic structures subject to continuous remodeling. They can remain silent for decades or evolve rapidly into complicated phases with rupture of the fibrous cap and formation of occlusive thrombi, leading to major clinical events such as myocardial infarction, ischemic stroke, as occurs in stenoses of coronary and carotid arteries, respectively. This critical step is strongly influenced by plaque composition: an extended lipid core, a thin fibrous cap, and high inflammatory infiltration are associated with instability and thrombotic risk (Holm Nielsen S. et al., 2020; Mallat Z. et al., 2022).

Recent molecular classifications propose an integration between the morphology, cell composition, and inflammatory plaque profile, suggesting that a more precise phenotypic definition of atherosclerosis may improve prognosis and therapeutic personalization (Mallat Z. et al., 2022).

1.1.2 Risk Factors, Epidemiology, Prevalence, and Costs

Atherosclerosis is a multifactorial disease driven by both modified and non-modified risk factors, or secondary to other diseases (Lusis AJ., 2000; Libby P., 2021; Kumar V., 2021):

Non-modifiable risk factors:

- Age: the predominant risk factor. While atherosclerosis initiates in youth, its clinical significance increases with individuals' age; in general, the prevalence of cardiovascular disease (CVD) in the elderly aged 70 exceeds 10%, and this burden further increases with aging (Libby P., 2021). Ageing is characterized by progressive biological alterations that impair vascular homeostasis. According to the recent "Roadmap for alleviating the manifestations of ageing in the cardiovascular system" (Liberale L. et al., 2025), vascular ageing encompasses processes such as endothelial senescence, mitochondrial dysfunction, low-grade chronic inflammation ("inflammageing"), and extracellular matrix remodeling, all of which synergistically accelerate atherosclerosis and plaque instability.

- Gender: Males exhibit a higher vulnerability to this pathology compared to females until menopause. Indeed, female hormones exert protection (Jebari-Benslaiman S. et al., 2022).

- Family history: a higher risk exists when close relatives have experienced atherosclerotic complications at an early age (Jebari-Benslaiman S. et al., 2022).

- Hyperhomocysteinemia: this condition involves an elevated homocysteine level due to a genetic defect. Homocysteine works as an effective activator of the endothelium (Cimmino G. et al., 2023)

Modifiable risk factors (lifestyle-related):

- Cigarette smoke: smoking directly impacts endothelial function through nicotine, reducing the activity of endothelial nitric oxide synthase and subsequently limiting the availability of nitric oxide, a vasodilator. Smoking also triggers the production of reactive oxygen species (ROS) via NADPH oxidase activation and induces chemical damage due to the toxic pollutants (Jebari-Benslaiman S. et al., 2022).

- Hyperlipidic diet: factors contributing to obesity and metabolic syndrome are pivotal risk factors in atherosclerosis progression.

- Physical inactivity: sedentary habits lead to an unfavourable pro-atherogenic lipid profile, as high-density lipoproteins (HDL) are more efficiently synthesized in individuals engaged in physical activity. (Lechner K. et al., 2020).

- Anxiety-prone personality: this personality trait promotes increased blood pressure, causing mechanical damage to the endothelium (Paterniti S. et al., 2001).

Risk factors secondary to other pathologies:

- Hypertension: increased blood pressure mechanically damages the endothelium. Blood normally flows in a laminar pattern, but turbulence at arterial bifurcations (where atherosclerotic plaques often form) leads to endothelial activation and dysfunction (Poznyak AV. et al., 2022).

- Hyperlipidaemia: exceeding the physiological range of lipid concentration accelerates plaque formation. Hyperlipidaemia is often a genetic disease, as in familial hypercholesterolemia.

CVD is the leading cause of death globally, with approximately 17.9 million deaths each year, accounting for 32% of all deaths recorded worldwide. Of these, more than 85% are due to acute events such as myocardial infarction and ischemic stroke. A particularly relevant finding is that about 75% of cardiovascular deaths occur in low- and middle-income countries, where populations often lack timely access to adequate health services, preventive screening, and effective drug treatments. This unequal distribution highlights the marked global health disparities, with direct implications also for the sustainability of national health systems (Lusis AJ., 2000; Libby P., 2021).

In Europe, CVD remains one of the leading causes of death. According to Eurostat data from 2021, the standardised death rate for diseases of the circulatory system is 343.4 deaths per 100,000 inhabitants, with higher values in men (379.8) than in women (314.1) (https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Cardiovascular_diseases_statistics).

In Italy, CVD is the leading cause of death and disability. ISTAT data indicate that the prevalence of disability due to CVD is 4.4‰ of the population, a figure that highlights the high social and health impact. At the pharmacological level, drugs for the cardiovascular system represent the primary class prescribed, accounting for 23.5% of public pharmaceutical expenditure, followed by those for the nervous system and the digestive system (IAS, 2025).

In 2021, the overall economic impact of CVD in the 27 member states of the European Union was estimated at €282 billion. Of this sum, 46% is represented by direct health care costs, while the rest includes costs for informal care (28%), productivity losses (17%), and social care services (9%). In Italy, hospital spending on cardiac surgery amounts to about 650 million euros per year, representing 1% of the entire national health expenditure. These data highlight how

CVDs represent not only a health challenge, but also a major economic problem for the community (IAS, 2025).

Epidemiological projections suggest that, in the absence of effective interventions, deaths from CVDs could reach 24 million per year by 2030, an increase of 34% from current levels. According to the International Atherosclerosis Society (IAS), there are still numerous unmet clinical needs in the primary prevention of atherosclerosis, which represents the main pathological substrate of CVDs. The 2025 IAS document highlights how current approaches are often insufficient in reaching high-risk individuals and recommends strengthening early screening strategies, personalization of therapeutic interventions, and promoting a global alliance between health professionals, institutions, and communities (IAS, 2025).

1.1.3 Etiopathogenesis

Atherosclerosis is a process involving an intricate series of cellular and molecular events. At the basis of the initiation of the lesion, there is endothelial dysfunction, induced by mechanical factors (such as blood turbulence and low wall tension), chemical (smoking, blood sugar, HDL), and immunological factors. This condition facilitates endothelial permeability and promotes monocyte adhesion through the expression of adhesion molecules such as Vascular Cell Adhesion Molecule-1 (VCAM-1) and Intercellular Adhesion Molecule-1 (ICAM-1) (Gimbrone MA Jr. et al., 2016).

The lesion begins in the intima layer of the vessel and then progresses into the media layer. Specifically, it involves the accumulation of lipids, inflammatory cells (mainly monocytes), or fibrous material within the vessel beneath the endothelium. The monolayer of endothelial cells lines the inner surface of the vessel wall and possesses various properties. The endothelium can produce substances with vasodilatory activity, such as nitric oxide, or vasoconstrictive substances like endothelin (Vanhoutte PM et al., 1996). It also exerts antithrombotic and fibrinolytic properties and can regulate inflammatory processes by producing physiologically anti-inflammatory substances.

The accumulation of lipids beneath the endothelium is a process that begins at an early age, forming "fatty streaks" and progresses with advancing years to form plaques. This involves the accumulation of cholesterol droplets, calcium, and cell debris. A fibrous cap is formed beneath the endothelium, consisting of collagen fibers and VSMCs. Notably, VSMCs are not only structural components of the fibrous cap but also actively participate in plaque biology: they migrate from the media to the intima, undergo phenotypic switching from a contractile to a

synthetic state, and can even become foam cells, contributing up to 50% of the foam cell population in advanced lesions (Azar P. et al., 2025). Neo-angiogenic processes also occur particularly in the shoulder region (a site of the plaque more prone to rupture), leading to intra-plaque haemorrhages (Virmani R. et al., 2005).

Depending on the variable percentages of these components, there are different types of lesions with varying degrees of instability (Stary HC. et al., 1995).

The pathogenic process of atherosclerosis can be divided into several phases (Pontieri GM. et al., 2018):

1. Initial Phase: Endothelial cell activation triggered in response to external stimuli. During this process, endothelial cells can release various chemical mediators and signaling molecules that influence vasodilation, blood coagulation, platelet adhesion, and inflammation. Endothelial activation can be caused by factors such as hypertension, LDL cholesterol oxidation, and inflammation. This process leads to the recruitment of inflammatory cells, like monocytes, into the sub-endothelial area, thereby promoting the development of atherosclerotic lesions, which begin with fat accumulation beneath endothelial cells appearing as "fatty streaks." The trigger for the accumulation of these lipid-rich macrophages is the oxidation—both enzymatic and free radical-mediated—of LDL, which transports cholesterol from the liver to peripheral tissues. Oxidation causes LDL to be recognized not by normal receptors but by scavenger receptors on macrophage or VSMC membranes. Components of oxLDL also increase the expression of adhesion molecules on the endothelial surface, such as VCAM-1 and ICAM-1 (Singh V. et al., 2023). Monocytes adhere to and infiltrate the sub-endothelial layer, where they gather as macrophages along with oxLDL. Recent evidence has highlighted that VSMCs, through the same scavenger receptors, can also internalize oxLDL and become foam cells, thereby linking lipid retention not only to immune cells but also to vascular wall cells (Azar P. et al., 2025).

2. Progressive Phase: OxLDL internalization by macrophages starts with internalization in endosomes. Then cholesterol passes into the endoplasmic reticulum, and through the enzyme, is re-esterified, leading to foam formation. Moreover, macrophages assist the migration of VSMCs into the intima layer and sub-endothelial zone. VSMCs can also internalize oxidized LDL components by expressing scavenger receptors like SRA, CD36, and SR-B1, thereby acquiring foam cell characteristics. During plaque progression, lymphocytes and neutrophils interact, indicating the presence of an immune response throughout the plaque's evolution. Plaque formation proceeds through the development of a lipid core formed by foam cells, with

a necrotic core surrounded by a fibrous cap (Virmani R. et al., 2005). VSMCs play a dual role in this context: while they contribute to plaque stabilization by secreting ECM and thickening the fibrous cap, their apoptosis, defective efferocytosis, and acquisition of inflammatory or osteochondrogenic phenotypes can promote necrotic core expansion, microcalcification, and instability (Azar P. et al., 2025).

The thickness of the fibrous cap classifies the plaque as more or less stable; a thicker cap indicates a more stable plaque compared to a thinner cap. An elevated density of microvessels has been linked to intraplaque bleeding and plaque rupture, according to pathologic evaluation of unstable lesions (Burke AP. Et al.,1999; Lusis AJ., 2000; Kong P. et al., 2022).

The adaptive immune system plays an important role: Th1 lymphocytes promote plaque progression via interferon-γ, while Tregs have a protective function; B lymphocytes contribute biphasically: B1 cells secrete natural IgM with a protective function, while B2 cells can promote inflammation and plaque instability (Mallat Z. et al., 2022).

Plaque instability is a critical phase. Vulnerable plaques have a thin fibrous cap, a high density of macrophages, and an abundant necrotic area. They can rupture or undergo endothelial erosion, triggering the coagulation cascade and the formation of a thrombus. While disruption is associated with MMPs and acute inflammation, erosion is most often related to endothelial dysfunction and neutrophilic activation mediated by Toll-Like Receptors (TLR) 2 and hyaluronic acid (Holm Nielsen S. et al., 2020; Kong P. et al., 2022). In this setting, VSMCs have also been implicated, as they can produce hyaluronan that, once degraded, activates TLR2 and promotes endothelial activation, linking VSMC biology to the erosion pathway (Azar P. et al., 2025).

3. Plaque Rupture: Plaque rupture can be a rapid and often unpredictable process, and macrophages play a crucial role in plaque destabilization. Indeed, they produce proteolytic enzymes, particularly MMPs, which contribute to the breakdown of the ECM within the plaque (Johnson JL. 2017). This process can weaken the plaque's structural integrity and increase the risk of rupture. When a plaque rupture occurs, the underlying tissue components, such as collagen, lipids, and VSMCs, are exposed to the blood flow, triggering a series of events involving platelets and the coagulation cascade.

- Platelet Activation: platelets are activated when they encounter the exposed collagen and other substances in the plaque, leading to platelet aggregation, plug formation at the site of rupture.

- Coagulation Cascade: It involves a sequence of enzymatic reactions and ultimately results in the conversion of fibrinogen to fibrin, forming a stable blood clot or thrombus. The thrombus can completely block the vessel within a few minutes, causing ischemia or necrosis, in situ or in downstream vessels, when embolization occurs.

1.1.4 Prevention, Management, and Therapy

The prevention of atherosclerosis is based on a multifactorial approach.

In primary prevention, lifestyle modifications represent the first line of intervention: the adoption of a balanced diet (e.g., Mediterranean diet), regular physical activity, smoking cessation, and the management of psychophysical stress are all crucial elements to prevent the progression of the disease (IAS, 2025). Numerous studies have shown that these behavioral interventions can significantly reduce the incidence of cardiovascular events (Libby P., 2021).

In secondary prevention, in subjects at high risk or with established CVD, lipid-lowering therapy is the cornerstone of pharmacological treatment. Statins are the first-line treatment for LDL cholesterol reduction, and they are also effective for their pleiotropic anti-inflammatory effects. In high-risk patients, therapy can be intensified with ezetimibe or PCSK9 inhibitors, while in selected patients, new molecules such as inclisiran (a six-monthly siRNA) and bempedoic acid are available (Mallat Z. et al., 2024; Kong P. et al., 2022).

A field of rapid development is the immunomodulatory prevention: studies such as CANTOS and COLCOT have shown that selective reduction of inflammation, with canakinumab and colchicine, respectively, can reduce the incidence of cardiovascular events independent of lipid levels (Mallat Z. et al., 2024). However, the issue of long-term immune safety remains.

To improve the identification of subjects at risk, new tools are being deployed, combining predictive algorithms, non-invasive imaging (e.g., carotid intima-media thickness (IMT), coronary calcium score), and inflammatory biomarkers (PCR-us, IL-6). The use of carotid ultrasound for IMT measurement and for the identification of vulnerable plaques is an important diagnostic parameter, although its routine use is still a matter of debate (Nezu T. et al., 2016).

The management of atherosclerosis is based on an integrated and personalized approach, which includes lifestyle modifications, drug therapy, clinical monitoring, and, in selected cases, revascularization interventions to reduce the global cardiovascular risk (IAS,2025).

The treatment of high blood pressure, diabetes, and obesity is equally critical. Antihypertensive drugs (ACE inhibitors, sartans, calcium channel blockers, diuretics), antidiabetics (metformin, SGLT2 inhibitors, GLP-1 agonists), and weight loss strategies help modulate overall risk. Antiplatelet agents, such as acetylsalicylic acid, are recommended for secondary prevention in patients with a history of atherothrombotic events (Libby P. et al., 2021).

When drug therapy fails to contain the risk or in the presence of critical stenosis, interventional revascularization procedures such as percutaneous coronary intervention or coronary artery bypass grafting surgery are indicated. In cerebrovascular diseases, carotid endarterectomy (CEA) and carotid stenting (CAS) are valid options for stroke prevention in selected patients (Halliday A. et al., 2004).

Finally, the integration of personalized medicine and digital technology in the management of atherosclerosis represents a promising frontier. Remote monitoring of clinical parameters, therapy management apps, artificial intelligence (AI) -based predictive models, and the use of molecular biomarkers to personalize therapy enable increasingly accurate risk stratification and targeted interventions (Mallat Z. et al., 2024; Kong P. et al., 2022).

While atherosclerosis is a systemic process, its clinical impact depends on the affected vascular bed and on plaque biology. In the carotid arteries, atherosclerotic lesions developing at the bifurcation are a major substrate for ischemic stroke, mainly through artery-to-artery embolization. Importantly, stroke risk is not dictated solely by the degree of luminal narrowing, but by the presence of “vulnerability” features reflecting inflammation, extracellular matrix remodeling, and microvascular changes. Therefore, an integrated view connecting systemic mechanisms of atherosclerosis with carotid-specific clinical phenotypes and plaque composition is essential to guide risk stratification and therapeutic decisions.

1.2 Carotid stenosis

1.2.1 Definition

Carotid stenosis is the narrowing of the lumen of the carotid artery, mainly due to atherosclerotic plaques that develop at the level of the carotid bifurcation between the internal and external carotid arteries. This narrowing impairs cerebral blood flow and represents one of the main pathogenetic mechanisms of ischemic stroke, particularly on an embolic basis. Plaques can be stable, with a thick fibrous capsule and small lipid core, or unstable, characterized by a thin

capsule, large lipid core, and high inflammatory activity, which makes them more susceptible to rupture and emboli formation (Kolodgie FD. et al., 2017).

For a correct clinical and therapeutic framework, it is essential to distinguish between two clinical entities: symptomatic carotid stenosis and asymptomatic carotid stenosis (Kolodgie FD. et al. 2017)

According to the most up-to-date guidelines (Lanza G. et al., 2022; Naylor et al., 2023), a carotid stenosis is defined as symptomatic when the patient experiences a transient or permanent cerebral ischemic event, such as a Transient Ischemic Attack (TIA), amaurosis fugax, or non-disabling stroke, within 90 days before diagnosis. Notably, research cohorts and institutional clinical frameworks may adopt different operational time windows (e.g., up to 6 months) when recording "recent symptoms," reflecting variability in study design and data collection. (Barisione C. et al., 2025).

These events, typically located in the cerebral hemisphere supplied by the affected carotid artery, are indicative of a high risk of early recurrence, particularly in the first two weeks, making the correction of the stenosis urgent.

A carotid stenosis is defined as asymptomatic in the presence of significant narrowing of the internal carotid artery but without recent neurological symptoms. Such strictures are often diagnosed during ultrasound checks or screenings in patients at high cardiovascular risk (Lanza G. et al., 2022).

Stenosis severity is estimated following two criteria: the European Carotid Surgery Trial (ECST), or the North American Symptomatic Carotid Endarterectomy Trial (NASCET) (Naylor R. et al., 2023).

According to this classification, mild stenosis is defined as a lumen occlusion <50%, moderate between 50-69%, and severe if ≥70%. A stenosis is defined as critical when it reduces the lumen beyond 90%, with possible significant hemodynamic impairment (Saba L. et al., 2024).

In symptomatic patients, rapid and aggressive treatment (CAS or CEA within 14 days) is indicated.

In asymptomatic patients, optimized medical therapy is preferred, and the intervention is reserved for subjects with predictive factors of high embolic risk and good life expectancy.

The degree of severity of the stenosis, the morphology of the plaque, and the individual vulnerability must always be contextualized to the presence or absence of recent ischemic symptoms, to guide therapeutic choices based on consolidated evidence and personalized risk-benefit balance (Bonati LH. et al., 2022).

Histopathologically, carotid plaques display heterogeneous characteristics. Recent studies indicate that the presence of intraplaque hemorrhage, neovascularization, and perivascular inflammation is associated with significantly increased embolic risk. The morphology of the plaque, more than just the degree of stenosis, is now a central element in risk assessment and therapeutic decision (Kolodgie FD. et al., 2017).

The most recent guidelines recommend an integrated approach to diagnosis and management, which considers not only the percentage of stenosis but also the patient's clinical profile, plaque stability, cardiovascular comorbidities, and the presence of signs of subclinical cerebral microembolization (Lanza G. et al., 2022; Müller MD. et al., 2022).

1.2.2 Diagnostic

Kolodgie and colleagues in 2017 (Kolodgie FD. et al., 2017) studied the cellular, molecular, and structural mechanisms that make carotid plaques vulnerable and capable of triggering acute cerebrovascular events, such as stroke or TIA. Vulnerability is not based exclusively on the degree of stenosis, but on a complex set of morphological and biological characteristics that determine the instability of the plaque. One of the central factors is macrophage-mediated inflammation. This process, already well described by Virmani and colleagues in 2000 (Virmani R. et al., 2000), is accentuated by local pro-inflammatory signals, such as cytokines, and by the presence of dead cells that release necrotic contents, promoting a condition of intraplaque hypoxia. As a response, intraplaque neoangiogenesis develops, leading to the formation of new blood vessels. These are fragile, immature, and poorly structured, permeable, and prone to rupture; they also cause intraplaque hemorrhages that further fuel the enlargement of the necrotic core, amplify local inflammation, and contribute to asymmetric plaque growth. Studies such as that of Kolodgie and his group in 2003 (Kolodgie FD. et al., 2003) and Moreno and collaborators in 2006 (Moreno PR. et al., 2006) have shown that these hemorrhages represent a key event in the transformation of the plaque from stable to vulnerable. Another distinguishing feature of vulnerable plaques is the presence of microcalcifications, which are located within the fibrous cap and act as foci of mechanical stress concentration. Unlike macrocalcifications, which are considered more stable, these microcalcifications increase the risk of fissure, an event

that can precede the actual rupture. This mechanism has been described by the laboratory of Kelly-Arnold (Kelly-Arnold A. et al., 2013), who have shown that calcium microdeposits are associated with focal stress peaks capable of overcoming the mechanical resistance of the cap. Taken together, these processes contribute to the transformation of a stable plaque predominantly fibrotic, into a high-risk configuration, characterized by a thin fibrous cap, large central lipid area, local bleeding, and intense inflammatory activity.

