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IDENTIFICATION OF A MOLECULAR SIGNATURE AS POTENTIAL BIOMARKER IN BLOOD OF SYMPTOMATIC AND ASYMPTOMATIC PATIENTS WITH CAROTID ATHEROSCLEROTIC PLAQUE

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Abstract

Background and Objectives: Cardiovascular diseases (CVDs) have become one of the major social, political, and economic issues of the last few decades and a leading cause of disability and premature mortality globally. Carotid revascularization to prevent future vascular events is reasonable in some patients with high-grade carotid stenosis. However, the degree of stenosis alone is not sufficient to decide upon the best clinical management in some situations. The discovery of reliable circulating biomarkers may complement the data obtained through carotid imaging. Currently, several biomarkers to predict carotid plaque development and progression have been investigated, among which microRNAs (miRs) are promising tools for the diagnosis of atherosclerosis.

The present thesis is focused on the study of tissue and circulating (c-) miRs as evaluable molecular drivers and markers in carotid pathology. This study attempted to answer three main questions: i) May c-miRs profile distinguish between asymptomatic and symptomatic patients? ii) May the simultaneous evaluation of tissue miRs and c-miRs in two districts (i.e.plaque tissue and blood) give results about a circulating miR-based signature and a specific plaque-phenotype? iii) May circulating biomarkers (c-miRs signatures) be predictive of the development of carotid disease in outpatients with asymptomatic stenosis?

Methods and Results

A total of 49 participants were included in the study, divided into two main populations: Population 1 comprising symptomatic and asymptomatic inpatients, and Population 2 comprising asymptomatic outpatients. All subjects were tested for hemato-biochemical analyses.

The study consisted of two main phases: a preliminary discovery phase and a validation phase, applying different techniques. MiR-profiles were performed on plasma and plaque tissue samples obtained from 4 symptomatic and 4 asymptomatic inpatients. MiRs emerging from profiling comparisons, i.e. miR-126-5p, miR-134-5p, miR-145-5p, miR-151a-5p, miR-34b, miR-451a, miR-720 and miR-1271-5p, were subjected to validation through RT-qPCR analysis in the total cohort of donors. Comparing asymptomatic and symptomatic inpatients, significant differences were reported in the expression levels of c-miRs for miR-126-5p and miR-1271-5p in blood, being more expressed in symptomatic subjects. In contrast, simultaneous evaluation of the selected miRs in plaque tissue samples did not confirm data obtained by the miR profiling, and no significant differences were observed between asymptomatic and symptomatic inpatients.

To further characterize the phenotype, histological analyses of endarterectomy specimens were evaluated. No significant differences in tissue and circulating miR expression levels were reported when comparing hemorrhagic and non-hemorrhagic plaques.

Using Receiver-Operating Characteristic (ROC) analysis, a circulating molecular signature (mir-126-5p, miR-1271-5p, albumin, C-reactive protein, and monocytes) was identified, allowing the distinction of the two groups in Population 1 (AUC = 0.795). The identified molecular signature was also tested in Population 2. A mathematical prediction method developed provided a similarity value towards one of the two groups in Population 1. The results obtained were consistent with the observation that no outpatients were found to have significantly changed their clinical condition at the one-year follow-up.

Conclusions

Data emerging from this thesis suggest that c-miRs (i.e. miR-126-5p, miR-1271-5p) combined with selected haemato-biochemical parameters (albumin, C-reactive protein, and monocytes) produced a good molecular 'signature' to distinguish asymptomatic and symptomatic inpatients. The strategy adopted in the current thesis to validate the miRs shared between blood and plaque in the discovery phase did not reveal significant difference in the miRs expression levels between asymptomatic and symptomatic inpatients. C-miRs in blood do not necessarily reflect the expression levels of the same miRs in carotid plaque tissues since different mechanism can influence their expression. The molecular signature may characterize the trajectories of outpatients by indicating a similarity to the symptomatic or asymptomatic phenotype.

Unfortunately, the COVID-19-related health emergency affected the cohort size and a predictive model could not be developed for outpatients. However, the mathematical prediction method developed allowed us to give a level of similarity to one of the two phenotypes present in Population 1 for each outpatient.

Studies based on larger sample sizes are required to determine the potential use of miR-126-5p, miR-1271-5p, albumin, C-reactive protein, and monocytes circulating molecular signature. Future perspectives will involve a larger panel of c-miRs in an enlarged cohort of patients and their combination with other histological parameters in a more sensitive signature will attempt to disentangle the complexity of the carotid atherosclerotic development process. Further data collected

could be used in more complex analysis, to create algorithms for modeling in the context of personalized and precise medicine.