It is therefore highlighted how the vulnerability of the plaque must be interpreted in a dynamic and multidimensional perspective, overcoming the paradigm centered on the degree of stenosis alone. The clinical implications are significant: patients with apparently "non-critical" plaques from a hemodynamic point of view may still be at high risk of ischemic events if the plaque is structurally and biologically unstable. This justifies the increasing use of advanced imaging techniques aimed at detecting plaque composition and indirect signs of instability, as suggested by Otsuka et al. (Otsuka F. et al., 2014).

In the 2022 study, Bir and colleagues highlighted the precise characteristics that induce plaque vulnerability; among these are (Stary HC. et al., 1995; Bir SC. et al., 2022):

(1) structural features: large lipid-rich necrotic core, thin fibrous cap, matrix remodeling, and adventitial inflammation and angiogenesis

(2) cellular characteristics: lack of VSMCs and accumulation of macrophages in plaque

(3) functional characteristics: impairment of VSMC-induced matrix synthesis and disruption of macrophage-derived MMPs.

Based on these characteristics, the vulnerabilities of the plaque were classified:

(1) plaque rupture with intraplaque hemorrhage (IPH),

(2) plaque rupture plus thrombosis,

(3) plaque rupture with IPH and thrombosis, and

(4) IPH without plaque breakdown.

On the contrary, uncomplicated, i.e., "stable", plaques tend to be smooth and calcified (Stary HC. et al., 1995; Bir SC. et al., 2022).

From these studies, it is therefore necessary to integrate histopathological analysis with molecular imaging tools for a more precise identification of plaques at risk and to guide therapeutic decisions, both medical and surgical (Kolodgie FD. et al., 2017; Lanza G. et al., 2022).

EchocolorDoppler (ECD) of supra-aortic trunks is the tool of choice for screening, monitoring, and non-invasive evaluation of stenosis. It provides detailed information on flow velocity, plaque morphology, turbulence, and ulceration. Peak systolic and end-diastolic velocities, along with the ratio of flows, are used to estimate the degree of stenosis according to international standards. However, ECD sensitivity may be limited in patients with short necks, extensive calcifications, or multiple plaques (Lanza G. et al., 2022).

In the presence of ambiguous ultrasound results or in view of interventional treatment, second-level techniques such as computed tomography (CT) angiography and magnetic resonance angiography (MRA) are used. CT angiography has the advantage of providing a detailed view of the vascular lumen and arterial walls, also allowing a semiquantitative evaluation of parietal calcium. MRA angiography is free of exposure to ionizing radiation and allows the study of soft tissues and plaque composition, but it is more sensitive to movement and less accurate in the evaluation of very tight stenosis. Digital subtraction angiography remains the gold standard for precise quantification of stenosis, but is reserved for selected cases due to the procedural risk of stroke (Bir SC. et al., 2022).

Emerging technologies such as radiomics and AI are gaining space in predicting individual risk. Through machine learning algorithms, clinical, biochemical, and imaging data can be integrated to obtain predictive models of stenosis evolution and embolic risk. Some pilot studies have shown how deep learning can distinguish symptomatic from asymptomatic plaques with high accuracy based on morphological parameters extracted from CT angiography (Saba L. et al., 2025).

Finally, an integration between vascular imaging and brain neuroimaging is essential. Brain MRI can show silent microinfarcts, white matter hyperintensities, and cortical atrophy, parameters related to long-term cognitive risk. A multidisciplinary neurological and cardiological assessment allows for optimal risk stratification and personalized therapeutic planning (Saba L. et al., 2024).

1.2.3 Therapy and Surgery

The therapeutic strategy in carotid stenosis is complex and is based on an integrated assessment of the degree of stenosis, the presence or absence of recent ischemic symptoms, the characteristics of the plaque, and the patient's risk profile. The main treatment options are Best Medical Therapy (BMT), CEA, and CAS (Gaba K. et al., 2018).

BMT is the basic treatment for all patients, both symptomatic and asymptomatic. It includes: (1) blood pressure control with a target of <140/90 mmHg;

(2) the reduction of LDL cholesterol below 55 mg/dL (or 70 mg/dL in moderate-risk patients) by high-intensity statins and possibly ezetimibe or PCSK9 inhibitors;

(3) the treatment of diabetes mellitus according to the most recent guidelines;

(4) the use of antiplatelet agents, in particular acetylsalicylic acid or clopidogrel;

(5) smoking cessation and the adoption of a healthy lifestyle (Lanza G. et al., 2022).

Numerous studies have shown that aggressive BMT can reduce the risk of stroke in patients with asymptomatic carotid stenosis to levels comparable to those achieved with surgery, making strict medication adherence and structured follow-up critical (Gaba K. et al., 2018).

CEA is indicated in symptomatic patients with ≥70% stenosis according to NASCET criteria, ideally within 14 days of the ischemic event, a period in which the benefit of the intervention is maximum. In patients with 50-69% stenosis, CEA may be proposed in the presence of additional risk factors (unstable lesion, ulceration, intraplaque hemorrhage, cerebral microemboli detected on transcranial ECD). The guidelines emphasize the importance of performing the surgery in high-volume centers with a low incidence of perioperative complications (<3%) (Howell SJ. et al., 2007).

In asymptomatic patients, CEA is indicated only in subjects with ≥60% stenosis who present characteristics of high embolic risk (e.g., rapid progression of stenosis, hyperechoic or ulcerated plaque, signs of silent embolization on imaging). In these cases, the net clinical benefit is expected only in patients with a good life expectancy (>5 years) and low operative risk. In recent years, in the face of increasing efficacy of medical therapy, the selection criteria for CEA in asymptomatic patients have become more restrictive (Müller MD. et al., 2022).

CAS represents a valid alternative to CEA, especially in patients at high surgical risk (e.g., previous cervical surgery, post-radiotherapy stenosis, severe cardiopulmonary comorbidities). However, the risk of periprocedural stroke tends to be higher in patients over 70 years of age than in CEA. CAS, on the other hand, is particularly effective in young patients, in whom vascular manipulation is safer and cranial complications are rare (Gaba K. et al., 2018).

Ultimately, the choice of the therapeutic strategy must take place within a multidisciplinary approach, which includes neurologists, cardiologists, vascular surgeons, and interventional radiologists, to adapt the intervention to the clinical, anatomical, and prognostic profile of the individual patient (Gaba K. et al., 2018).

1.3 Biomarkers

In recent years, interest in the identification and validation of cardiovascular biomarkers has grown exponentially, driven by the need to improve risk stratification in patients with atherosclerotic disease or at risk of developing it. Primary and secondary prevention strategies have traditionally been based on established clinical risk factors, such as hypertension, diabetes, dyslipidemia, and smoking, which, however, fail to fully explain the individual variability of cardiovascular events. In fact, many patients who experience myocardial infarction or stroke do not have a high-risk profile according to traditional models, suggesting that more sensitive tools are needed to intercept those who are really at risk at an early stage (Neumann JT. et al., 2023).

In this context, biomarkers represent a valuable resource because they offer additional information on the biological processes underlying atherosclerotic disease, going beyond the simple quantification of classical risk factors. An ideal biomarker should not only reflect the activity of specific pathological pathways, but also correlate robustly with the presence, progression, and vulnerability of atherosclerotic plaque. In fact, it is increasingly clear that it is not just the anatomical severity of the stenosis that determines the clinical risk, but rather the vulnerability of the plaque: apparently moderate lesions can rupture and cause acute events, while severe stenosis can remain clinically silent if the fibrous cap remains stable (Libby P. et al, 2019).

From this point of view, the identification of biomarkers of inflammatory activity, oxidative stress, neoangiogenesis, and endothelial remodeling processes is crucial and could allow not only earlier diagnosis but also dynamic monitoring of the disease, providing valuable insights for personalized therapeutic interventions and identification of patients needing more aggressive preventive strategies.

In addition, biomarkers offer a unique window into the link between local and systemic phenomena: while plaque vulnerability is a local process, it reflects immune and metabolic activations that can be intercepted in the bloodstream. For this reason, the identification of multimarker panels capable of integrating different aspects of vascular biology could represent the key to improving risk prediction and reducing the global burden of CVD.

In clinical practice, cardiovascular risk is commonly informed by validated systemic biomarkers such as LDL-C and triglycerides (TG) as lipid-related drivers, and inflammatory markers such as high-sensitivity C-reactive protein (hsCRP); lipoprotein(a) [Lp(a)] provides

additional genetically driven risk information. While these markers robustly predict overall cardiovascular risk, they do not specifically capture the local biology of carotid plaque vulnerability (e.g., intraplaque hemorrhage, microvascular remodeling, inflammatory activation within the fibrous cap/shoulder), and their relationship with individual plaque destabilization is variable. This gap is highlighted by outcome trials demonstrating that targeting inflammation can reduce events independently of lipid lowering, such as CANTOS (IL-1β inhibition) (Ridker PM. et a., 2017) and COLCOT (colchicine) (Tardif JC. Et al., 2019). Consistently, cardiovascular outcome trials targeting the IL-6 pathway are ongoing, reinforcing the translational relevance of immuno-inflammatory biology for risk stratification (Ridker PM. Et al., 2026).

In this thesis, the proposed panel aims to complement established systemic biomarkers by capturing stress-response, macrophage activation, extracellular matrix remodeling, and angiogenesis-related signatures more closely linked to plaque-level processes; in detail:

• macrophage activation phenotypes related to complicated lesions (CD163), particularly in the context of intraplaque hemorrhage and neovascularization;
• cellular stress sensing and adaptive responses (HO-1 and AhR), linking environmental/metabolic cues to inflammatory and redox programs;
• extracellular matrix remodeling and inflammatory amplification (CD147/EMMPRIN), relevant to fibrous cap weakening; and
• pathological angiogenesis and matrix remodeling niches within the plaque microenvironment (Fn-EDB).

The overarching hypothesis is that a multi-marker, multi-compartment approach (PBMC readouts combined with plaque tissue analyses and region-of-interest histology) may provide a more informative representation of vulnerability than any single marker alone.

Emerging evidence suggests that plaque vulnerability is also influenced by a neuro-immune axis, in which adventitial nerve fibers interact with immune and vascular cells to modulate local inflammation and remodeling (Ortona S. et al., 2026).

Therefore, in addition we explored neural markers (NF200 and TH) to assess the potential contribution of the adventitial neuro-immune interface to vulnerability-related inflammation and remodeling. Beyond classical lipid-driven mechanisms, the arterial wall hosts a bidirectional crosstalk between peripheral nerves, immune cells, and the microvasculature; neural cues can modulate leukocyte recruitment and activation, cytokine production, and

vascular tone, while inflammatory and angiogenic signals can promote neural remodeling and sprouting. This interaction is particularly relevant in the adventitia, where immune aggregates and vasa vasorum may colocalize with nerve fibers, providing an anatomical substrate for local neuro-immune modulation of plaque biology (Mohanta SK. et al., 2022).

1.3.1 Cluster of Differentiation 163

Macrophages are highly plastic cells of the innate immune system, capable of adopting different functional phenotypes in response to microenvironmental cues: their polarization is commonly distinguished into M1 subsets, which are pro-inflammatory and sustain tissue damage and immune activation, and M2 subsets, generally associated with reparative and angiogenic processes. However, in atherosclerosis, their activity may paradoxically contribute to plaque progression. Among the main receptors expressed by M2-like macrophages is Cluster of Differentiation 163 (CD163), a scavenger receptor belonging to the scavenger receptor cysteine-rich proteins superfamily (Guo L. et al, 2018).

Its main function is to bind and internalize the hemoglobin-haptoglobin (Hb-Hp) complex, contributing to the removal of free hemoglobin and limiting the oxidative damage it can cause. For this reason, CD163-positive macrophages were initially considered atheroprotective, involved in the processes of inflammation resolution and in the prevention of oxidative stress with reduced production of pro-inflammatory cytokines, resistance to transformation into foam cells, and ability to counteract free radicals (Martin-Ventura JL. et al., 2012).

In recent years, however, the role of CD163 in atherosclerosis has been proven to be much more complex and, in some contexts, even opposite to that originally hypothesized. In particular, Guo and colleagues in 2018 demonstrated that, in lesions complicated by IPH, $CD163^{+}$ macrophages promote instability. The endocytosis of Hb-Hp complexes by CD163 profoundly modifies intracellular iron homeostasis: the reduction of labile iron inhibits prolyl-hydroxylases and stabilizes the hypoxia-inducible factor 1α (HIF-1α), even in the absence of hypoxia. This leads to increased production of vascular endothelial growth factor A (VEGF-A), which stimulates the formation of intraplaque neovessels, which, being immature, fragile, and permeable, further increase episodes of IPH, fueling disease progression and compromising the stability of the fibrous cap (Guo L. et al, 2018).

These mechanisms have been confirmed both in mouse models and in clinical cohorts. In Apolipoprotein E knockout (ApoE-/-) mice, the genetic deletion of CD163 results in a significant reduction in neoangiogenesis, vascular permeability, and lesion growth. In parallel,

human genetic studies have shown that CD163 gene variants associated with increased receptor expression (in particular the SNP rs7136716) correlate with increased intraplaque microvascular density, increased VEGF-A expression, and increased risk of coronary artery disease and myocardial infarction (Guo L. et al, 2018).

Mori and collaborators in 2024 demonstrated that, stimulated by IPH, CD163+ macrophages promote the release of a wide repertoire of pro-inflammatory cytokines, activating intracellular pathways in the endothelial cells that culminate in the endothelium-mesenchymal transition (EndMT). This process is characterized by the loss of endothelial markers, the acquisition of mesenchymal traits, and the predisposition of endothelial cells to apoptosis. The result is a structural weakening of the endothelial lining and the fibrous cap, leading to increased vulnerability of atherosclerotic plaque (Mori M. et al., 2024).

This study also highlighted the role of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway, as the central node through which IPH-induced $CD163^+$ macrophages promote pro-apoptotic EndMT within the fibrous cap of advanced plaques. In this scenario, the CD163 receptor acts as a free hemoglobin sensor. Still, its activation does not lead to a protective outcome: on the contrary, it promotes disease progression through thinning of the fibrous cap, making the plaque more prone to rupture (Mori M. et al., 2024).

From a clinical point of view, these observations are extremely relevant. IPH is now recognized as one of the main features of plaque instability, associated with an elevated risk of ischemic events. Thus, the presence of CD163 in hemorrhagic areas of the plaque should not be interpreted as a protective signal, but as an indicator of vulnerability. Looking ahead, the detection of IPH using advanced imaging techniques and the assessment of the presence of $CD163^+$ macrophages could help identify plaques at higher risk of clinical complications. In addition, therapeutic interventions aimed at inhibiting the $CD163^+$-NF-κB-EndMT pathway could represent an innovative approach to prevent atherosclerotic progression and destabilization (Mori M. et al., 2024).

The article by Plevriti and colleagues emphasizes the importance of the soluble receptor CD163 (sCD163), which, if released into the circulation by macrophages, can represent an indicator of macrophage activation and systemic inflammatory status. Elevated levels of sCD163 have been associated with the presence and progression of atherosclerotic plaque, an increased risk of cardiovascular events, and mortality, suggesting a potential role as a prognostic biomarker.

However, although it is easily measurable with enzyme immunoassays, it remains uncertain whether it specifically reflects unstable plaque activity or rather a more general inflammatory condition (Plevriti A. et al., 2024).

In summary, CD163 represents a complex and biphasic biomarker: in physiological conditions or in the absence of intraplaque haemorrhage, its activation is associated with protective and antioxidant responses, while in the presence of IPH, it transforms into a driver of vulnerability, promoting inflammation, endothelial apoptosis, and thinning of the fibrous cap. This dual role makes it a particularly interesting indicator to study in the atherosclerotic field, since its function is not limited to reflecting the state of the plaque, but actively participates in the mechanisms that determine its stability.

1.3.2 Heme oxygenase-1

The redox imbalance within the vascular microenvironment is one of the many factors on which the vulnerability of atherosclerotic plaque depends. In this context, Heme oxygenase-1 (HO-1) has established itself as a key biomarker and modulator of plaque stability, due to its antioxidant, anti-inflammatory, and cytoprotective properties (Durante W. et al., 2020; Alonso-Piñeiro JA. et al., 2021; Kishimoto Y. et al., 2018).

HO-1 is the inducible isoform of the heme oxygenase family. Its primary function is the degradation of heme, a potentially toxic and pro-oxidant molecule, into three biologically active products: carbon monoxide (CO), biliverdin (immediately converted to bilirubin), and ferrous iron (Durante W. et al., 2020). Each of these catabolites exerts vasoprotective effects: CO acts as a gas transmitter, with vasodilatory, anti-apoptotic, and anti-proliferative properties; Biliverdin and bilirubin are powerful antioxidants that can neutralize ROS and protect cell membranes from lipid peroxidation; The liberated iron, although potentially hazardous, is rapidly sequestered by ferritin, reducing the risk of oxidative damage (Alonso-Piñeiro JA. et al., 2021). In this way, the activity of HO-1 is not limited to heme detoxification but actively contributes to the protection of the vascular wall.

The transcriptional regulation of HO-1 is complex. The main producer is the nuclear factor erythroid 2-related factor 2 (Nrf2), which in basal conditions is sequestered in the cytoplasm by Keap1 and directed to proteasomal degradation. In the presence of oxidative stress, Nrf2 is released, translocates to the nucleus, and activates antioxidant genes, including HO-1 (Alonso-Piñeiro JA. et al., 2021). In contrast, Bach1 acts as a transcriptional repressor of HO-1, competing for binding sites on the promoter. At the genetic level, polymorphisms in the HO-1

promoter affect its inducibility. In particular, the length of the microsatellite sequence (GT)n is inversely related to transcriptional activity: short alleles (short repeats) are associated with higher expression and a reduced incidence of cardiovascular complications, while long alleles (long repeats) correlate with lower expression and greater susceptibility to vascular diseases (Exner M. et al., 2004). This makes HO-1 not only a functional biomarker but also a candidate gene for cardiovascular risk.

Studies in animal models have provided conclusive evidence. In Male ApoE-/- mice lacking HO-1, an accelerated formation of atherosclerotic lesions is observed, with high macrophage infiltration, increased oxidative stress, intraplaque necrosis, and a thinner fibrous cap, conditions that favor vulnerability (Yet SF. et al., 2003). On the contrary, overexpression of HO-1 or its pharmacological induction reduces atherogenesis and stabilizes plaques, demonstrating the protective role of the enzyme (Stocker R. et al., 2006). Already in the 90s, Poss and Tonegawa had shown that the lack of HO-1 drastically reduces the ability of cells to defend themselves against oxidative stress, confirming its importance as an endogenous adaptive mechanism (Poss KD. et al., 1997).

At the cellular level, HO-1 affects different cell types of the vascular wall. In VSMCs, this enzyme restricts proliferation and migration, two processes that contribute to vascular remodeling and neointima growth (Durante W. et al., 2020). CO and bilirubin mediate many of these effects, interfering with intracellular mitogenic pathways and blocking the cell cycle. In addition, HO-1 is induced by some of the well-established cardiovascular risk factors and appears to have a protective role in the vascular wall against atherogenesis through several pathways (Idriss NK. et al., 2008).

Another mechanism by which HO-1 stabilizes plaque is MMP modulation. These enzymes degrade the collagen of the fibrous cap, increasing the risk of breakdown. HO-1 reduces MMP activity, contributing to the maintenance of plate structural integrity (Alonso-Piñeiro JA. et al., 2021). In addition, HO-1 catabolites exert additional vasoprotective effects: CO inhibits platelet activation and reduces vasoconstriction, while bilirubin acts as a free radical scavenger (Durante W. et al., 2020).

From a clinical point of view, the interest in HO-1 as a biomarker is growing. Despite being an intracellular enzyme, it can be released into plasma under cellular stress conditions. Studies have shown that plasma levels of HO-1 are elevated in patients with diabetes mellitus, acute coronary syndrome, and hypertension (Idriss NK. et al., 2008). Crucial work by Kishimoto and

colleagues showed that in patients with carotid plaque, plasma levels of HO-1 are significantly higher than in those without plaque (0.56 vs 0.44 ng/mL), and that values above 0.50 ng/mL increase the probability of plaque finding by more than two times, regardless of other risk factors (Kishimoto Y. et al., 2018). In addition, HO-1 levels correlated with the severity of the lesions, measured by plaque ultrasound score, suggesting its value as an indicator of vulnerability.

The prognostic importance of HO-1 has also been confirmed by other studies. Cheng and collaborators showed that the presence of HO-1 determines whether an atherosclerotic lesion evolves to a stable plaque or to a vulnerable one, characterized by intraplaque necrosis, macrophage infiltration, and a thin fibrous cap (Cheng C. et al., 2009). The expression of HO-1 within the plaque, therefore, seems to play a crucial role in modulating the transition to high clinical risk phenotypes.

Despite the consensus on the protective role of HO-1, its potential ambivalence should be emphasized. In some circumstances, excessive activation of the enzyme can promote the accumulation of free iron, with increased lipid peroxidation and cell damage (Poss KD. et al., 1997).

Therapeutically, pharmacological induction of HO-1 represents an interesting prospect. Hempin is one of the most potent inducers of HO-1, although its clinical use is limited. Statins, in addition to the lipid-lowering effect, activate the Nrf2/HO-1 pathway, contributing to plaque stabilization (Stocker R. et al., 2006). Natural compounds such as curcumin, resveratrol, and sulforaphane also stimulate the expression of HO-1 through Nrf2, suggesting a possible nutraceutical role (Alonso-Piñeiro JA. et al., 2021). Other innovative strategies include gene transfer of HO-1 and controlled administration of its catabolites, such as CO or bilirubin. However, clinical application requires further studies to balance the protective benefits with possible side effects.

In summary, HO-1 is a crucial enzyme in vascular defense against oxidative stress and inflammation. Experimental and clinical data converge in indicating that its expression contributes to plaque stability, while its plasma levels may serve as a biomarker of atherosclerotic vulnerability. Promoter genetic polymorphisms modulate individual susceptibility, and drug induction represents a promising therapeutic strategy. Overall, HO-1 is confirmed as a central node in atherosclerotic pathophysiology and a potential ally in the prevention of its clinical complications.

1.3.3 Aryl Hydrocarbon Receptor

The Aryl Hydrocarbon Receptor (AhR) is a ligand-dependent nuclear receptor belonging to the family of basic helix-loop-helix/Per-Arnt-Sim transcription factors (bHLH/PAS). Initially identified in the seventies as an intracellular "sensor" for environmental xenobiotics, in particular polycyclic aromatic hydrocarbons (PAHs) and dioxins, AhR has long been considered a receptor dedicated exclusively to the adaptive response against exogenous toxic substances (Nebert DW. et al., 2017). Over time, however, scientific interest has shifted beyond the toxicological dimension, recognizing AhR as having a pleiotropic role in numerous physiological and pathological processes. Today, AhR is considered an essential regulator of immune response, lipid and glucose metabolism, cell proliferation and differentiation, as well as vascular physiology (Li K. et al., 2023; Hao N. et al., 2013; Stockinger B. et al., 2014). Its ability to integrate environmental, endogenous, and nutritional stimuli makes it a central node in cardiovascular pathophysiology, and in particular in the vulnerability of atherosclerotic plaque.

From a molecular point of view, AhR is a protein of about 848 amino acids, characterized by three main functional domains: the bHLH domain, involved in DNA binding and heterodimerization with AhR Nuclear Translocator (ARNT); the two PAS domains (A and B), respectively responsible for the interaction with ARNT and binding with ligands; and the C-terminal domain of transactivation, responsible for the transcriptional regulation of target genes (Nebert DW. et al., 2017; Bock KW. et al., 2019). In basal conditions, AhR is localized in the cytoplasm in an inactive complex with molecular chaperones such as Hsp90, p23, and the AhR-interacting protein.

Among the signaling modalities that AhR can activate, there is the canonical pathway, which involves binding to the ligand, binding to ARNT, and activating the transcription of genes containing a specific DNA regulatory sequence called xenobiotic response elements (XREs), especially those involved in xenobiotic metabolism (Sondermann NC. et al., 2023).

Alongside this, there are non-canonical pathways in which AhR acts independently of ARNT, interacting with other transcription factors such as NF-κB or nuclear receptors, regulating genes lacking XRE, or functioning as a component of ubiquitin-ligase complexes. These alternative mechanisms give AhR a broader role in modulating inflammation, cell cycle, and differentiation, in addition to traditional xenobiotic metabolism (Sondermann NC. et al., 2023).

In the context of the canonical pathway, ligand binding induces conformational modification that exposes the nuclear localization sequence, allowing the receptor to translocate into the nucleus. Here, AhR heterodimerizes with ARNT, and the resulting complex binds to XRE, activating the expression of a set of genes known as the "AhR gene battery", comprising phase I (CYP1A1, CYP1A2, CYP1B1) and phase II (UGT1A1, NQO1, GST) enzymes (Nebert DW. et al., 2017; Bock KW. et al., 2019). AhR activation is finely regulated by feedback mechanisms, including proteasomal degradation, AhR repressor induction, and ligand removal via enzymatic metabolization (Vogel CFA. et al., 2017).

The variety of AhR ligands underlines its nature as a receptor sensitive to heterogeneous stimuli. They include exogenous substances, such as TCDD, PCBs, and PAHs, endogenous metabolites, such as tryptophan derivatives (e.g., 6-formylindole[3,2-b] carbazole, FICZ), bilirubin, and lipoxins, as well as nutritional compounds such as indole-3-carbinol and flavonoids (quercetin, resveratrol, curcumin) (Li K. et al., 2023; Hao N. et al., 2013; Bock KW. et al., 2019). This heterogeneity allows AhR to act as a "bridge" between environment, metabolism, and immunity, with effects that can be protective or harmful depending on the biological context. Moreover, in addition to the canonical pathway mediated by ARNT and XRE, AhR can act via non-canonical pathways, interacting with NF-κB, STAT, and endoplasmic reticulum, influencing inflammatory, proliferative, and differentiative processes (Hao N. et al., 2013; Ohtake F. et al., 2009).

The role of AhR in atherosclerosis has been progressively clarified thanks to epidemiological and experimental observations. Chronic exposure to air pollutants, cigarette smoke, and environmental toxic compounds, all known ligands of AhR, is associated with increased cardiovascular risk and acceleration of atherosclerotic progression (Li K. et al., 2023). In animal models, persistent activation of AhR elicits endothelial dysfunction, oxidative stress, and lipid accumulation in the vascular wall, promoting plaque formation and instability (Wu D. et al., 2011). From an immune point of view, AhR controls the balance between CD4+ lymphocyte subpopulations (Hao N. et al., 2013): its activation can promote either Th17 polarization, with the production of IL-17 and pro-inflammatory cytokines, or the differentiation of immunosuppressive Treg cells, which produce IL-10 and TGF-β, helping to reduce inflammation (Hao N. et al., 2013; Stockinger B. et al., 2014; Bessede A. et al., 2014). This dual capacity reflects the plasticity of the receptor, which can exert opposite effects depending on the cytokine microenvironment and the ligand type. In macrophages, AhR regulates the expression of genes involved in the production of ROS and cytokines such as IL-1β and TNF-

α, facilitating ECM remodelling and necrotic core formation (Li K. et al., 2023; Shinde R. et al., 2018).

At the metabolic level, AhR influences crucial pathways of lipid and carbohydrate metabolism. It regulates transporters such as ABCA1 and nuclear receptors such as PPARγ and Liver X receptor alpha, which are implicated in cholesterol efflux and lipid homeostasis (Li K. et al., 2023; Bock KW. et al., 2019).

Its hyperactivation leads to increased LDL oxidation, foam cell formation, and plaque progression (Wu D. et al., 2011). In addition, AhR modulates cellular energy metabolism through interaction with AMPK and HIF-1α, conditioning VSMCs proliferation and fibrous capsule mechanical resistance (Li K. et al., 2023; Hao N. et al., 2013).

Mouse models with AhR deletion show a reduction in lesion size and inflammatory response, while pharmacological inhibition of the receptor limits foam cell formation and promotes plaque stability (Wu D. et al., 2011). These data suggest that AhR is not a simple passive sensor, but an active regulator of vulnerability.

As a biomarker, AhR has several potential uses. Its expression and activity dynamically reflect the inflammatory and metabolic state of the plaque, as well as the impact of environmental factors. The analysis of its gene or protein expression can be conducted both at the tissue level and in circulating cells, such as monocytes and lymphocytes, paving the way for possible minimally invasive diagnostic approaches (Li K. et al., 2023; Stockinger B. et al., 2014). In addition, polymorphisms of the AhR gene have been associated with different cardiovascular risk profiles, suggesting a use also in genetic stratification (Nebert DW. et al., 2017; Bock KW. et al., 2019). However, it remains a challenge to distinguish protective effects from pathogenic ones, given the complexity of ligands and downstream pathways (Bessede A. et al., 2014; Shinde R. et al., 2018).

Overall, the AhR emerges as a multifactorial and dynamic biomarker and target to treat atherosclerotic vulnerability for its ability to integrate immune-inflammatory, metabolic, and environmental signals. Selective modulation of AhR by agonists or antagonists could, in the future, steer the immune response towards protective phenotypes and stabilize atherosclerotic lesions (Li K. et al., 2023; Hao N. et al., 2013; Wu D. et al., 2011).

1.3.4 Brain and Muscle ARNT-Like 1

In the last decade, the circadian system has gained importance among the main factors regulating cardiovascular homeostasis; it is made up of a network of genes and proteins that generate rhythmic oscillations with a periodicity of about 24 hours, synchronizing physiological processes such as energy metabolism, blood pressure, immune activity, and response to oxidative stress. At the heart of this system is the Brain and Muscle ARNT-Like 1 (BMAL1), also known as aryl hydrocarbon receptor nuclear translocator-like protein 1 (ARNTL), a transcription factor that, heterodimerizing with the CLOCK gene, drives the expression of numerous target genes containing E-boxes in their regulatory regions. The transcriptional activity of BMAL1 is counteracted by inhibitory proteins such as PER and CRY, which close the negative feedback loop, ensuring the cyclicity of the circadian rhythm (Cox KH. et al., 2019). The discovery of BMAL1 as a master regulator of the circadian clock has opened up new perspectives for understanding how biological temporality influences chronic diseases, particularly cardiovascular and neurodegenerative diseases.

At the vascular level, BMAL1 is crucial for the maintenance of endothelial homeostasis and VSMCs, as well as for the regulation of innate and adaptive immune responses. Experimental studies have shown that the reduction or absence of BMAL1 leads to a loss of circadian rhythmicity of key processes, resulting in increased oxidative stress, inflammatory dysregulation, and alterations in lipid metabolism, all factors directly implicated in the formation and destabilization of atherosclerotic plaque (Xie M. et al., 2020). In particular, in the progression of atherosclerotic disease, biological rhythms and the temporal interaction between immune cells and vascular tissues contribute to plaque vulnerability. The expression of BMAL1 can therefore determine time windows of greater susceptibility to vascular damage or thrombosis.

Clinical and experimental evidence support the role of BMAL1 in atherosclerosis and CVD. A study conducted by Xie and colleagues showed that downregulation of BMAL1 aggravates atherosclerosis induced by Porphyromonas gingivalis, an oral pathogen implicated in periodontitis and associated with cardiovascular risk. Mice with reduced BMAL1 expression had more extensive atherosclerotic lesions, accompanied by increased oxidative stress and activation of inflammatory pathways (Xie M. et al., 2020). The downregulation of BMAL1 in this context promotes an imbalance between the production of ROS and antioxidant systems, with the accumulation of oxidative damage that accelerates the destabilization of the plaque. It

is therefore conceivable that BMAL1 functions as an endogenous modulator of vascular resilience to inflammatory and microbial insults.

BMAL1 not only acts in the cardiovascular system, but also regulates other biological districts whose impairment is linked to aging and chronic diseases. Fan and colleagues emphasized the importance of BMAL1 in neurodegenerative diseases, particularly Alzheimer's disease (Fan R. et al., 2022). The loss or reduction of BMAL1 alters brain circadian rhythms, increases the production of free radicals, and promotes the accumulation of β-amyloid. These mechanisms share strong similarities with those observed in atherosclerosis: oxidative stress, chronic inflammation, and metabolic dysregulation. The convergence of Alzheimer's disease and atherosclerosis, two chronic-degenerative conditions typical of aging, reinforces the idea that BMAL1 is a crucial node in the biological stress resistance network. Thus, alterations in BMAL1 may not only aggravate cardiovascular risk, but also predispose to a systemic phenotype of vulnerability associated with premature aging.

The role of BMAL1 also extends to processes of angiogenesis and ischemic response. Xu and collaborators have shown that reducing BMAL1 worsens critical limb ischemia through a dual mechanism: (Xu L. et al., 2021) reduced expression of pro-angiogenic genes and increased production of inflammatory cytokines, with consequent impairment of tissue reperfusion and healing. This finding is also relevant for atherosclerosis: in vulnerable plaques, dysfunctional neovascularization is a risk factor for IPH and instability. The loss of BMAL1-mediated circadian control may therefore contribute to aggravating this phenomenon, reducing the ability of vascular tissue to respond in an orderly manner to ischemic stimulus.

Another central aspect concerns the circadian regulation of blood pressure. Xie and colleagues showed that BMAL1 expressed in VSMCs contributes to the circadian rhythm of blood pressure (Xie Z. et al., 2015). The deletion of BMAL1 in these cells leads to a loss of diurnal variability, resulting in the appearance of a "non-dipper" blood pressure profile, a known risk factor for organ damage and cardiovascular complications. This data underlines how BMAL1 is fundamental not only at the molecular level, but also for the integrated physiology of the cardiovascular system. Circadian regulation of blood pressure directly influences hemodynamic stress on the vascular wall and thus the risk of plaque rupture. The impairment of BMAL1 in VSMCs could therefore result in a condition of chronic stress that accelerates atherosclerotic vulnerability.

The contribution of BMAL1 to innate and adaptive immune responses was further investigated by Yang and colleagues, who analyzed the effect of BMAL1 deletion in myeloid cells (Yang G. et al., 2020). In hyperlipidemic mouse models, the absence of BMAL1 in macrophages attenuated the development of atherosclerotic lesions and reduced the formation of abdominal aortic aneurysms. This result, apparently at odds with other studies, reflects the complexity of the role of BMAL1, which may have cell-specific functions. In macrophages, in fact, BMAL1 seems to favor a pro-inflammatory response that fuels atherogenesis, while its absence reduces leukocyte infiltration and vascular inflammation. This ambivalence suggests that BMAL1 should not be considered simply as "protective" or "harmful", but as a contextual modulator whose function varies according to cell type and microenvironment.

While experimental studies demonstrate a complex role of BMAL1 in vascular biology, broader reviews of the circadian clock underline its centrality in systemic regulation. Cox and Takahashi described in detail the transcriptional architecture of the circadian clock, with BMAL1 at the center of the network of oscillations that synchronize metabolism and immunity (Cox KH. et al., 2019). Other review work has linked the loss of circadian rhythmicity with an increased incidence of CVD, diabetes, and metabolic syndrome (Maury E. et al., 2010). In addition, epidemiological studies have shown that sleep disorders, night work shifts, and chronic jet lag, conditions that impair BMAL1 function, are associated with a significant increase in cardiovascular risk (Vetter C. et al., 2016).

From a translational point of view, BMAL1 is proposed as an innovative biomarker. Its expression can be measured both in tissues and in peripheral cells, such as leukocytes, in which it reflects the systemic circadian state. Alteration of its expression pattern could be an early indicator of vascular vulnerability and predisposition to acute events. However, the complexity of its regulation suggests caution: as demonstrated by Yang et al., the function of BMAL1 may be protective in some contexts (e.g., endothelium and smooth muscle) and pro-atherogenic in others (macrophages) (Yang G. et al., 2020). For this reason, the interpretation of BMAL1 levels will necessarily have to consider cellular and tissue specificity.

On the therapeutic level, interest in BMAL1 is growing. The possibility of pharmacologically modulating circadian rhythms or synchronizing BMAL1 activity with chronotherapy strategies opens up new perspectives. Some compounds capable of influencing circadian clock activity, such as REV-ERB or the receptor-related orphan receptors nuclear receptor agonists, have shown positive effects on metabolism and inflammation (Solt LA. et al., 2012; Kojetin DJ. Et al., 2014). In addition, non-pharmacological interventions such as temporal restriction of

feeding and regulation of light-dark cycles have been proposed as tools to restore circadian function and thus improve BMAL1 activity (Manoogian ENC, et al, 2022). The integration of these approaches into the management of cardiovascular disease could represent a future field of clinical application.

Therefore, BMAL1 represents a key factor in the intersection between circadian biology, metabolism, and cardiovascular pathophysiology. Considering this evidence, BMAL1 is configured as an emerging biomarker of atherosclerotic vulnerability and as a potential target for new therapeutic strategies based on chronobiology.

Finally, the observations emerged in the review of Salminen in 2023, highlighted the pivotal role of BMAL1 in maintaining circadian and metabolic homeostasis, and suggested that its functional interaction with the AhR may represent a critical node linking environmental sensing to age-related metabolic disruption (Salminen A. et al., 2023).

1.3.5 Cluster of Differentiation 147

Among the various biomarkers taken into consideration in atherosclerotic disease, there is the Cluster of Differentiation 147 (CD147), also known as ECM Metalloproteinase Inducer (EMMPRIN) or Basigin, a membrane glycoprotein belonging to the immunoglobulin superfamily. CD147 is widely expressed in different cell types, including leukocytes, endothelial cells, VSMCs, and platelets, and participates in multiple disease processes, from chronic inflammation to tumorigenesis to CVD (Lv JJ. et al., 2024; Wang C. et al., 2015).

The main function of CD147, initially identified, is the ability to induce the expression and activation of MMPs, mainly MMP2 and MMP9, also in macrophages and VSMCs (Wang C. et al., 2015; Schmidt R. et al., 2008). These enzymes degrade components of the ECM, such as collagen and elastin, promoting tissue remodeling processes. In the arterial wall, excessive activity of MMPs leads to thinning of the fibrous cap and increased risk of plaque rupture.

A recent study by Lv and colleagues highlighted the direct role of CD147 in macrophage polarization and in the impairment of efferocitosis, i.e., the ability of macrophages to eliminate apoptotic cells (Lv JJ. et al., 2024): in mouse models, CD147 overexpression favored polarization toward a pro-inflammatory M1 phenotype, with increased production of cytokines such as TNF-α and IL-6, at the expense of the reparative M2 phenotype. In addition, CD147 reduced the efficiency of efferocitosis, leading to the accumulation of apoptotic bodies and intraplaque necrosis.

Additionally, CD147 interacts with numerous ligands and coreceptors, thereby modulating leukocyte activation, cell migration, and intercellular adhesion.

In human specimens, CD147 is strongly expressed in vulnerable human plaques, particularly at the level of the fibrous cap and in macrophage infiltration zones. By increasing collagen degradation, CD147 promotes the formation of "thin-cap fibroatheroma" plaques, typical of patients at risk of acute coronary syndrome. It is therefore evident that CD147 is not only a passive marker but an active player in atherosclerotic progression.

Finally, this dual effect of CD147, enhancing the inflammatory response and reducing the ability to resolve, accelerates the vulnerability of the plaque.

Genetic deletion of macrophage CD147 protected against foam cell formation by impeding cholesterol uptake.

CD147 is also involved in intercellular communication. Macrophage-derived microvesicles and platelets have been shown to carry CD147, and their release increases under conditions of oxidative stress and inflammation (Millimaggi D. et al., 2017). These vesicles can transfer CD147 to other vascular wall cells, amplifying MMP expression and aggravating pathological remodeling.

The regulation of CD147 is complex and multifactorial. Pro-atherogenic factors such as oxidized LDL, hyperglycemia, and inflammatory cytokines (e.g., IL-1β, TNF-α) increase its expression in endothelial cells and macrophages (Yang SH. et al., 2013). In addition, oxidative stress induces CD147 transcription via MAPK and NF-κB pathways. Hypoxia is an additional stimulus, mediated by HIF-1α, that increases CD147 expression in plaques with dysfunctional neovascularization (Ke X. et al., 2012).

Evidence from clinical settings indicates that CD147 could represent a promising biomarker of instability. Clinical and histological studies have shown that CD147 levels are significantly higher in vulnerable carotid plaques and in patients with ischemic events compared to controls (Xu B. et al.,2018). In parallel, elevated plasma CD147 levels have been observed in patients with acute coronary syndrome compared to those with stable coronary artery disease (Yan J. et al., 2015).

CD147 may also represent a novel therapeutic target. Preclinical studies have explored the use of monoclonal antibodies or specific inhibitors such as SP-8356, which blocks the CD147-

CypA interaction. In ApoE-/- mouse models, these approaches reduce plaque progression and promote plaque stabilization, decreasing MMP activity and macrophage infiltration (Pahk K. et al., 2019). However, its wide distribution in tissues and participation in many physiological functions pose the problem of systemic side effects, necessitating the development of more selective approaches.

In summary, CD147 is configured as a biomarker and an active player of atherosclerotic vulnerability. Its ability to promote M1 polarization, reduce efferocitosis, induce MMP, and facilitate thrombosis places it in a key position in the pathogenic cascade that leads from subclinical atherosclerosis to acute events.

1.3.6 Extra B domain of Fibronectin

In the complex architecture of atherosclerotic plaque formation, an emerging role has been attributed to some isoforms of fibronectin (Fn), in particular to the variant containing the extra B domain (Fn-EDB), an alternative splicing product of the pre-mRNA of Fn (Nicolò G. et al., 1990; Vaidya A. et al., 2020).