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1. INTRODUCTION

1.1 CAROTID ARTERY DISEASE

1.1.1 Background of Cardiovascular Disease

Cardiovascular diseases (CVDs), mainly of atherosclerotic origin, are the leading cause of disability and premature mortality worldwide (Virani et al., 2020).
By 2030, approximately 23.6 million people are predicted to die from CVDs annually (Mozaffarian, 2017; Roth et al., 2017; Song et al., 2020). The huge and still growing burden of CVDs on individuals, families, and health care systems indicates an urgent need for research on atherosclerotic diseases and the implementation of preventive measures (Song et al., 2020). Even though huge resources are utilized for the study and treatment of CVDs, almost half of all patients have a major cardiovascular event, i.e., myocardial infarction (MI) or stroke without a prior diagnosis of CVD.

Large-artery atherosclerosis is one of the main causes of ischemic stroke and transient ischemic attack (TIA). The commonest form of large-artery atherosclerosis causing ischemic stroke is carotid artery stenosis.
Stroke is defined as a sudden onset of clinical signs of a focal or global cerebral deficit, with the clinical symptoms related to the anatomic location of the lesion, lasting more than 24 hours or until earlier death, and with no apparent non-vascular cause (Hatano, 1976). It is characterized by symptoms such as a unilateral limb or facial motor weakness or sensory loss, speech disturbance, visual disturbance, and ataxia. Ischemic stroke occurs when blood flow to the brain is compromised by reduction of the hemodynamics or occlusion by embolism, causing lack of oxygenation to a region of the brain.

TIA follows the same pathophysiology as ischemic stroke and is defined as “a transient episode of neurological deficit caused by a focal brain, spinal cord, or retinal ischemia without acute infarction” (Easton et al., 2009). Stroke can be either hemorrhagic or ischemic of origin, with approximately 85% of the strokes classified as ischemic. Retinal artery occlusion and *amaurosis fugax* are also considered a form of ischemic stroke (Hayreh, 2011). These involve occlusion of the retinal artery, causing monocular blindness. *Amaurosis fugax* is a transient episode of unilateral visual loss, whereas retinal artery occlusion causes symptoms for more than 24 hours.

An early diagnosis allows individuals at higher risk or prone to stroke to be identified and targeted for specific therapies or interventions (Goldstein et al., 2006).
Early detection of atherosclerosis in apparently healthy people has mainly focused on peripheral arteries and carotid arteries (Piepoli et al., 2016). Using ultrasonography, carotid intima-media thickness (IMT) can be assessed non-invasively (O'Leary and Bots, 2010; Touboul et al., 2012).

1.1.2 Carotid artery stenosis

The presence of atherosclerotic lesions at the carotid bifurcation and the presence of ischemic cerebral lesions are the main factors leading to cerebrovascular events.

There are two main mechanisms described of carotid stenosis causing ischemic stroke:

1. Forming of thrombus in the carotid plaque and distal embolization.
2. Hemodynamic failure due to the stenosis and consequently hypoperfusion of the brain.

The main mechanism of carotid stenosis causing ischemic stroke is due to the forming of embolism after rupture of the unstable carotid plaque.

Especially, the risk of major events such as death or stroke is further increased in patients presenting unstable plaque (Hafiane, 2019; Wang and Connolly, 2010). The degree of stenosis of carotid arteries, as determined by carotid artery imaging techniques, is considered the major risk factor for strokes and contributes to the selection of the management of patients with carotid atherosclerosis. More specifically, in patients with symptomatic carotid artery stenosis higher than 70%, carotid endarterectomy (CEA) is strongly recommended, whereas in individuals with cerebral ischemia and carotid stenosis higher than 50%, as well as in asymptomatic individuals with carotid stenosis over 70%, CEA or carotid artery stenting should be discussed (Messas et al., 2020). Weerd and colleagues (de Weerd et al., 2010) reported prevalence estimates for moderate stenosis of 50% or more and severe stenosis of 70% or more through a systematic literature review and meta-regression analysis of 40 studies. The pooled prevalence of moderate stenosis was 4.2% (95% CI 3.1–5.7) and of severe stenosis was 1.7% (0.7–3.9). However, in their study, Song and colleagues showed the prevalence of carotid stenosis of 50% or more was 1.5% (95% CI 1.1–2.1) (Song et al., 2020). This discrepancy might be related to differences in lesion definition and inclusion criteria for the meta-analyses. Prevalence data according to the

degree of stenosis might be more useful to show the global burden on the health care system and to develop effective strategies for the prevention and management of carotid atherosclerosis.