Fibronectin is a multidomain glycoprotein of the ECM involved in cell adhesion, migration, differentiation, and tissue repair processes. A single gene encodes it, but it can occur in different isoforms generated by alternative splicing at three sites: the type III connective sequence (IIICS), the extra domain A (EDA), and the extra domain B (EDB). The latter is a complete 91-amino acid type III repeat, rare in normal adult tissues, but typically expressed during embryonic development, angiogenic processes, and pathological conditions characterized, such as neoplastic tissue, by intense matrix remodeling (Nicolò G. et al., 1990; Vaidya A. et al., 2020; Nicolò M. et al., 2003).

From a structural point of view, the insertion of the EDB domain between repeats III7 and III8 of the FN induces a conformational rearrangement that alters the interdomain interfaces. This leads to the exposure of cryptic epitopes, as demonstrated by the monoclonal antibody C6, which recognizes a specific loop of repeat III8 only when EDB is present (Ventura E. et al., 2010; Schiefner A. et al., 2012; Carnemolla B. et al., 1992; Ventura E. et al., 2016). These structural modifications expand the functional repertoire of Fn, giving it new properties of interaction with the ECM and with cellular receptors.

In recent years, numerous studies have extended the interest in Fn-EDB to the cardiovascular field as well. In particular, Yu et al. showed that Fn-EDB is significantly increased in advanced atherosclerotic lesions of type III, IV, and V compared to normal arteries and early lesions (Yu M. et al.,2018). Fn-EDB expression correlates with macrophage accumulation and intraplaque neovascularization, two histopathological features closely associated with instability. In addition, the use of nanoparticles functionalised specifically for Fn-EDB enabled non-invasive plaque visualisation by magnetic resonance imaging, as well as selective drug delivery in the atherosclerotic microenvironment. This approach paves the way for a "theranostic" strategy, combining molecular diagnostics and targeted therapy.

In parallel, molecular biology studies have elucidated the role of alternative splicing in regulating the functional availability of EDB. Ventura and colleagues showed that the inclusion of the EDB domain modifies the accessibility of the B-C loop of repeat III8, creating a new otherwise cryptic functional site (Ventura E. et al., 2010). This observation is particularly relevant for angiogenic processes, since the conformational remodeling of FN containing EDB can modulate the interaction with integrins and other matrix molecules, promoting endothelial proliferation and migration.

Fn-EDB was initially characterized in oncology, but the principles that supported its validation as a tumor biomarker also apply to atherosclerosis. For example, Lieverse and colleagues have pointed out that Fn-EDB is an ideal target for antibody and immunoconjugate therapies, thanks to its selective expression in areas of pathological angiogenesis (Lieverse RIY. et al., 2020). The same concept can be translated to vulnerable atherosclerotic plaques, in which angiogenic processes and ECM remodelling are central.

In the cardiovascular field, the interest in Fn-EDB is part of the broader context of the role of fibronectin isoforms in atherosclerotic pathogenesis. Studies in mouse models have demonstrated differential roles of EDA-containing variants produced by EC and VSMCs, respectively, in the early and late stages of atherogenesis (Doddapattar P. et al., 2020). This suggests that FN splicing is a dynamic mechanism, capable of finely modulating the cellular response to hemodynamic and inflammatory stress signals.

In addition to the diagnostic value, Fn-EDB offers therapeutic perspectives. Anti-EDB monoclonal antibodies, such as the well-known L19 developed by Neri's group, have already found clinical application in oncology, also in the form of immunoconjugates or radiopharmaceuticals. Hooper and colleagues have shown that an anti-Fn-EDB antibody-

conjugate increases therapeutic efficacy against tumors, especially in combination with checkpoint inhibitors (Hooper AT. et al., 2022). These data suggest that similar approaches could be explored to stabilize high-risk atherosclerotic plaques by delivering anti-inflammatory or anti-angiogenic drugs directly into the lesion microenvironment.

The translational potential of Fn-EDB as a cardiovascular biomarker is therefore double; it enables advanced molecular imaging of vulnerable plaques, and it represents a target for in situ therapies acting selectively on prone-to-vulnerability lesions. Compared to traditional biomarkers, Fn-EDB offers the advantage of spatial specificity (associated with sites of angiogenesis and pathological remodeling) and temporal specificity (different expression during lesion progression).

1.3.7 Neurofilament 200

The 200 kDa heavy neurofilament (NF200) is a subunit of the neurofilament, the main component of the axon cytoskeleton.

NF200 is particularly abundant in large-caliber axons and myelinated fibers, and is used as a marker of peripheral nerve fibers in tissues. As highlighted by the review by Khalil, neurofilaments — and in particular NF200 — are robust biomarkers of axonal integrity in numerous neurological diseases, due to their specificity and the possibility of being detected both histologically and in biological fluids (Khalil M. et al., 2018).

This marker helped to describe the neural component of adventitia of the arteries (Mohanta SK. et al., 2022; Mohanta SK. et al., 2023); studies on athero-prone mouse models have shown an increased density of NF200-positive fibers in adventitia of diseased vessels compared to healthy ones; these fibers tend to concentrate around areas of neovascularization and near immune cell aggregates, suggesting that innervation is not a random element but part of a related topographic organization to the local inflammatory response (Mohanta SK. et al., 2022; Busnelli M. et al., 2024; Gerhardt T. et al., 2025).

Increase NF200 in adventitia can be read through multiple interpretative lenses: on the one hand, it reflects the activation of axonal sprouting processes guided by chemoactive and neurotrophic signals released in an inflamed microenvironment; on the other hand, hyperinnervation can be functionally relevant because it places nerve endings in proximity to immune cells (macrophages, T lymphocytes) and vasa vasora, creating the potential anatomical

basis for neurochemical modulation of immune response and wall perfusion (Mohanta SK. et al., 2022; Mohanta SK. et al., 2023)

Denervating interventions or neurochemical manipulations in animal models have induced changes in the local inflammatory response and in the progression of atherosclerotic plaque, indicating that the nerve component contributes to the pathological dynamics of the plaque. The fibers highlighted by NF200 thus provide not only a map, but imply an ability to influence the processes of cell recruitment, pro-inflammatory activation, and remodeling of the ECM through the local release of neuropeptides and neurotransmitters (Mohanta SK. et al., 2022; Noller CM. et al., 2017).

From a pathogenetic point of view, the spatial connection between NF200 and immune clusters lays the foundations for the hypothesis of bidirectional mechanisms: inflammatory and angiogenic signals favor sprouting and the attraction of new fibers, while innervation, through the release of neuroactive molecules, modulates macrophage function, cytokine production, and matrix stability. This circuit can become self-reinforcing in chronic local inflammation, contributing to the progression of plaque vulnerability (Mohanta SK. et al., 2022; Mohanta SK. et al., 2023).

1.3.8 Tyrosine hydroxylase

Tyrosine hydroxylase (TH) is the limiting enzyme in catecholamine biosynthesis and catalyses tyrosine hydroxylation to L-DOPA, the first obligatory step for the formation of dopamine, norepinephrine, and adrenaline (Waløen K. et al., 2016). For this reason, it is considered a highly specific marker of catecholaminergic nerve endings and is used in immunohistochemistry as an indicator of sympathetic innervation. The study of its distribution in the arterial wall helps to realize that sympathetic innervation not only regulates vascular tone and perfusion, but is closely involved in the immune and inflammatory processes of atherogenesis (Mohanta SK. et al., 2022).

The clinical evidence linking TH and vascular pathology was enriched with the study by Cañes and collaborators in 2021, which documented how TH is strongly up-regulated not only in vascular innervation, but also in aortic smooth muscle and immune cells of both murine and human abdominal aortic aneurysms. This work demonstrated that pharmacological inhibition of TH by α-methyl-p-tyrosine significantly reduces aneurysm formation and progression, attenuating inflammation, oxidative stress, and elastic matrix degradation. In addition, it highlighted a pathogenetic axis involving angiotensin II and the transcription factor Neuron-

derived orphan receptor 1, which promotes TH expression, underlining its central role as a neurochemical regulator of the disease. While focusing on aneurysms, these data have a clear parallelism with atherosclerotic stenosis, where sympathetic and catecholaminergic activity contributes to both the recruitment of inflammatory cells and the structural destabilization of the wall. Cañes' work, therefore, represents direct evidence that modulating TH is not just a theoretical concept, but a potential pharmacological target with promising effects on vascular disease progression (Cañes L. et al., 2021).

In perspective, the possibility of using TH as a diagnostic marker and as a therapeutic target appears concrete. From a diagnostic perspective, immunodetection of TH in human tissues and the prospect of developing in vivo molecular imaging techniques targeting catecholamines could help identify vulnerable plaques. Therapeutically, the experience of Cañes and colleagues with pharmacological inhibition of TH in experimental models provides proof of concept that biochemical neuromodulation can modify the natural history of the disease. However, serious side effects on blood pressure control and stress response associated with a systemic suppression of catecholaminergic synthesis represent the risk that must be considered.

1.4 The Interaction Between the Nervous, Immune, and Cardiovascular Systems

Atherosclerosis is now interpreted not only as a lipid disorder but as a real chronic inflammatory disease of the arterial wall, characterized by a complex and dynamic interaction between the cardiovascular, immune, and nervous systems. Understanding how these three systems interact with each other is not only theoretical but also has enormous clinical implications. In this context, adventitia, long considered a secondary compartment of the wall, has emerged as the true crossroads of neuroimmune interactions capable of determining the vulnerability or stability of plaque (Mohanta et al., 2022).

The autonomic nervous system, and particularly the sympathetic, traditionally plays a crucial role in regulating vascular tone and cardiac function. However, recent studies have shown that nerve fibers do not limit themselves to modulating hemodynamic parameters but establish direct contact with immune and stromal cells within the arterial wall (Carnevale D. et al., 2021). This observation introduced the concept of neuroimmune cardiovascular interface (NICI), defined by Mohanta and colleagues as a real functional synapse between nerves, immune cells, and vascular structures, which organizes itself in the adventitia near the plaques (Mohanta et al., 2022). The presence of NICIs implies that the arterial wall can send afferent signals to the central nervous system and, in turn, receive efferent signals, creating a circuit called the artery-brain circuit (ABC). This, once activated in pathological conditions, feeds a sympathetic loop that promotes the progression of the disease.

The work of Mohanta et al. has clarified how atherosclerotic progression is accompanied by a marked adventitial innervation, with sensory fibres that transmit information about the local inflammatory state to the brain and sympathetic fibres that return, resulting in an enhancement of inflammation and plaque instability. A key finding is that celiac ganglionectomy (CGX), i.e., the surgical interruption of sympathetic nerve flow through the celiac ganglion, results in a drastic reduction in atherosclerotic progression and the collapse of the lymphocyte structures that form in the adventitia, the Artery Tertiary Lymphoid Organs (ATLOs) (Mohanta et al., 2022). ATLOs have been extensively characterized in murine models of advanced atherosclerosis, whereas in humans adventitial lymphoid aggregates are reported with variable degree of organization, and their equivalence to murine ATLOs remains under investigation. This observation is of extraordinary importance because it suggests a causal link between

sympathetic activity and the maintenance of adventitial immune structures, which in turn contribute to chronic plaque inflammation (Mohanta et al., 2022).

ATLOs have been described in detail by Yin and colleagues as true centers of local immunity (Yin et al., 2016). They typically appear near advanced plaques in ApoE-/- mouse models and have peculiar features: distinct compartments of T and B lymphocytes, follicular dendritic cells, classical dendritic cells, plasma cells, aberrant lymphatic vessels, and high endothelial venules. They represent the equivalent of an ectopic non-encapsulated lymph node located directly in the arterial wall, capable of supporting localized adaptive immune responses. ATLOs thus become an interaction point where resident immune cells, circulating cells, fibroblasts, and nerves converge to orchestrate the inflammatory response. The observation that CGX determines their collapse indicates that the maintenance of ATLOs depends on sympathetic tone, probably through the modulation of chemokine secretion and survival factors for lymphocytes (Mohanta et al., 2022).

The clinical implications of these findings are significant. The possibility of modulating the sympathetic nervous system to influence atherosclerosis opens up new therapeutic strategies. Modulation of the nervous system, for example, through sympathetic denervation procedures or the use of drugs that reduce adrenergic tone, could represent an additional way to control vascular inflammation. Finally, ATLOs or ATLO-like adventitial aggregates could represent a new biomarker of vulnerability: their presence or collapse could indicate disease progression or regression, respectively.

2. Materials and Methods

2.1 Biobanking and selection of the two study populations of carotid stenosis patients

This project has been conducted in the Laboratory of Clinical and Experimental Vascular Biology at Ospedale Policlinico San Martino in Genoa; it was approved by the Ethics Committee of the University of Genova (Comitato Etico Regionale Liguria, Italy - cod. Genoa MC-Bio-AAA) and adhered to the Declaration of Helsinki Principles.

As part of the laboratory team, I took part in the Genoa Tissue Bank - Vascular Division (GTB-VD); the GTB-VD comprises the Vascular and Endovascular Surgery Unit, the Laboratory of Experimental and Clinical Vascular Biology (BioVasc Lab), and the Anatomic Pathology Unit within the Department of Surgical and Diagnostic Sciences at the University of Genoa.

The GTB-VD recruits patients undergoing surgical repair for carotid artery stenosis (CS), enrolled based on selection criteria, upon informed consent, and it provides biological specimens and data collection of the two study populations, symptomatic carotid stenosis (SCS) and asymptomatic carotid stenosis (ACS) patients, the object of this study.

In the GTB-VD, symptomatic carotid stenosis (SCS) is defined by an ipsilateral cerebral or retinal ischemic event (e.g., TIA, amaurosis fugax, non-disabling stroke) occurring within the previous 6 months, whereas asymptomatic carotid stenosis (ACS) refers to severe carotid narrowing in the absence of recent neurological symptoms (Barisione et al., 2025). We acknowledge that clinical guidelines commonly define “symptomatic” carotid stenosis using a 90-day window; however, different time windows are adopted across clinical frameworks and research cohorts (Lanza G. et al., 2022).

In this thesis, plaque vulnerability was approached as a multidimensional phenotype integrating

1. the clinical presentation (SCS vs ACS) as a proxy of unstable behavior,
2. the local plaque microenvironment assessed through region of interest analyses of the surgical specimens (e.g., fibrous cap and plaque shoulder), and
3. molecular and cellular readouts measured both systemically and locally. Specifically, peripheral blood-derived PBMCs were used to capture systemic immune/stress-response signatures, while carotid tissue analyses (gene/protein

expression and immunohistochemistry on FFPE sections) were used to characterize plaque inflammation, remodeling, and stress-response pathways in situ.

This integrated design was intended to capture plaque heterogeneity beyond luminal stenosis and to support the identification of vulnerability-associated biomarker patterns.

The 4-step workflow is:

1. Recruitment of Participants and Biospecimen Collection:

Patients admitted to the Vascular and Endovascular Surgery Unit for elective surgical correction of CS are selected one day before the intervention. If eligible for biobanking, they can be enrolled upon informed consent.

Criteria of inclusion: patients admitted to the Vascular and Endovascular Surgery Unit for open or endovascular surgery of both symptomatic and severe-asymptomatic CS.

Criteria of exclusion: serological positive for HIV, HBV, HCV; history of recent or ongoing neoplasia or anti-tumor therapy in the previous year; myocardial infarction in the 6 months before surgery; presence of autoimmune diseases.

Participants undergo peripheral blood sampling the day before the intervention; tissue samples are available when open surgery is performed.

The hospital transport team is responsible for delivering specimens, at room temperature, within two hours from the Vascular and Endovascular Surgery Unit to the BioVasc Lab.

2. Reception and Processing of Samples:

Upon sample arrival at the BioVasc Lab, we assess its quality, including factors such as the elapsed time between collection and delivery and the presence of hemolysis in plasma or serum. An alphanumeric code is electronically assigned to each patient and recorded on labels for cryovials containing frozen specimens and jars with 10% formalin for tissue biopsies. Samples that do not meet the criteria for biobanking are disposed of after informing the patient, who is required to sign a specific form. Blood, urine, and tissue samples are processed in a dedicated room under a sterile laminar flow hood.

- PBMCs isolation

Once blood samples are accepted, serum, plasma, and peripheral blood mononuclear cells (PBMC) are collected.

Plasma and sera are processed by centrifugation for 15 minutes at 3500g. After centrifugation, they are stored in 500 µL aliquots. For isolation of PBMCs, 6 mL of blood is first diluted with an equal volume of saline in 15 mL tubes. Density centrifugation gradient is then performed over 3 mL of Lympholyte separation medium (Cedarlane, Canada) for 20 minutes at 1800g. The interface ring, which contains PBMCs, is carefully recovered and rinsed with saline through centrifugation. The resulting pellet is divided into four aliquots, subjected to a brief high-speed centrifugation (30 seconds), and subsequently dry stored at -80°C.

- Tissue samples collection

Tissue samples are obtained during open repair surgeries. Depending on the size, they are processed in consecutive segments. Two aliquots are immediately stored at -80°C for molecular experiments. The remaining part is fixed in 10% formalin and then sent to the Anatomy Pathology Unit of Ospedale Policlinico San Martino. Subsequently, they are processed and included in paraffin to obtain histologic slides for immunohistochemistry analysis.

3. Storage:

Peripheral blood-derived and frozen tissue samples are stored in monitored and alarmed facilities at -80°C, managed by the CRB-HSM. Formalin-fixed, paraffin-embedded (FFPE) samples are stored in histotheques of the Anatomic Pathology Unit, in cardboard boxes at room temperature, protected from dust, light, and heat.

4. Data collection:

Clinical data collections are housed in the electronic database system of the Italian Society of Vascular and Endovascular Surgery (https://www.sicvereg.it/). Patients are registered with the study number and electronically assigned to prevent subject identification.

2.2 RNA extraction from tissue samples and PBMCs, reverse transcriptase, and Polymerase Chain Reaction

Only for tissue samples, a tissuelyser was used for 4 minutes to obtain a tissue homogenate.

Total RNA from tissue homogenate and PBMCs was extracted using TRiFast II reagent (Euroclone, Milan, Italy) following the chloroform addition and centrifugation at 12000g for

15 min at 4°C, enabling phase separation. The organic phase (transparent) contains RNA. It was collected and moved to a new tube with isopropanol, mixed by inversion, and incubated for 10 min at room temperature. After centrifugation for 10 min at 12000 g at 4°C, an RNA pellet was obtained. The supernatant was discarded, and the RNA pellet was washed in 70 % ethanol, allowed to dry, and resuspended in 40 µL DEPC-treated water.

The RNA was quantified by using a Tecan Spark multimode microplate reader (TECAN, Männedorf, Switzerland).

Total µg of RNA was transcribed into cDNA using iScript Reverse Transcription Supermix (Bio-Rad Laboratories, Milan, Italy).

Subsequently, to every sample is added 40 µL of H2O nuclease-free and then stored at 4°C.

For each analyzed gene, a mix was prepared by adding H2O, Luna universal qPCR Master Mix containing a fluorescent dsDNA binding dye, and the specific primers mix (Tib MolBioL, Genoa, Italy) at a final concentration of 0,025 µM.

In a 96-well plate for each gene and sample, 8 µL of the mix and 2 µL of the sample cDNA were loaded. Then the plate was analysed by the LightCycler 96 Instrument (Roche).

Preliminary validation of housekeeping genes was performed to ensure expression consistency for relative quantification, in line with the MIQE guidelines. At the end of this, we chose β2-microglobulin as housekeeping.

The genes analyzed in this study were:

Gene and ID number	Primer Forward 5'→ 3'	Primer Reverse 3' →5'
AhR	TAC CCC AGA CCA GAT TCC TC	GCA AAC AAA GCC AAT TGA G
BMAL-1	AAG GGA AGC TCA CAG TCA GAT	GGA CAT TGC GTT GCA TGT TGG
HO-1	TCC TGG CTC AGC CTC AAA TG	CGT TAA ACA CCT CCC TCC CC
CD147	CCCTTCCTGGGCATCGT	CGGCGTCGTCATCATCC
CD163	GGATCTGCTGACTTCAGAAG	CTCCTTGTCTGTTCCTCCAA
Fn-EDB	TCAAGGATGACAAGGAAAGTG	AATAATGGTGGAAGAGTTTAGC
β2-microglobulin (housekeeping)	CCA GCG TAC TCC AAA GAT TCA	TGC TCC ACT TTT TCA ATT CTC TC

2.3 Histology

2.3.1 Haematoxylin & Eosin Staining

The use of haematoxylin and eosin staining allows for the visualization of cellular structures within histological specimens. Haematoxylin imparts a blue-violet colour to negatively charged cellular components, such as the nucleus, cell membrane, and elastin. In contrast, eosin stains positively charged cellular components, including the cytoplasm, collagen, and cellular and mitochondrial proteins, with a pink colour (Haematoxylin and Eosin Staining - Manual Protocol from Baylor College of Medicine).

The staining procedure comprises the following steps:

De-paraffinization and Hydration: The slides containing the tissue sections are sequentially immersed in xylene (3 times, 3 minutes each), absolute ethanol (3 times, 3 minutes each), 95% ethanol (3 minutes), 80% ethanol (3 minutes), and deionized water (5 minutes).

Haematoxylin Staining: The sections are stained with haematoxylin for 3 minutes and then washed in water for 5 minutes. Following this, the sections are immersed in acidified ethanol (HCl + 70% ethanol, diluted 1:400) to remove excess stain, followed by rinsing in running water and deionized water.

Eosin Staining: The sections are stained with eosin for 5 minutes and subsequently dehydrated through a series of increasing ethanol concentrations: 95% ethanol (5 minutes), absolute ethanol (5 minutes), and xylene (5 minutes) to dehydrate the tissue.

The slides are then mounted using the synthetic mounting medium, Eukitt.

2.3.2 Movat Pentachrome Staining

Movat pentachrome staining is employed to distinguish five histological components (collagen, elastin, muscle cells, mucin, and fibrin), and is used in the study of cardiac tissues, blood vessels, and connective tissues to detect alterations of the ECM and its interaction.

The procedure, according to the manufacturer's datasheet, is the following (ScyTec Laboratories MPS-2-IFU). Through this technique, different tissue components are distinctly stained, each displaying a unique colour. The colorimetric references are detailed in the table below.

It provides the following colours based on the tissue type:

Colour	Tissue type
Black	Nuclei; elastic fibers
Yellow	Collagen fibers; reticular fibers
Blue	Ground substance; mucin
Bright red	Fibrin
Red	Muscle

2.3.3 Immunohistochemistry

Immunohistochemistry of formalin-fixed paraffin tissue sections (4 μm) from carotid stenosis samples was conducted.

Histological sections were deparaffinized through a graded series of alcohols and subsequently rehydrated in phosphate-buffered saline for 5 minutes.

Then, the antigen was unmasked by immersion in an acidic solution of citric acid and sodium citrate at a high temperature. This step is crucial because the fixation process and paraffin embedding can obscure or alter the three-dimensional structure of protein antigens in FFPE tissues. Fixation can induce cross-linking between proteins, making epitopes less accessible to antibodies. Antigen unmasking restores the three-dimensional conformation of epitopes.

To quench the content of endogenous peroxidases, which may react non-specifically with the chromogen 3,3'- diaminobenzidine (DAB), the slides were immersed twice in quenching solution of methanol and hydrogen peroxide (H2O2) for 15 minutes.

To reduce non-specific bindings, samples were incubated for 20 minutes at room temperature with a PBS block buffer containing 2,5 % Normal Goat Serum (Vector Laboratories), which is the serum from the animal in which the secondary antibody was produced.

After removal of the blocking solution, the sections were incubated overnight at 4 °C with the primary antibody diluted in the same block buffer. The next day, the slides were washed in PBS for 10 min, and then incubated for 40 minutes with Goat-Anti Mouse or Goat- Anti-Rabbit

(Vector Laboratories, Newark, CA, USA) conjugated with Horseradish peroxidase (HRP). The incubation with the DAB produces an insoluble brown-coloured precipitate.

Counterstaining of nuclei with haematoxylin.

Only for double staining with Emerald Chromogen (NeoBiotech), the sections underwent a second quenching to block endogenous peroxidases, then incubated overnight at 4 °C with the second primary antibody diluted in the same block buffer. The next day, the slides were washed in PBS for 10 min, and then incubated for 40 minutes with Goat-Anti Mouse or Goat- Anti-Rabbit (Vector Laboratories, Newark, CA, USA) conjugated with Horseradish peroxidase (HRP) (Vector Laboratories, Newark, CA, USA). The incubation with the Emerald produces a green-coloured precipitate.

Finally, they are sealed with glycerol gel or Eukitt in case of double staining, since Emerald is water-soluble.

Negative controls were included in each staining run by omitting the primary antibody (secondary-only control) to monitor non-specific background and chromogen deposition.

Antibodies used for protein detection in IHC were:

Protein	Ab	Brand	Dilution
AhR	Mouse anti-AhR	Santa Cruz	1:150
CD147	Rabbit anti-CD147	BiOrigene	1:100
NF200	Mouse anti-NF200	Millipore	1:400
TH	Rabbit anti-TH	Millipore	1:750
CD3	Rabbit anti-CD3	Abcam	1:100
CD163	Mouse anti-CD163	Santa Cruz	1:200
Anti-Mouse	Goat-Anti Mouse	Vector Laboratories	Ready to use
Anti-Rabbit	Goat- Anti-Rabbit	Vector Laboratories	Ready to use

2.4 Image analysis

The quantitative evaluation of immunohistochemistry (IHC) has been performed on images acquired with the Aperio AT2 DX System, Leica DX (Leica, Cambridge, UK). Detection of DAB chromogen positivity was performed through a systematic computational analysis using the Fiji ImageJ software (https://imagej.net/software/fiji/).

For each carotid section, three distinct regions of interest (ROIs) - media, fibrous cap, and shoulder-neovasa- were identified. For each ROI, 3 to 5 fields were acquired and evaluated. These ROIs were selected to capture plaque heterogeneity and to focus on compartments with distinct biological roles, including regions commonly associated with inflammatory infiltration and microvascular remodeling (e.g., the shoulder/neovessels area), thereby supporting a region-specific assessment of vulnerability-related signals.

The analysis was conducted adjusting the protocol from Bio Protocol (Crowe AR, et al., 2019) to set up a threshold value for each ROI on a reference specimen, chosen as “calibrator,” and repeated in each work session. This calibrator-based normalization was adopted to minimize inter-session variability (e.g., illumination, staining intensity, thresholding) while maintaining ROI-specific quantification.

Firstly, the image was split using the "Colour Deconvolution" option, selecting the "HDAB" vector tool, which enables the identification of immunopositivity in relation to DAB staining.

Subsequently, the percentage of the positive area was calculated by adjusting the saturation of the threshold in a range between 140 and 160 for each ROI calibrator; each working session was then normalized with respect to the calibrator sample, arbitrarily set at 1000.

Data for statistical analysis, expressed as Arbitrary Unit (AU), were thus obtained by applying this algorithm for each session: (Mean value ROI specimen/Mean value ROI calibrator) x 1000

2.5 Statistical analysis

Continuous clinical and laboratory parameters are presented as mean (SD) or median (interquartile range, IQR) depending on their normal or skewed distribution, while categorical data are reported as absolute and relative frequency. Comparisons were performed by the Mann-Whitney test. Spearman's correlation was used to demonstrate the relationship between two variables. Statistical significance was set at $p<0.05$.

Among clinical parameters, randomly missing data are indicated as unknown and limited to a maximum of 1 data point in each population of patients.

RT-qPCR values with threshold cycle > 33 have been excluded, and the number of values or pairs used is indicated in the figure legends.

All statistical analyses were performed using GraphPad Prism (v. 8 for Windows, GraphPad Software, San Diego, California, USA).

3. Results

3.1 Demographics and medical history of the two study populations

Table 1 summarizes demographics and clinical characteristics of the two study groups.

Variable	**overall, n = 68**	**asymptomatic, n = 46**	**symptomatic, n = 22**	***p***
Male gender	68 (100.0%)	46 (100.0%)	22 (100.0%)	
Age	75.03 (6.29)	75.70 (6.33)	73.64 (6.09)	0.209
Smoke				0.115
ex	33 (48.5%)	26 (56.5%)	7 (31.8%)	
no	19 (27.9%)	12 (26.1%)	7 (31.8%)	
yes	16 (23.5%)	8 (17.4%)	8 (36.4%)	
Hypertension	55 (80.9%)	35 (76.1%)	20 (90.9%)	0.146
Type II diabetes	23 (33.8%)	17 (37.0%)	6 (27.3%)	0.430
Dyslipidemia	46 (67.6%)	33 (71.7%)	13 (59.1%)	0.297
Heart conditions	7 (10.3%)	5 (10.9%)	2 (9.1%)	0.821
Leukocytes x10E9/L	7.14 (1.68)	7.19 (1.81)	7.04 (1.42)	0.728
Erythrocytes x10E12/L	4.40 [4.08, 4.80]	4.50 [4.13, 4.80]	4.20 [3.63, 4.70]	0.033*
Hemoglobin g/L	131.13 (19.14)	133.28 (18.00)	126.64 (21.05)	0.182
Platelets x10E9/L	204.03 (54.05)	199.15 (46.93)	214.23 (66.63)	0.285
Ultrasound calcification	41 (61.2%)	31 (68.9%)	10 (45.5%)	0.065
Unknown	1	1	0	
Creatinine mg/dL	1.07 [0.90, 1.30]	1.00 [0.80, 1.30]	1.10 [0.90, 1.30]	0.247
Unknown	2	1	1	
eGFR mL/m^2/1,73mq	66.50 [52.25, 85.00]	70.00 [55.00, 87.00]	64.00 [51.00, 79.00]	0.220
Stage 1 of CKD	8 (12.1%)	8 (17.8%)	0 (0.0%)	0.039*
Stage 2 of CKD	33 (50.0%)	21 (46.7%)	12 (57.1%)	0.428
Stage 3a of CKD	13 (19.7%)	8 (17.8%)	5 (23.8%)	0.566
Stage 3b of CKD	12 (18.2%)	8 (17.8%)	4 (19.0%)	0.901
Stage 4 of CKD	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Stage 5 of CKD	0 (0.0%)	0 (0.0%)	0 (0.0%)	

Table 1. Demographics and laboratory tests in the two study groups.

The two study populations did not differ for modifiable risk factors such as smoking habits, hypertension, type II diabetes, dyslipidaemia, and cardiac history.

Laboratory parameters displayed no significant difference in haemoglobin, leucocytes, and platelet levels. Red blood cell count was slightly reduced in symptomatic patients (p= 0.033).

Plaque calcification, evaluated echographically by measuring the typical hyper-echogenicity and/or posterior shadow cone induced by ultrasound reflection, tended to be higher in asymptomatic patients (p= 0.065). However, a bias may reside in the comparison of values obtained by different operators.

No differences were observed in terms of renal function (creatinine, eGFR) between the two cohorts.

In this preliminary study, to avoid sex-dependent biases, only male patients were selected, and no differences in the age range were observed between asymptomatic and symptomatic patients (Figure 1).

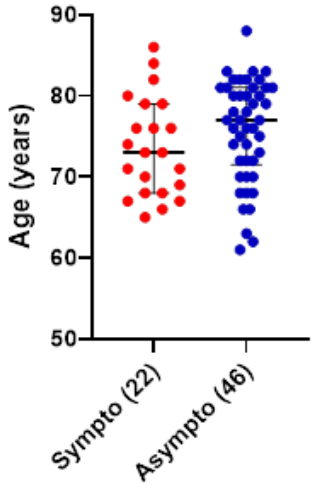

Figure 1. Age distribution in years of the two study groups.

As shown in Table 2, pre-surgery therapies in the two groups appear substantially homogeneous, except for significant differences in the use of dicoumarol or heparin anticoagulants and ACE inhibitors, which are of uncertain clinical significance.

Variable	Overall, n = 68	asymptomatic, n = 46	symptomatic, n = 22	*p*
Statins	50 (73.5%)	35 (76.1%)	15 (68.2%)	0.489
Antiaggregants/aspirin	48 (70.6%)	32 (69.6%)	16 (72.7%)	0.789
Antiaggregants/ tienopiridine/inibitori fosfodiesterasi (ticlopidine, clopidrogel)	29 (42.6%)	18 (39.1%)	11 (50.0%)	0.397
anticoagulants (heparin, warfarin)	11 (16.2%)	4 (8.7%)	7 (31.8%)	0.015*
type anticoagulant				0.264
apixaban	2 (18.2%)	2 (50.0%)	0 (0.0%)	
dabigatran	1 (9.1%)	0 (0.0%)	1 (14.3%)	
edoxaban	1 (9.1%)	0 (0.0%)	1 (14.3%)	
enoxaparin	6 (54.5%)	2 (50.0%)	4 (57.1%)	
rivaroxaban	1 (9.1%)	0 (0.0%)	1 (14.3%)	
Diuretics	17 (25.0%)	12 (26.1%)	5 (22.7%)	0.765
antidiabetics (sulphonylureas-biguanides- glitazoni-inh DDP)	20 (29.4%)	16 (34.8%)	4 (18.2%)	0.160
antidiabetics (insulin)	5 (7.4%)	5 (10.9%)	0 (0.0%)	0.108
beta-blockers	30 (44.1%)	22 (47.8%)	8 (36.4%)	0.373
ACE I	21 (30.9%)	18 (39.1%)	3 (13.6%)	0.033*
ARBs	13 (19.1%)	8 (17.4%)	5 (22.7%)	0.601

*p<0.05; **p<0.01; ***p<0.001

Table 2. Pre-surgery therapies in the two study groups.

3.2 Gene expression in PBMCs

The mRNA levels of CD163, AhR, HO-1, and BMAL-1 were analyzed by RT-qPCR in PBMCs collected from 46 asymptomatic and 22 symptomatic patients; then, correlation analysis among gene expression in PBMCs was performed.

3.2.1 CD163 and AhR mRNA are significantly higher in PBMCs of symptomatic patients.

As shown in Figure 2, CD163 (Figure 2a, p= 0.0295) and AhR (Figure 2b, p= 0.0394) expression were significantly higher in symptomatic patients than in asymptomatic ones.

No differences were observed in the two populations of patients for HO-1 (Figure 2c) and BMAL-1 (Figure 2d) expression.

We then ascertained the absence of possible confounders among demographic and clinical parameters, as no association was found between each of them and CD163, AhR, HO-1, and BMAL-1 gene expression, both in each study population and the whole cohort of patients.

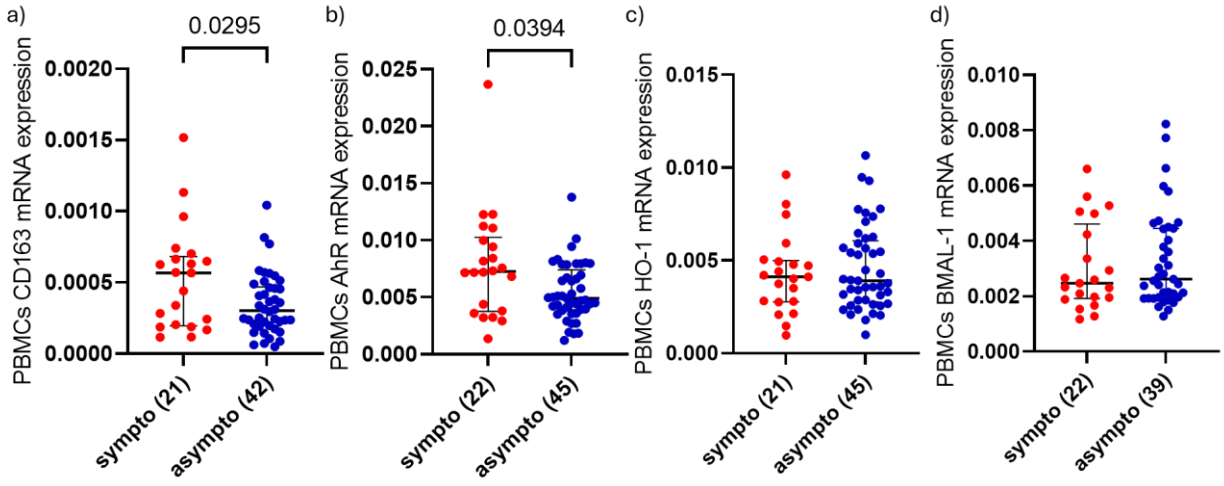

Figure 2. Gene expression in the PBMCs in the two study populations (symptomatic in red and asymptomatic in blue).

3.2.2 CD163 mRNA correlates with both AhR and HO-1 mRNA in PBMCS.

As shown in Figure 3, a correlation was found between CD163 and AhR when including the whole cohort (symptomatic and asymptomatic) (Figure 3a, r= 0.4546, p= 0.0002, n= 62); and the asymptomatic subjects (Figure 3b, r= 0.3565, p= 0.0221, n= 41); a similar trend was found in symptomatic population although not reaching the significance (r= 0.3805, p= 0.09, n= 21).

Similarly, as shown in Figure 3, the correlation between CD163 and HO-1 reached the statistical significance in both population (Figure 3c, r= 0.3332, p= 0.0081, n= 62), and in symptomatic patients only(Figure 3d, r= 0.5338, p= 0.0127, n= 21) while it failed to reach asymptomatic population significance (r=0.167 p= 0,2949 n= 41).

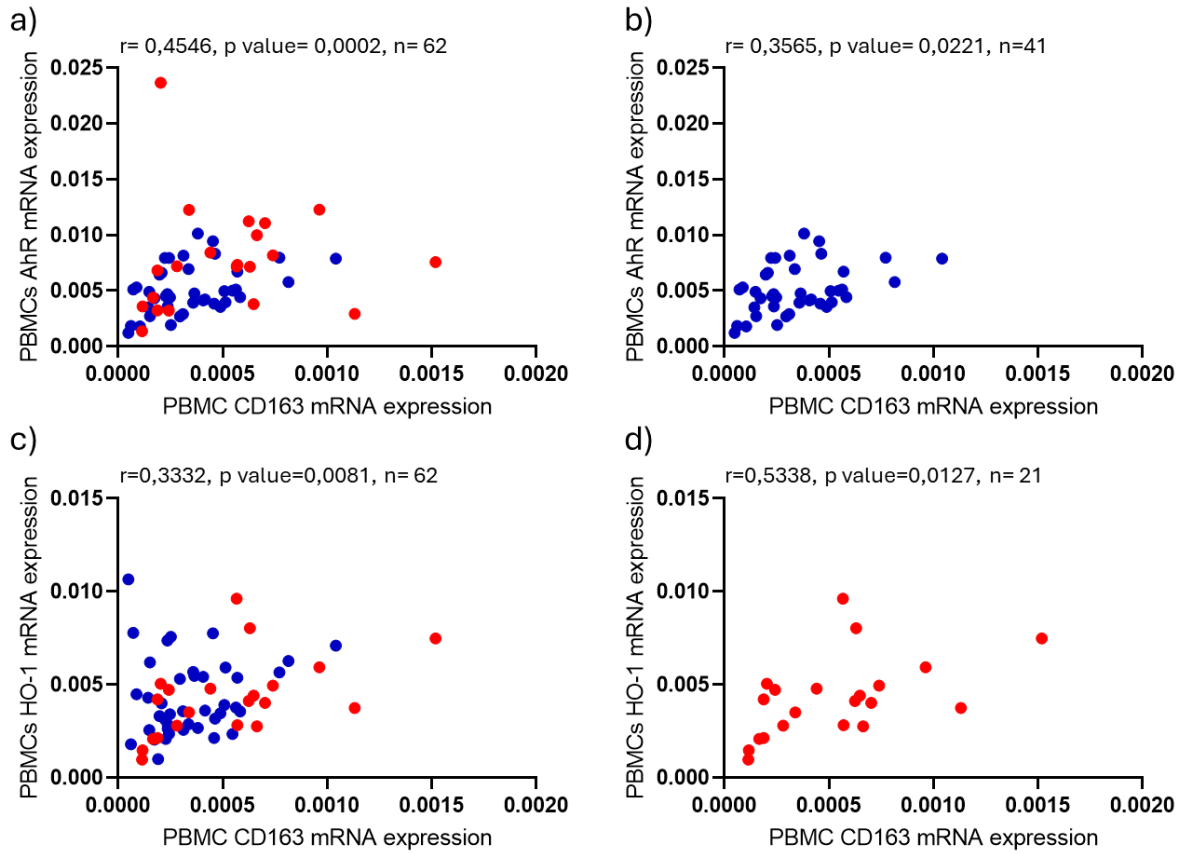

Figure 3. CD163 correlation between: a) AhR in the two study populations; b) AhR only in asymptomatic patients; c) HO-1 in the two study populations; d) HO-1 only in symptomatic patients (symptomatic in red and asymptomatic in blue).

3.2.3 HO-1 mRNA is associated with BMAL-1 mRNA in PBMCS of the whole population.

As shown in Figure 4, when including all subjects (symptomatic and asymptomatic), a significant correlation between HO-1 and BMAL-1 was observed (= 0.2987, p= 0.0204, n=60); when taking the two separate populations, no significance was found.

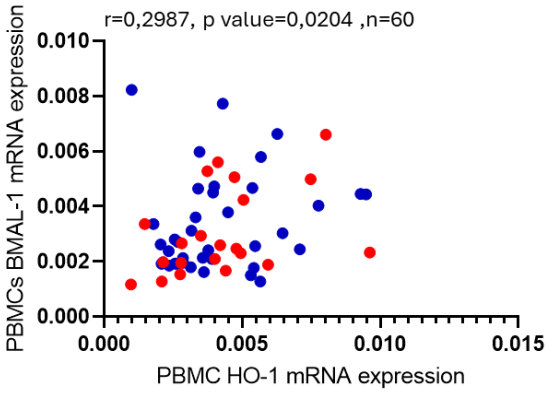

Figure 4. HO-1 correlates with BMAL-1 in the two study populations (symptomatic in red and asymptomatic in blue).