The evidence base for CEA for symptomatic stenosis is considerable (Rothwell et al., 2004, 2003), but guidelines on surgery for asymptomatic stenosis have been largely based on the results of the Asymptomatic Carotid Atherosclerosis Study (ACAS) (Rothwell and Goldstein, 2004) in conjunction with other smaller trials (Hobson et al., 1993). However, the early intervention aims to avoid embolization caused by a vulnerable lesion at the carotid district, nevertheless, there is a big issue to recognize "young" vulnerable plaque non-invasively before an acute clinical event occurs (Setacci et al., 2010).

Different studies estimate the individual risk of stroke such as the Framingham risk score, the ABCD2, or the ABCD3 score systems (Bhatt and Jani, 2011; Song et al., 2013). The prognostic value of all these score systems improves if completed with clinical information, vascular imaging data, and brain imaging data of patients (Kelly et al., 2016). Cerebro-ischemic symptoms, such as TIA or *amaurosis fugax*, increased the risk of major events such as death or stroke (Liapis et al., 2001). In magnetic resonance imaging prospective assessment studies, it has been shown, that the occurrence of cerebrovascular events in asymptomatic patients is associated with the characteristics of carotid plaque; thinned or ruptured fibrous caps, intraplaque hemorrhage, larger lipid-rich necrotic cores, and larger maximum wall thickness (Takaya et al., 2006). According to Ota et al. (Ota et al., 2009), hemorrhage, and larger percentage of lipid-rich/necrotic core were independently associated with a thin or ruptured fibrous cap status at an early to advanced stage of carotid atherosclerosis. Rapid advances in non-invasive carotid artery imaging provide important pathophysiological insights (Zhu et al., 2020).

1.1.3 Risk factors of carotid atherosclerosis

Atherosclerosis is a heterogeneous disease with multiple risk factors from genetics to lifestyle factors. Epidemiological studies in recent decades have revealed a complex etiology of atherosclerosis and highlighted certain risk factors. These factors can be divided based on a large environmental component from those with mainly uncontrollable non-environmental factors.

Confirmed conventional risk factors of cardiovascular disease are smoking, diabetes, dyslipidemia, and hypertension. All these risk factors are preventable, and good results have been achieved in many western countries in the reduction of smoking and limiting the effects of hypertension and hypercholesterolemia by medication. Still, according to WHO, the number of patients with dyslipidemia, diabetes, and high blood pressure is increasing in the world.

Predisposing risk factors contain non-modifiable factors such as age, gender, ethnicity, family history, and genetics. Many genetic alterations have been shown to make individuals more prone to develop atherosclerosis. Studies have shown that within the population, the heritability of atherosclerosis is over 50% (Lusis et al. 2004).

Modifiable predisposing risk factors include overweight and obesity, physical inactivity, insulin resistance (IR, without diabetes), and socioeconomic-behavioral factors (Liapis et al. 2009). In addition to similar contributing factors with atherosclerosis (diet, low activity), obesity is related to CVDs through lipid contribution and the role of inflammation. Lipid accumulation and modification is important in both obesity and atherosclerosis, but also the inflammatory burden caused by obesity can promote atherosclerosis (Rocha and Libby. 2009).

It is interesting to note that inflammaging may be accelerated by age-related comorbidities (Franceschi et al., 2018a), favoring atherosclerosis onset and plaque progression acceleration. Thus, the biological age could be considered the most important atherosclerosis risk factor, associated with artery-specific molecular and physiological characteristics which interact with many variables and may have a role in atheroma development, such as gender, genetic makeup, aging process, regenerative intrinsic abilities, lifestyles, and socioeconomic status (Collura et al., 2020).

In The Lancet Global Health, Peige Song and colleagues report their systematic review, meta-analysis, and modeling study of the prevalence and risk factors of carotid atherosclerosis by means of increased carotid IMT, carotid plaque, and carotid stenosis, in the general global population. They found that approximately 28% of individuals aged 30–79 years had increased carotid IMT. Risk factors that significantly contributed to this condition included older age, male sex, smoking, diabetes, and hypertension (Song et al., 2020).

1.1.4 Carotid plaque: pathogenesis

Atherosclerosis has a long, slow asymptomatic phase, starting early in life, several years before the onset of clinical signs (Libby et al., 2019; Rea et al., 2018) and in many patients becoming manifest at a relatively advanced age. Increased carotid IMT, decreased elasticity of carotid artery, and decreased endothelial function are seen as early atherosclerotic changes and predict future cardiovascular disease (Lorenz et al., 2012; Shimbo et al., 2007). Especially carotid IMT is widely believed to indicate the presence of early-stage atherosclerosis (Bisoendial et al., 2002).