3.3 Gene expression in plaque specimens

In plaque specimens collected from 46 asymptomatic and 18 symptomatic patients, the mRNA levels of CD163, AhR, CD147, and Fn-EDB were analyzed by RT-qPCR.

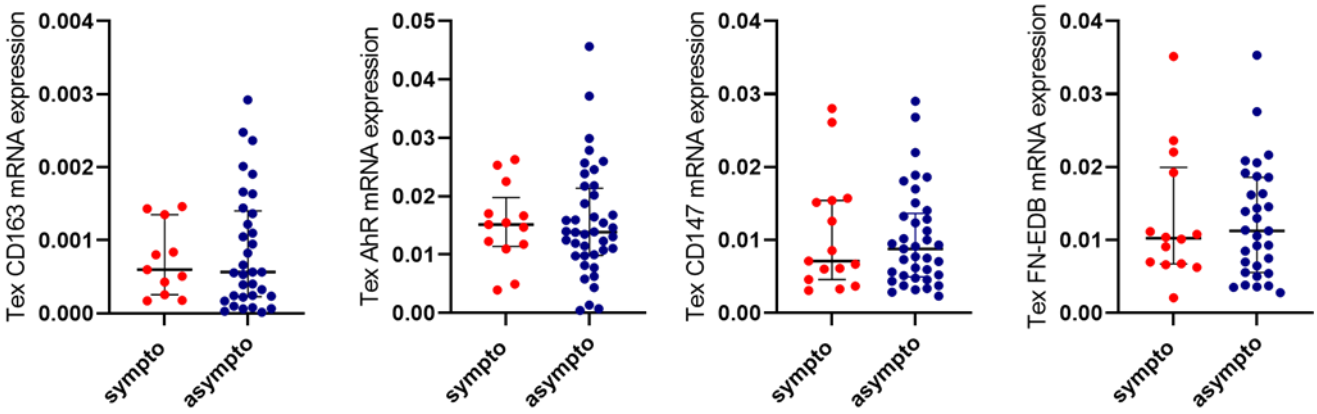

Figure 5. Gene expression in the plaque specimens in the two study populations (symptomatic in red and asymptomatic in blue).

No differences were observed between the two populations of patients for gene expression; then, correlation analysis among genes was performed (Figure 5).

3.3.1 AhR mRNA correlates with CD147 mRNA in plaque specimens.

As shown in Figure 6, when including all patients, a significant correlation between AhR and CD147 was observed (Figure 6a, r= 0.5119, p= 0.0002, n= 47); considering the two populations separately, the correlation was statistically significant only in asymptomatic subjects (Figure 6b, r= 0.6034, p= 0.0002, n= 34). A similar trend, although not significant, was found in the symptomatic population (r=0.3681, p=0.2167, n=13).

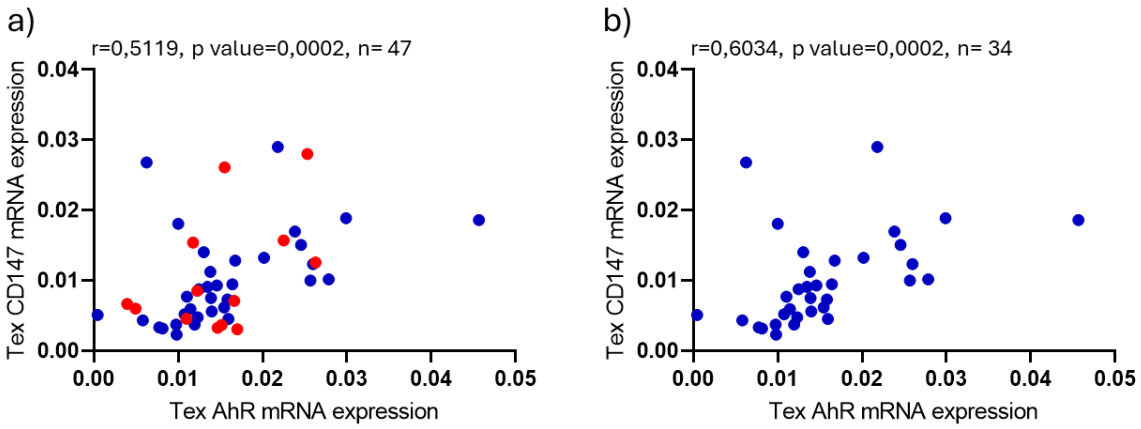

Figure 6. AhR correlation between a) CD147 in the two study populations; b) CD147 only in asymptomatic patients (symptomatic in red and asymptomatic in blue).

3.3.2 Fn-EDB mRNA is associated with both AhR and CD147 mRNA in plaque specimens of the whole population.

As shown in Figure 7, in plaque specimens, there was a significant correlation between AhR mRNA and Fn-EDB mRNA when including all patients (Figure 7a, r= 0.5395, p= 0.0002, n= 44), and in asymptomatic patients, there was a strong significant correlation (Figure 7b, r= 0.5859, p= 0.0005, n= 31). No significance was found in the symptomatic population (r=0.4451, p=0.1299, n=13).

A similar correlation has also been observed between FN-EDB mRNA and CD147 mRNA, considering together the two study populations (Figure 8a, r= 0.5387, p= 0.0002, n= 43) and separately, symptomatic (Figure 8b, r= 0.7055, p= 0.0063, n= 14) and asymptomatic (Figure 8c, r= 0.4621, p= 0.0116, n= 29).

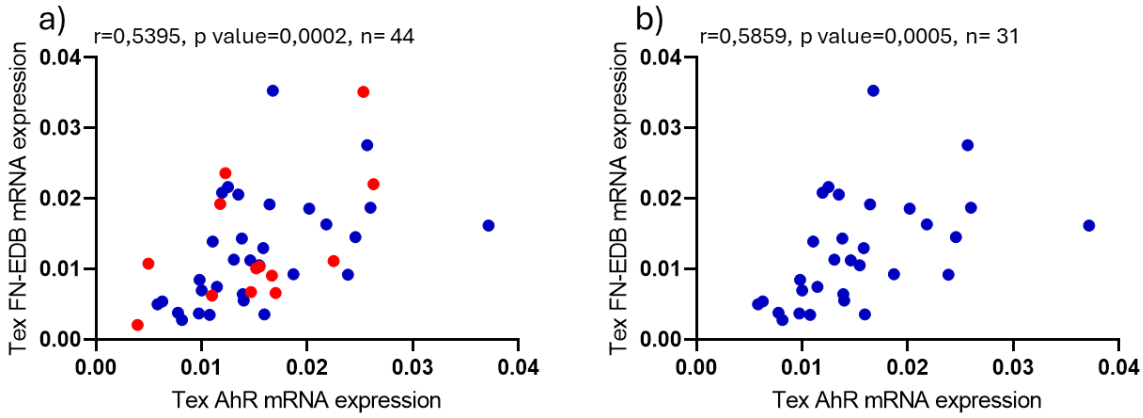

Figure 7. Fn-EDB mRNA correlation between a) AhR mRNA in plaque specimens of the entire study population and of asymptomatic patients (b) (symptomatic in red and asymptomatic in blue).

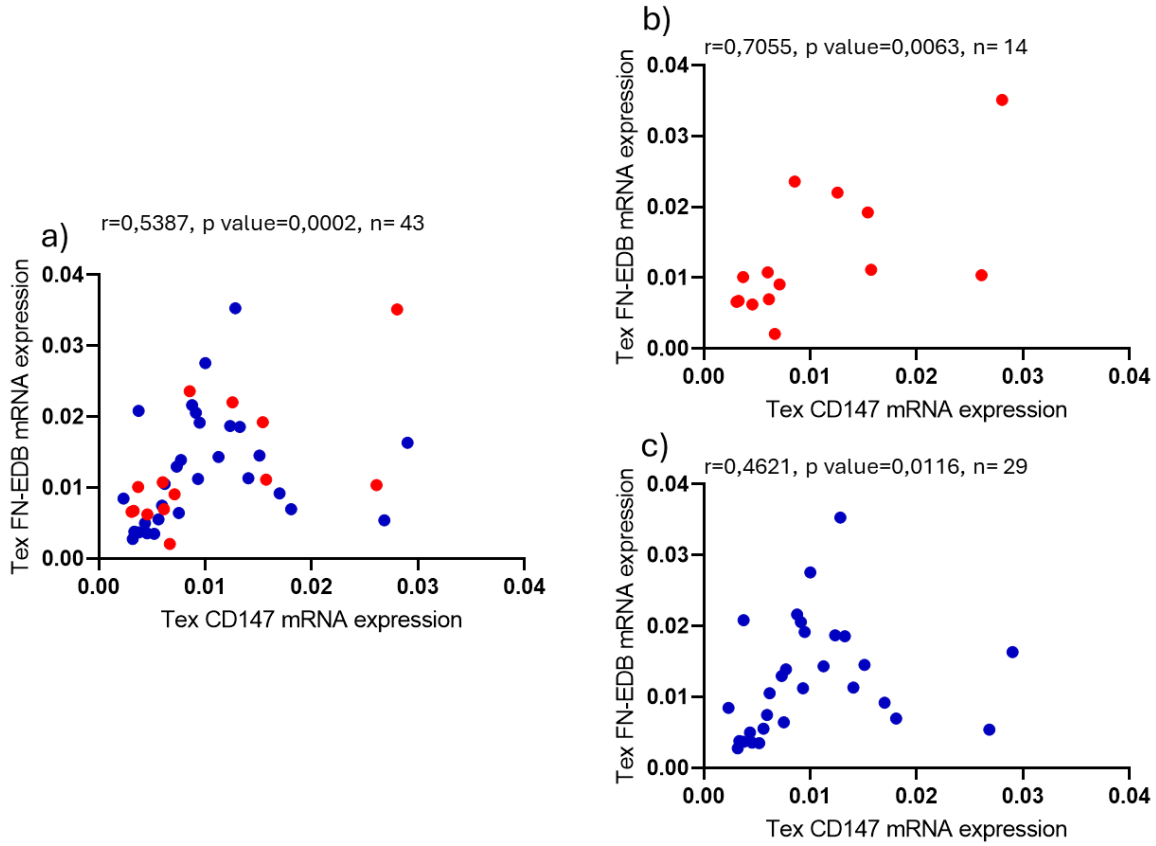

Figure 8. Fn-EDB mRNA correlation between a) CD147 mRNA in plaque specimens of the entire study population, b) CD147 mRNA in plaque specimens of symptomatic, and c) CD147 mRNA in plaque specimens of asymptomatic patients (symptomatic in red and asymptomatic in blue).

3.4 Immunopositivity in plaque specimens

To investigate a possible correlation of AhR mRNA levels in PBMCs and plaque instability, endarterectomies from 14 symptomatic and 34 severe asymptomatic patients were analyzed.

For technical reasons, plaque samples from patients who underwent CAS intervention were not available. Also, some samples were deemed not suitable for histological evaluation due to preanalytical issues (e.g., incorrect preservation, transport, or excessive calcification).

Within lesions, the following areas were identified as ROIs: media layer, fibrous cap (surrounding the lipid-rich necrotic core), and plaque shoulder.

As shown in Figure 9, in agreement to the severity assessed by in vivo imaging, the histological examination of specimens from both the study groups showed a high degree of complexity in terms of tissue remodeling, ECM deposition, infiltration of inflammatory cells, and morphological change of structural cells (VSMCs), recognizing in all the cases the main features of complicated atherosclerotic plaque.

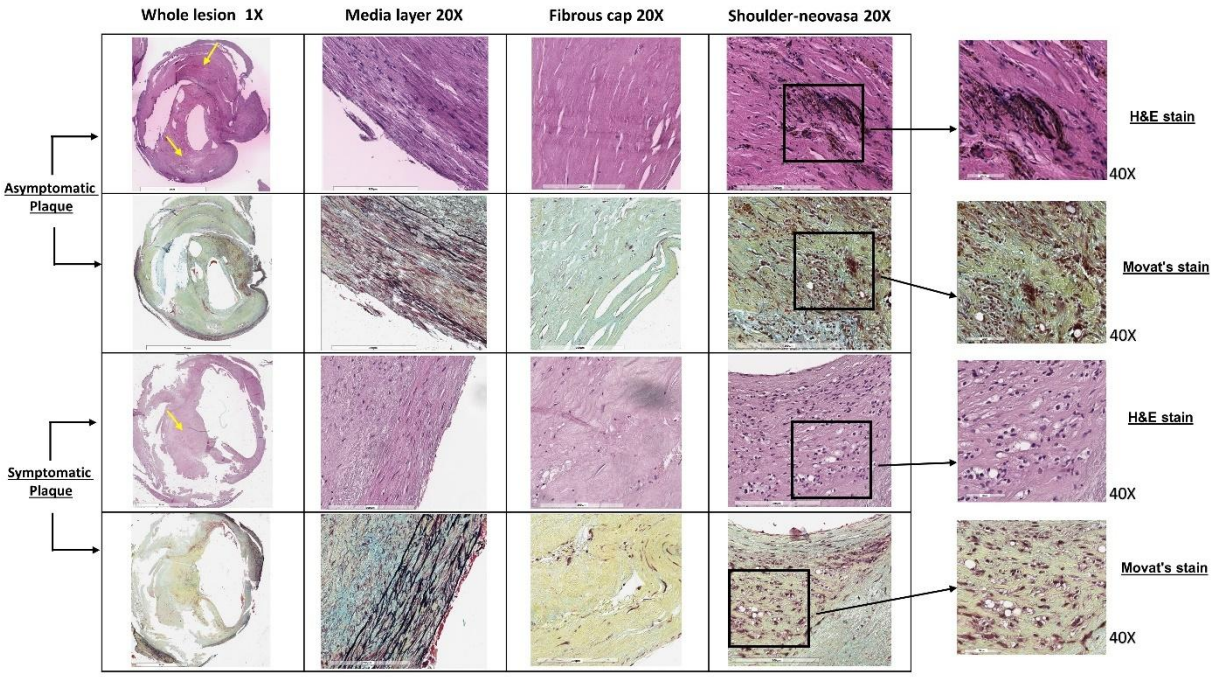

Figure 9. Representative 1X (scale bar= 3 mm) and 20X (scale bar= 200 µm) images of haematoxylin and eosin (H&E) and Modified Russel-Movat stained FFPE tissue samples of the two study populations, with the whole lesion, media layer, fibrous cap, and shoulder region with neovasa. The yellow arrows indicate the lipid-rich necrotic core. The black arrows indicate a 40X magnification (scale bar 60 µm) of cellular infiltrate and neovascularization.

3.4.1 AhR immunopositivity is significantly higher in the whole lesion of symptomatic patients

AhR expression was evaluated both as immunopositivity of the whole lesion and within each ROI (Figure 10a). Lesions from symptomatic subjects displayed a higher AhR positivity when considering the whole lesion with a difference close to the statistical significance (p= 0.064); a similar trend was observed mainly in the media and in the plaque shoulder (Figure 9b). AhR immunostaining localized mainly in the neovasa and inflammatory cell infiltration. Thus, the AhR overexpression pattern highlighted more active and vulnerable sites of the carotid stenosis, suggesting that AhR may be involved in the propagation of the arterial wall injury.

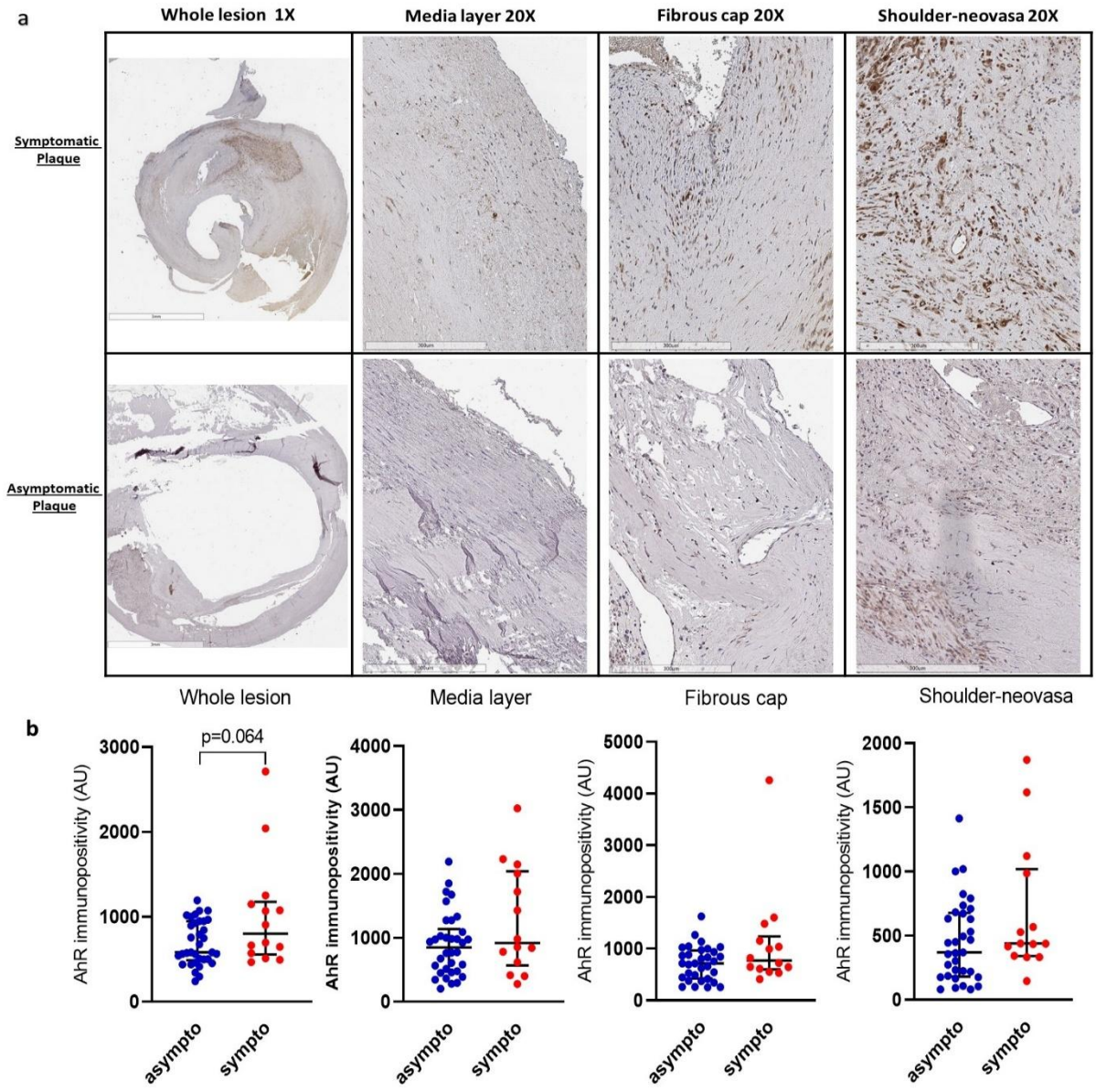

Figure 10. AhR immunopositivity in tissue samples from the two study populations. (a) IHC for AhR on tissue samples: whole lesion (scale bar= 3 mm), media layer, fibrous cap, and shoulder region (scale bar= 300 µm) are shown. (b) Quantification of AhR immunopositivity of the two study populations (asymptomatic, n= 34; symptomatic, n= 14) in the different ROIs.

3.4.2 CD147 immunopositivity and association with AhR

Figure 11 demonstrated that CD147 immunopositivity didn't differ between the two populations.

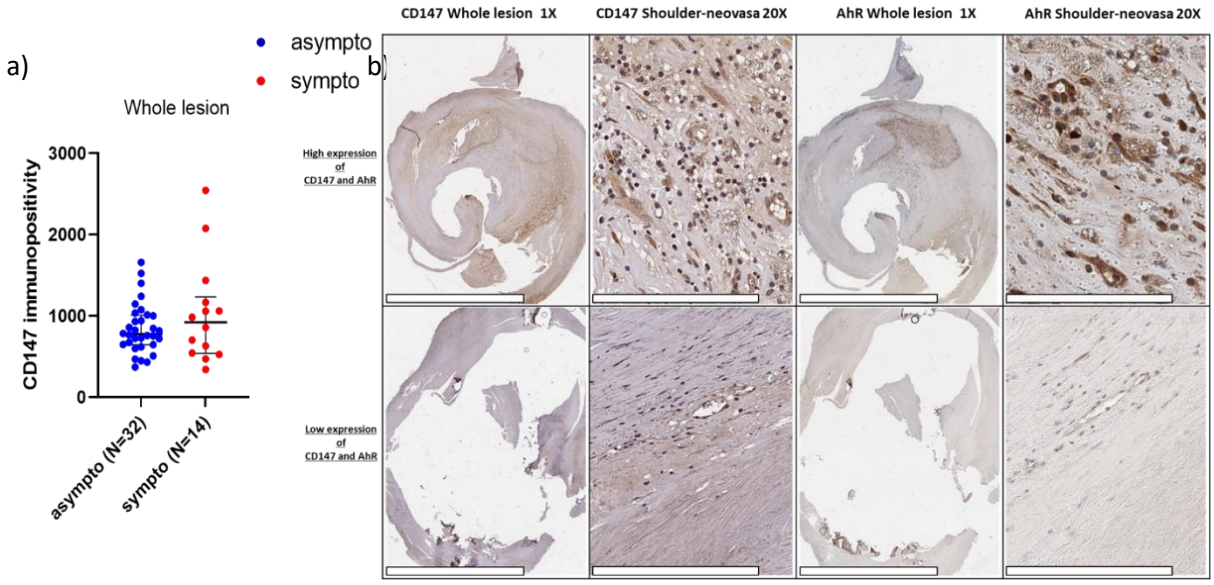

Figure 11. CD147 immunopositivity in tissue samples from the two study populations. (a) Quantification of CD147 immunopositivity in the two study populations in the whole lesion. (b) High and low expression of CD147 and AhR in tissue samples: Whole lesion (scale bar = 3 mm); shoulder region (scale bar = 200 µm).

As shown in Figure 12, when including both populations, a significant correlation between AhR and CD147 was found both in the whole lesion and in the plaque shoulder.

AhR positivity in the whole lesion correlated with CD147 in plaque shoulder in all patients (Figure 12a, r= 0.2760, p= 0.0698, n= 44). This trend was even stronger when considering asymptomatic subjects only (Figure 12b, r= 0.3357, p= 0.0697, n= 30). No significance was found in the symptomatic population (r=0.1692, p=0.5629, n=14).

As shown in Figure 12c, AhR and CD147 immunostaining in the plaque shoulder were strongly associated in both populations (r= 0.3187, p= 0.0350, n= 44), especially in the asymptomatic one (Figure 12d, r= 0.4278, p= 0.0184, n= 30). No significance was found in the symptomatic population (r=0.0901, p=0.7616, n=14).

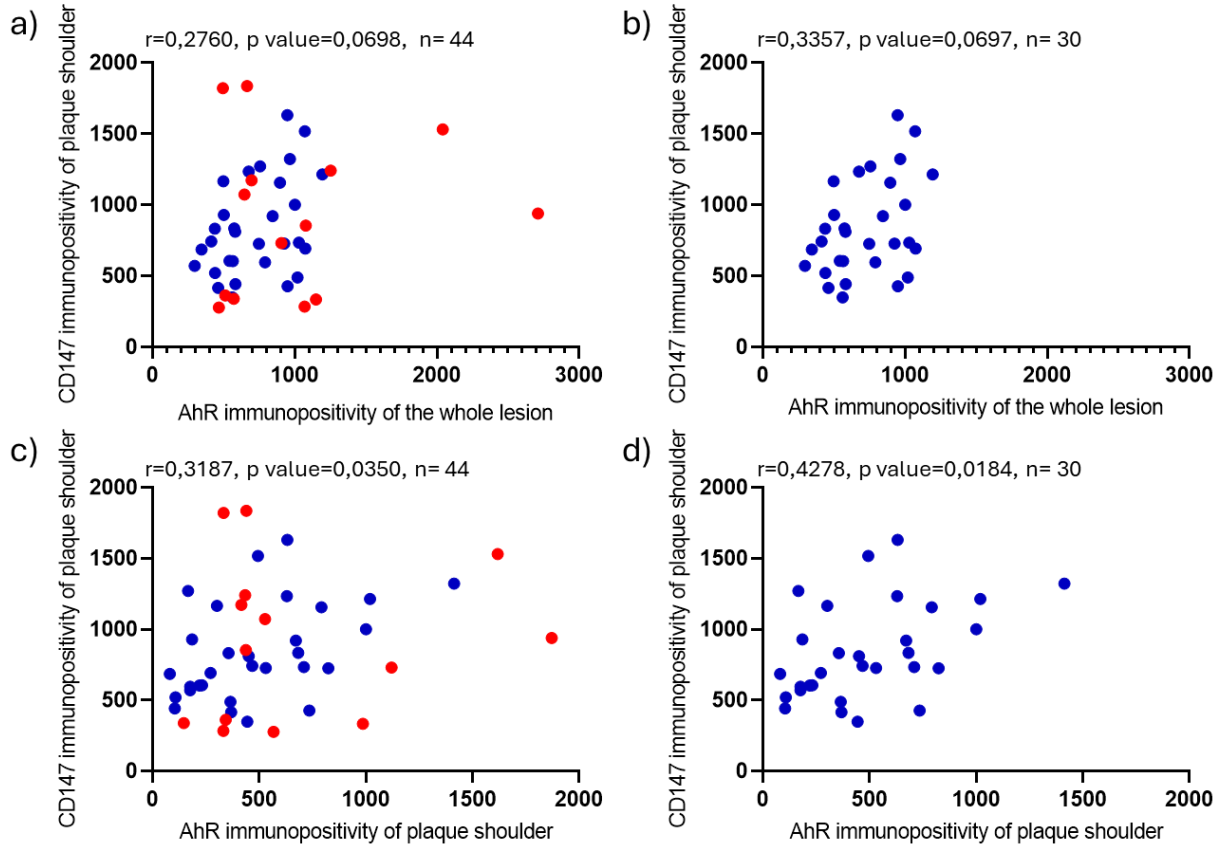

Figure 12. AhR immunopositivity of the whole lesion correlation between a) CD147 immunopositivity in plaque shoulder of all study population and b) CD147 immunopositivity in plaque shoulder of asymptomatic; AhR immunopositivity of plaque shoulder correlation between c) CD147 immunopositivity in plaque shoulder of all study population and d) CD147 immunopositivity in plaque shoulder of asymptomatic (symptomatic in red and asymptomatic in blue).

3.5 Correlation between gene expression in PBMCs and plaque specimens

3.5.1 AhR immunopositivity in the whole lesion correlates with HO-1 mRNA in PBMCs in the whole study population

HO-1 gene expression in PBMCs also displayed a close to significant association with AhR immunopositivity of the whole lesion when considering both populations, as shown in Figure 13.

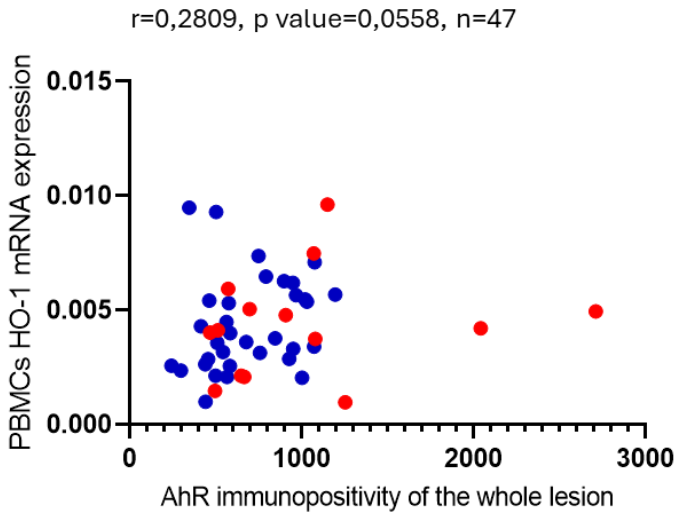

Figure 13. AhR immunopositivity of the whole lesion correlates with HO-1 mRNA in PBMCs of all study population (symptomatic in red and asymptomatic in blue).

3.5.2 AhR immunopositivity in the whole lesion correlates with BMAL-1 mRNA in PBMCs in asymptomatic patients

Figure 14 showed that AhR immunopositivity of the whole lesion correlated with BMAL-1 gene expression at the circulating level only in asymptomatic patients with a p= 0,0372.

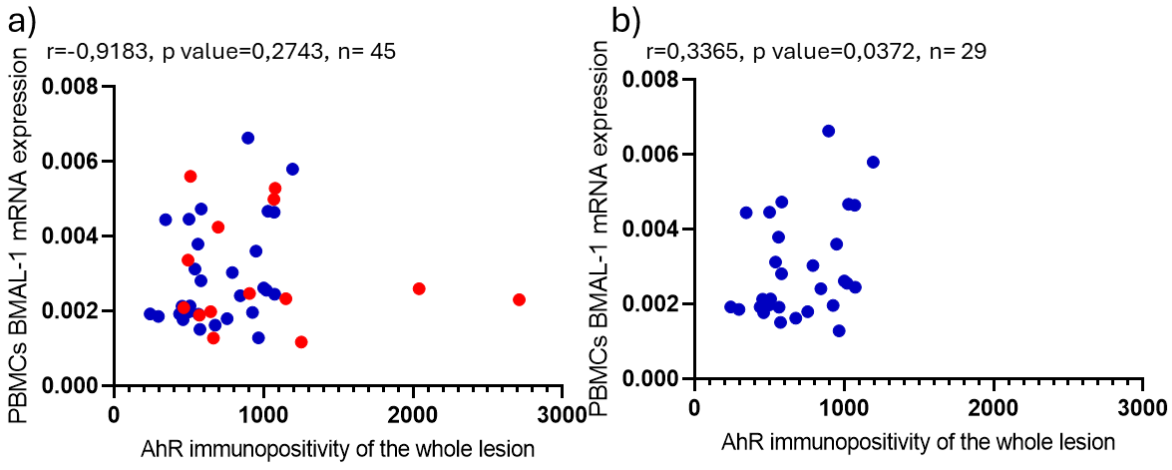

Figure 14. AhR immunopositivity of the whole lesion correlation between BMAL-1 mRNA in PBMCs, a) of all study population and b) of asymptomatic patients (symptomatic in red and asymptomatic in blue).

3.5.3 AhR immunopositivity in the whole lesion negatively correlates with Fn-EDB mRNA in plaque specimens of asymptomatic subjects

As shown in Figure 15, AhR immunopositivity of the whole lesion negatively correlates with B domain of the fibronectin at the circulating level only in asymptomatic subjects with a p= 0,0483.

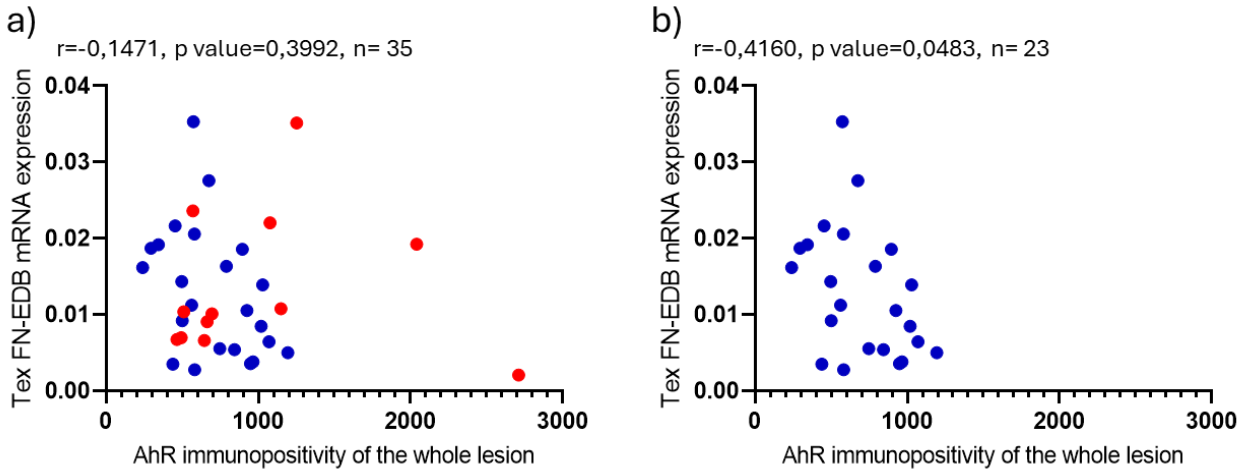

Figure 15. AhR immunopositivity of the whole lesion negatively correlated with Fn-EDB mRNA in tissue specimens, a) of all study population and b) of asymptomatic patients (symptomatic in red and asymptomatic in blue).

3.5.4 CD147 immunopositivity in the whole lesion negatively correlates with CD163 and HO-1 mRNA in PBMCs in symptomatic patients

As shown in Figure 16, CD147 immunopositivity of the whole lesion negatively correlated with CD163 (p= 0,0014) and HO-1 (p= 0,0274) expression in PBMCs only in symptomatic patients.

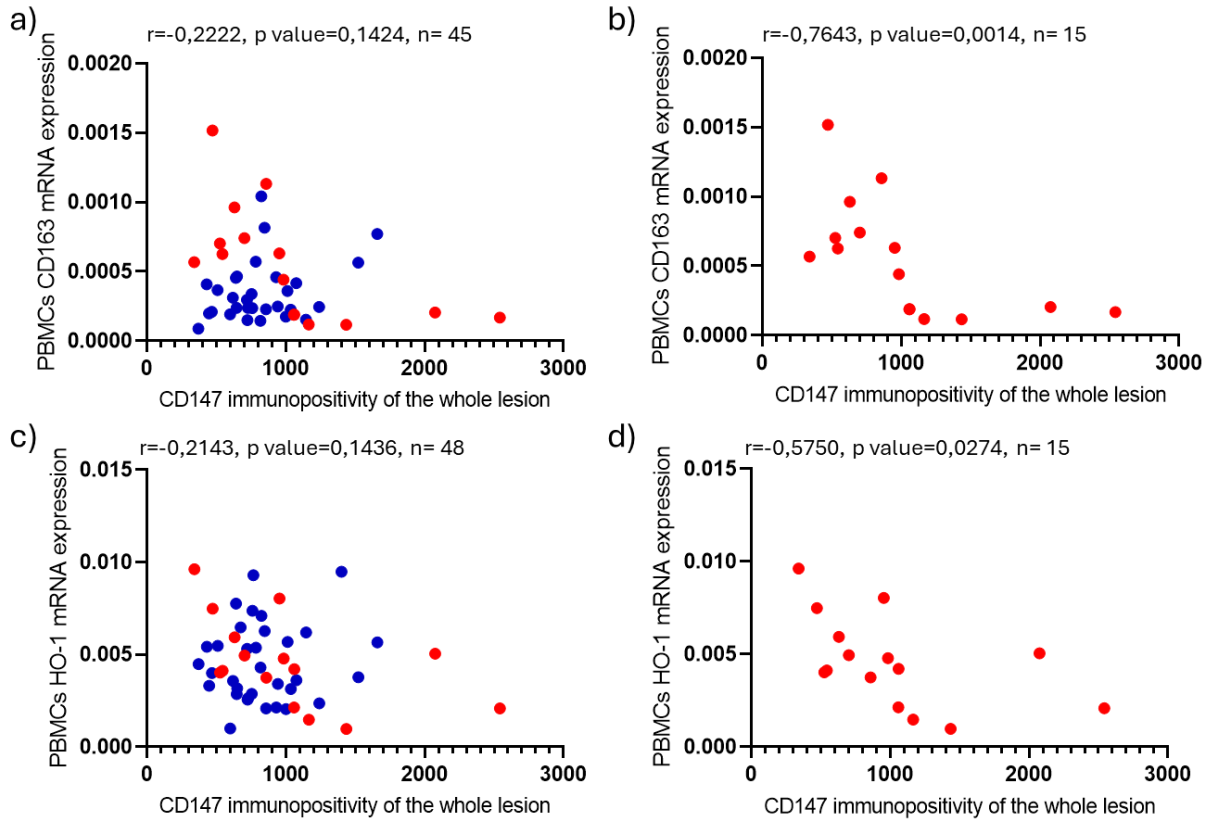

Figure 16. CD147 immunopositivity of the whole lesion negatively correlated with CD163 mRNA in PBMCs, a) of all study population and b) of symptomatic patients; and also, between HO-1 mRNA in PBMCs, c) of all study population and d) of symptomatic patients (symptomatic in red and asymptomatic in blue).

3.5.5 CD163 and HO-1 mRNA in PBMCs negatively correlate with CD147 immunopositivity of plaque shoulder

As shown in Figure 17, CD147 immunopositivity of plaque shoulder negatively correlated with CD163 expression in PBMCs in the whole study population (p= 0,0261) and in symptomatic subjects (p= 0,0015); no in asymptomatic subjects (p=0.4805). It also negatively correlated with HO-1 expression in PBMCs in the whole study population (p= 0,0350) and in symptomatic subjects (p= 0,0162); no in asymptomatic ones (p=0.8429)

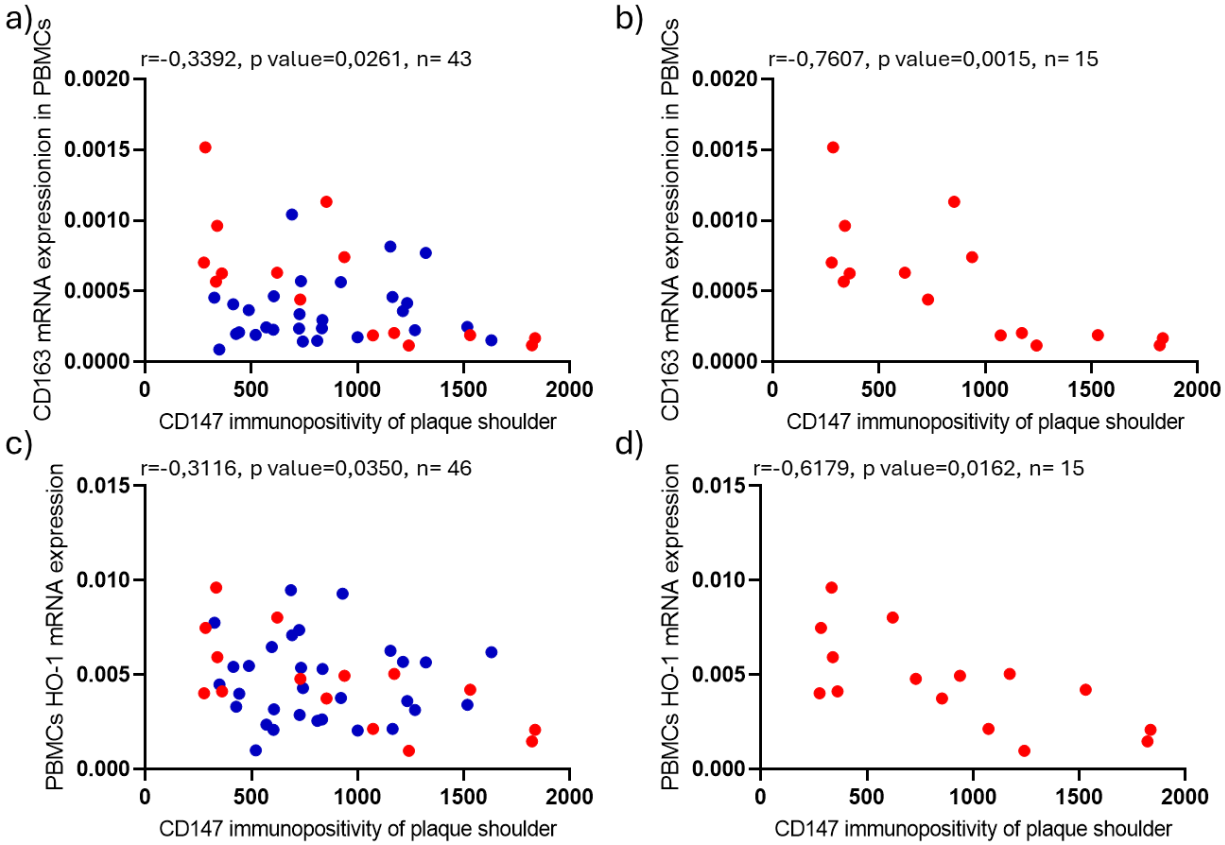

Figure 17. CD147 immunopositivity of plaque shoulder negatively correlated with CD163 mRNA in PBMCs a) of all study population and b) of symptomatic patients; and also, between HO-1 mRNA in PBMCs c) of all study population and d) of symptomatic patients (symptomatic in red and asymptomatic in blue).

3.5.6 CD147 immunopositivity of plaque shoulder negatively correlates with AhR in PBMCs

As shown in Figure 18, CD147 immunopositivity of plaque shoulder negatively correlates with AhR in the whole study population (p= 0,0008), in symptomatic patients (p= 0,0377), and in asymptomatic patients (p= 0,0470).

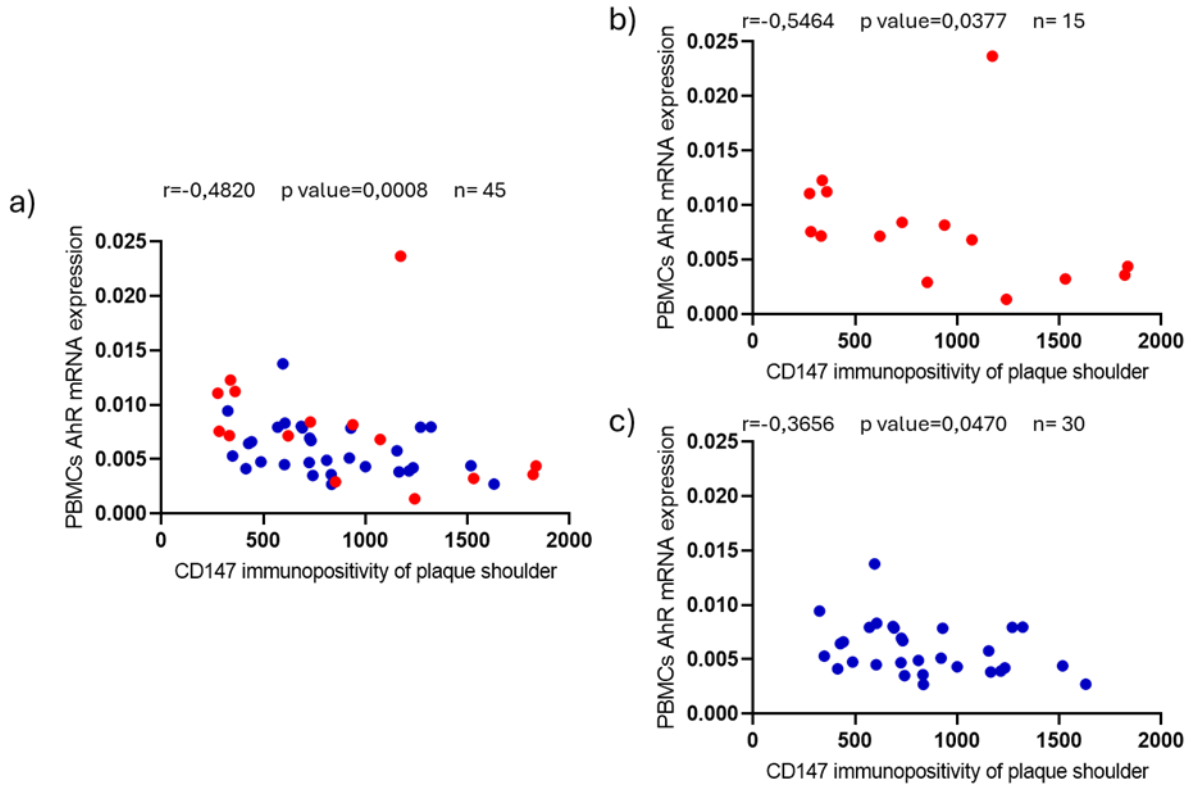

Figure 18. CD147 immunopositivity of plaque shoulder correlation between AhR mRNA in PBMCs, a) of all study population and b) of symptomatic patients, and c) of asymptomatic patients (symptomatic in red and asymptomatic in blue).

3.6 Preliminary data from immunohistochemistry for single and double staining biomarkers of Neuro, Immune, and Cardiovascular systems

Immunohistochemical analysis of carotid sections showed, in Figure 19, that the regions of interest (ROI), particularly in the plaque shoulder, were infiltrated by CD3+ T lymphocytes and CD163+ macrophages. In the same areas, immunopositivity for NF200, TH, and AhR was detected.

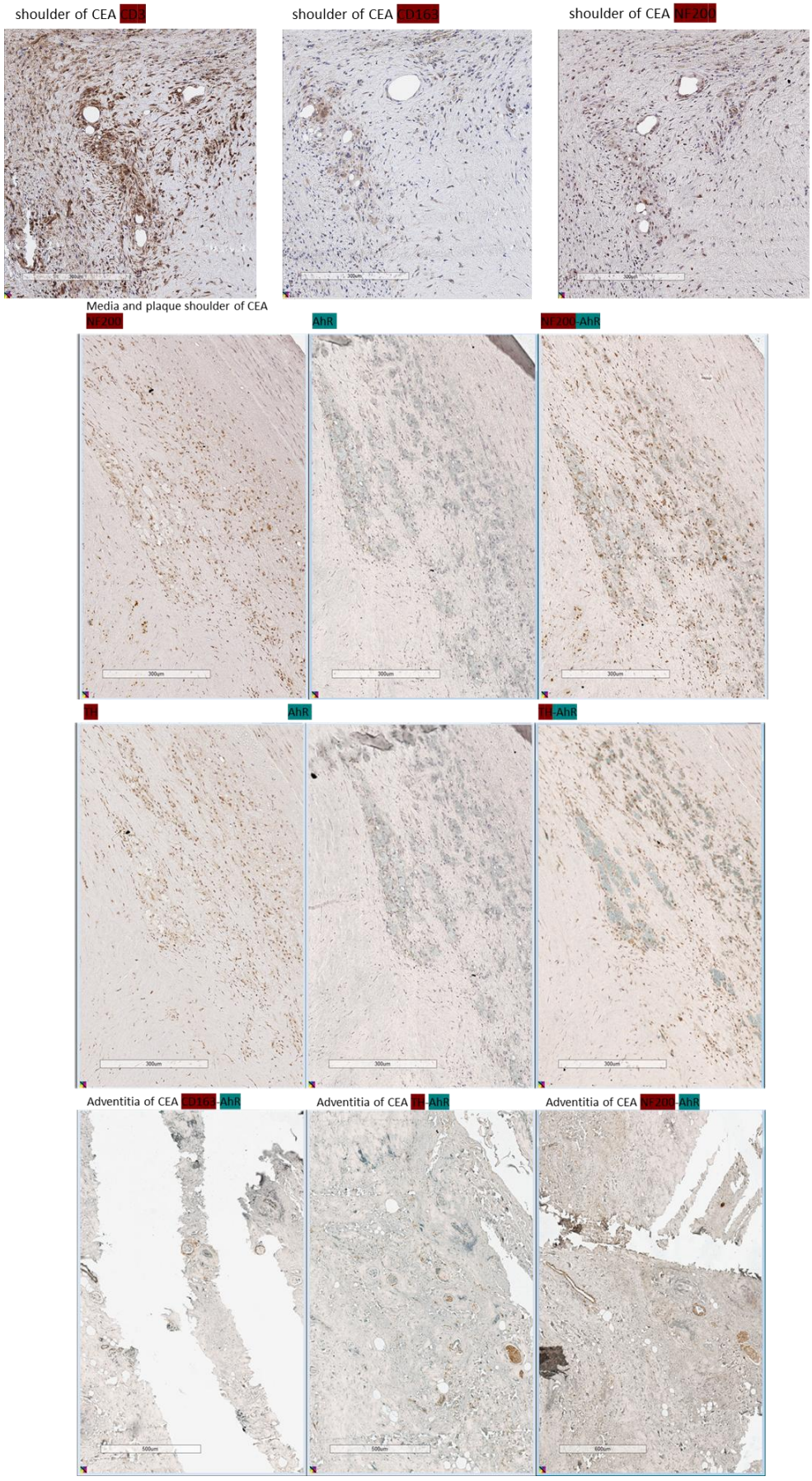

Figure 19.Neuro, Immune, and Cardiovascular biomarkers immunopositivity in tissue samples from the two study populations.

4. Discussion

Carotid stenosis is considered “severe” when the ultrasound imaging reveals the presence of atherosclerotic plaques occluding more than 70% of the vessel lumen. Indeed, at this stage, the plaque is statistically more likely to cause embolic and cerebral ischemic events (Chang C. et al., 2022), with consequent death or severe morbidity. Despite optimized medical therapy, management of severe carotid stenosis still relies on symptoms and luminal narrowing, while plaque biology largely determines embolic risk; therefore, robust biomarkers supporting vulnerability stratification remain an unmet clinical need. (Lanza G. et al., 2022). However, although plaque characteristics assessed by ultrasound (such as extension and presence of calcifications) are part of the clinical evaluation, there is currently no evidence that these features alone are sufficient to identify unstable and potentially symptomatic plaques: therefore, finding reliable biomarkers for risk stratification of plaque vulnerability is an urgent, so far unmet need in the clinical practice.

To this end, my three-year PhD project has investigated the relationship between stress-response pathways, such as AhR and its downstream molecules HO-1, CD147, and CD163, and plaque vulnerability, to identify novel panels of potential biomarkers for disease progression.

The study was conducted on a cohort of subjects with severe carotid stenosis, recruited at the Genoa Tissue Bank - Vascular Division (GTB-VD), Biological Resource Center, IRCCS Policlinico San Martino Hospital, Genoa, Italy, and further distinguished as “symptomatic” or “asymptomatic”, based on the occurrence of neurological manifestations within the previous 6 months. Only male patients were included to avoid sex-based differences in pathogenesis and risk factors, equally distributed in an age range of 62-86 years.

Although epidemiological data show a 5:1 ratio of asymptomatic to symptomatic individuals, we included 46 asymptomatic and 22 symptomatic patients to allow statistical comparison.

After thorough population analysis, we observed that the demographic and clinical characteristics of the two groups were largely comparable, with no statistically significant differences in terms of main modifiable vascular risk factors (hypertension, smoking, dyslipidemia, type II diabetes) and standard laboratory parameters, supporting the hypothesis that differences in plaque behaviour may be related to intrinsic biological factors rather than patients’ clinical profiles. A slight reduction in red blood cell count was observed in symptomatic patients ($p= 0.033$), potentially reflecting subclinical alterations in systemic

inflammatory status. We also observed a trend toward greater plaque calcification in asymptomatic patients (p= 0.065). Although not statistically significant, this finding aligns with literature suggesting that more calcified plaques may be more stable and less prone to rupture.

Differences observed in preoperative therapy, particularly the use of dicoumarol or heparin anticoagulants and ACE inhibitors, while statistically significant, are difficult to interpret and are unlikely to be directly linked to plaque vulnerability.

In this study, we analyzed PBMCs and tissue specimens, collected one day before and during the surgery, respectively, demonstrating the existence of a complex interaction between systemic and local compartments that differ significantly between symptomatic and asymptomatic patients.

Gene expression analysis in the two study populations showed a significant increase in CD163 and AhR mRNA levels in PBMCs of symptomatic patients compared to asymptomatic ones. CD163, a scavenger receptor expressed on M2 macrophages, is known for its role in anti-inflammatory responses and heme metabolism. AhR, a transcriptional regulator involved in various immunological and metabolic pathways, has recently been implicated in modulating innate immunity and atherogenesis, through interaction with environmental or endogenous ligands (e.g., tryptophan derivatives or oxidative metabolites) (Nebert DW. et al., 2017). This suggests an association between the activation of systemic immuno-mediated stress-response pathways and the presence of vulnerable plaques. As discussed by Gianopoulos and colleagues, differences in terms of infiltrating immune cells abundance and lineage have been demonstrated in carotid arteries retrieved from symptomatic patients, not only with respect to the timing of surgical intervention after the ischemic events but also to the severity of the symptoms. Moreover, when considering the heterogeneity in plaque-derived macrophages, the gene expression of the inflammasome pathway and foam cell formation is higher in asymptomatic patients, while for iron metabolism and storage-related genes, a profile committed towards clearance of iron and metabolites from intraplaque haemorrhage and healing after plaque rupture is higher in symptomatic patients (Gianopoulos I. et al., 2024).

The correlations observed between CD163 and AhR, and between CD163 and HO-1, suggest a shared functional network among these genes, potentially activated in response to oxidative or systemic inflammatory stimuli; while the one between CD163 and HO-1, so far, is evidence only in symptomatic subjects, might reflect an antioxidant response to ongoing cellular stress. HO-1 is a cytoprotective enzyme with antioxidant and anti-inflammatory activity, often co-

expressed with CD163 in macrophages (Barisione C. et al., 2016). These associations reinforce the hypothesis of an immune-activated state in patients with unstable plaques, which is also reflected systemically, in PBMCs, even in the absence of apparent clinical differences. However, this clue must be verified on a wider study population.

We also observed a significant association between HO-1 and BMAL-1 in PBMCs expression across the cohort, which is a novel finding with potential pathophysiological interest. BMAL-1 is a key gene in the circadian system but has also been implicated in the regulation of oxidative stress and vascular inflammation. Its correlation with HO-1 may reflect both a link between circadian mechanisms and antioxidant responses in PBMCs, suggesting a possible influence of circadian rhythms in the progression of atherosclerosis, or, disruptive effect of AhR overactivation on circadian-related gene expression

Looking at gene expression at the tissue specimens, no significant differences were observed in CD163, AhR, CD147, and Fn-EDB, possibly due to intrinsic plaque variability, the heterogeneous nature of the analyzed tissue, or the presence of different disease stages within the same group.

The same parameters were correlated within the different populations, and this analysis provided more relevant insights than direct group comparisons. The significant correlation between AhR and CD147 expression in plaques, particularly in asymptomatic patients, suggests a functional interaction potentially involved in vascular remodeling or local inflammation regulation. CD147 is an immunoglobulin family glycoprotein known for promoting ECM degradation through MMP induction and modulating immune activation. Its association with AhR could reflect a concerted response to local metabolic or inflammatory signals in the plaque, even in a non-symptomatic stage. This may represent an early index of potential instability, not yet resulting in clinical events.

We also observed a strong correlation between Fn-EDB expression and AhR and CD147 in both study populations. These results further support the existence of an active molecular network in plaque progression. Fn-EDB is a marker of neoangiogenesis and tissue remodeling, commonly expressed in growing tissues or pathological conditions such as tumors and unstable atherosclerosis (Lieverse RIY. et al., 2020). The correlation is stronger in asymptomatic subjects, but is often even reinforced when comprising also the symptomatic ones, confirming its biological relevance and providing a therapeutic target identification.

Protein expression was also localized. Although other studies have shown histological differences in plaque complexity between symptomatic and asymptomatic patients (Virmani R. et al., 2005), in our cohort, we observed a high complexity irrespective of whether from the symptomatic or asymptomatic group.

Immunohistochemistry confirmed increased AhR expression in atherosclerotic lesions of symptomatic patients compared to asymptomatic ones. Although the difference across the entire lesion approached statistical significance ($p = 0.064$), the observed trend and preferential immunopositivity localization in areas prone to vulnerability, such as the plaque shoulder and proximity to neovessel, suggest active involvement of AhR in mechanisms of vulnerability.

This finding is consistent with the transcriptional data observed in PBMCs, where AhR was more highly expressed in symptomatic patients, reinforcing the hypothesis that its activation is not limited to the systemic compartment, in an attempt to activate the cell-stress response pathway, but also occurs locally, potentially contributing to endothelial damage propagation and local inflammation amplification.

In contrast to AhR, CD147 immunopositivity levels did not differ significantly between symptomatic and asymptomatic patients. However, its tissue distribution and strong correlation with AhR, particularly in the plaque shoulder, indicate that these two markers may act synergistically at the local level. The plaque shoulder is a region rich in inflammatory infiltrate and frequently a site prone to rupture; interaction between AhR and CD147 in this area may thus represent a key element in the processes preceding plaque destabilization. The correlations between AhR and CD147 were stronger in asymptomatic patients, although the same trend was shown in symptomatic ones.

Beyond the concept of the vulnerable plaque, our data support the broader paradigm of the vulnerable patient, in whom systemic immune-inflammatory and stress-response signatures may reflect an underlying high-risk biological state associated with plaque destabilization, even if their prospective predictive value remains to be demonstrated (Ortona S. et al., 2026).

The analysis of gene expression in PBMCs and tissues, along with immunopositivity in the plaque, revealed complex interactions between systemic and local compartments that differ markedly between symptomatic and asymptomatic patients. These findings suggest that the investigated markers not only reflect but may also be associated with pathways that could contribute to atherosclerotic vulnerability.

The negative association between AhR protein levels in tissue and HO-1 and BMAL1 in PBMCs, and also Fn-EDB mRNA in plaque tissue, suggests a complex interaction between these markers, but further investigation is needed.

CD147 emerged as particularly noteworthy due to its multiple inverse correlations with circulating markers in symptomatic patients, both at the level of the overall plaque and within the shoulder region, a critical site for rupture. Its negative association with systemic CD163, HO-1, and AhR in symptomatic individuals, if confirmed in a wider population, may indicate that local overexpression of CD147 is linked to systemic suppression of anti-inflammatory and antioxidant defense.

Its negative correlation with AhR (observed in both patient groups) further supports the hypothesis of reciprocal regulation between these two markers, both involved in metabolic control of the inflammatory microenvironment.

No association was found between AhR mRNA expression in PBMCs and AhR immunopositivity in plaque tissue. This is likely because AhR immunopositivity in the plaque reflects a chronic process that leads to gradual protein overexpression over time. In contrast, AhR mRNA expression in PBMCs represents an acute activation of circulating monocytes, which may contribute to plaque progression, but the kinetics of AhR expression are plausibly distinct.

These findings reinforce the idea that transcriptional changes in peripheral immune cells can mirror the inflammatory and oxidative state associated with carotid plaque vulnerability. It is not the absolute expression of a single gene, but rather the concerted modulation of multiple molecular pathways that defines plaque status.

Per evidenziare la conclusione dell'argomento principale della tua tesi, e il fatto che i dati successivi sono una possibile prosecuzione – al momento osservazione preliminare e concatena al pregresso, qui metterei un richiamo al tema emergente dell'asse neuro-immuno-mediato; anche questo lo trovi in review.

Consistently, immunohistochemical analyses of carotid sections revealed that the plaque shoulder regions were infiltrated by $CD3^+$ T lymphocytes and $CD163^+$ macrophages, near $NF200^+$ and TH^+ neural fibers, and showed AhR immunopositivity.

Although key mechanistic insights on neuro-immune regulation of atherosclerosis derive from experimental models, our observations were obtained in human carotid endarterectomy

specimens and support the presence of neuroimmune proximity within plaque compartments associated with vulnerability. Importantly, this study is descriptive and does not establish causality; rather, it provides a human tissue-based framework that motivates future functional and spatially resolved studies to test whether local innervation actively shapes inflammatory remodeling in high-risk plaques (Mohanta SK. et al., 2022).

These observations support the existence of a neuro-immune interface within vulnerable areas of the plaque, where sympathetic and sensory innervation may interact with local immune populations to modulate inflammation, stress responses, and tissue remodeling. The co-expression of AhR in these regions further suggests the activation of a stress-related transcriptional program linking immune activation, oxidative pathways, and neuronal signaling. Interestingly, we also identified ganglia showing heterogeneous expression patterns, with some TH^{+}/AhR^{+} and others TH^{-}/AhR^{+}. This finding suggests that AhR expression may occur independently of catecholaminergic identity, possibly reflecting distinct neuronal subpopulations or different activation states in response to local inflammatory or oxidative cues within the plaque microenvironment.

This observation supports the hypothesis that AhR may be involved in regulatory programs linked to the local neuro-immune microenvironment in atherosclerotic progression.

Several limitations should be acknowledged.

- The study was conducted on a single-center cohort within a biobanking framework, with a moderate sample size; therefore, external validation in larger and multicenter cohorts is required to confirm robustness and generalizability.
- Only male patients were included to minimize sex-related biological variability; consequently, sex-specific effects cannot be inferred and the findings should not be directly extrapolated to females.
- The design is cross-sectional and relies on clinical presentation (symptomatic vs asymptomatic) as a proxy of vulnerability; thus, the results are associative, do not establish causality, and do not directly assess the prospective predictive value of the proposed biomarker panel.
- PBMC transcriptional profiling was performed using bulk measurements, which provide averaged signals and may mask differences in immune-cell subset distribution between groups; future studies should adopt cell-type resolved

strategies (e.g., leukocyte phenotyping, deconvolution approaches, single-cell and spatial methods).

- Carotid plaques are highly heterogeneous lesions and tissue-based readouts can be influenced by sampling and regional variability; although region-of-interest analyses were performed and IHC was quantified as DAB-positive area normalized to ROI area with negative controls, residual biological and technical variability may remain.
- We did not perform a standardized quantitative histopathological scoring of canonical vulnerability features (e.g., lipid-rich necrotic core size, fibrous cap thickness, and intraplaque hemorrhage) across the entire cohort; therefore, direct correlations between molecular readouts and validated morphometric measures of vulnerability could not be systematically assessed.
- Finally, symptom timing is embedded in the clinical definition of the symptomatic group (within the previous 6 months); therefore, heterogeneity within this window and other unmeasured clinical variables may contribute to variability in tissue and PBMC readouts.

In conclusion, our study has identified a pathway of stress-related cell responses that may be exploited as a novel panel of potential biomarkers for the detection of vulnerable atherosclerotic plaques, suggesting that a multi-marker approach enhances their discriminatory performance.

Moreover, it suggests the AhR pathway as a new therapeutic target to dampen in multiple cell lineages the combined cell events involved in atherosclerosis progression and complications. These results underscore the need for a multicentre study to evaluate these promising biomarkers in a larger population and to establish a reference database for defining normal ranges and cut-off values for identifying patients at risk of carotid plaque vulnerability, with possible ischemic consequences.

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