The anatomical details of the common carotid artery and its branches have attracted clinicians and researchers for their key role in the development of plaque (Uslu et al., 2016) and the stroke-associated risk. Despite the systemic nature of its risk factors, atherosclerosis is a geometrically focal disease. Turbulent blood flow and low shear stress contribute to the localization of atherosclerotic plaques at branching or curving regions. Arterial endothelial cells where the flow is normal are typically ellipsoid in shape and aligned in the direction of the flow. However, endothelial cells in regions where the flow is turbulent are often found to be polygonal in shape and not aligned to a particular direction. These regions under high physical force show an increased permeability to low-density lipoprotein (LDL) and other macromolecules compared to normal flow areas and are therefore more susceptible to plaque formation (Lusis, 2000; Malek et al., 1999).

The normal adult artery consists of three well-defined layers: the intima, media, and adventitia. These three layers are separated by layers of elastin. Internal elastic lamina separates intima from media and external elastic lamina separates media from the adventitia (Keaney. 2000). Intima is the innermost layer of the vessel wall and is directly in contact with the bloodstream. At birth, the intima is just a layer of endothelial cells abutting to the internal elastic lamina. During the growth, a subendothelial layer consisting of loose connective tissue with branched cells lying within the extracellular matrix (ECM), grows into the intima (Perrotta. 2013). The most widely accepted hypothesis is that the atherosclerotic plaque forms in the innermost layer, the intima (Ross, 1999). The endothelium is believed to play a fundamental role in the initial development of the plaque. Fatty streaks and lesions are found primarily in large and medium-sized muscular arteries in areas where the blood flow is turbulent and does not cause as much shear stress as elsewhere (Chatzizisis et al. 2007). This kind of area is found in arterial branches, bifurcations, and curvatures in the coronary (Asakura and Karino. 1990), cerebral, carotid (Ku

et al. 1985) or femoral arteries and in the aorta. There has been evidence that the blood flow affects whether the plaque progresses into a symptomatic or asymptomatic plaque (Chatzizisis et al. 2007).

Previously, atherosclerosis was thought primarily as a "plumbing" problem. The degree of stenosis on an angiogram, symptoms and signs of ischemia provided the main tools to assess atherosclerosis. In the last decades, studies on the pathogenesis of the disease have grown fast (Libby and Hansson, 2019). The understanding of the pathophysiology of this disease has now entered a new era based on the understanding of the biology and a critical reassessment of the pathobiology of atherothrombosis (Hansson and Libby, 2006; Libby, 2021).

The pathogenesis of atherosclerosis can be conveniently divided into three phases: initiation, progression, and complications.

According to Libby et al. (Libby et al., 2019) in the early stage of lesion initiation, LDL particles accumulate in the intima, where protected from plasma antioxidants, they can undergo oxidative and other modifications that can render them pro-inflammatory and immunogenic. Classic monocytes that exhibit a pro-inflammatory palette of functions then enter the intima. Monocytes circulate in the bloodstream and can bind to adhesion molecules expressed by activated endothelial cells. Chemoattractant cytokines, known as chemokines, can promote the migration of the bound monocytes into the artery wall. Once in the intima, monocytes can mature into macrophages, and attain characteristics associated with the reparative or less pro-inflammatory monocyte/macrophage population. These cells express scavenger receptors that permit them to bind lipoprotein particles and become foam cells (Figure 1 - A). In this phase, atherosclerotic plaques are mainly composed of cellular elements: smooth-muscle cells, macrophages and leukocytes, connective tissue of ECM–collagen, elastic fibers and proteoglycans, and intra- and extra-cellular lipid deposits. The overlying fibrous cap is composed of smooth-muscle cells, few leukocytes, and relatively dense connective tissue. The relative proportion of these components varies depending on the different plaque, which results in a wide spectrum of lesions with different degrees of instability. T lymphocytes, although numerically less abundant than monocytes, also enter the intima, and regulate functions of the innate immune cells as well as the endothelial and smooth muscle cells. Smooth muscle cells (SMCs) in the tunica media can migrate into the intima in response to mediators elaborated by the accumulating leukocytes. The smooth muscle cell chemoattractant platelet-derived growth factor arising from macrophages and deposited by activated platelets at sites of endothelial

breaches or intraplaque hemorrhage probably participates in this directed migration of medial SMCs into the intima (Libby et al., 2019). Especially at the periphery of the lesions, frequent aspects of neovascularization can be detected, and they are represented by the proliferation of blood vessels that can be differently mature. During the evolution of the atherosclerotic plaque, the resident and recruited SMCs produce ECM molecules that contribute to the thickening of the intimal layer. However, T cell mediators such as IFNγ can impair the ability of the SMC to synthesize interstitial collagen and thereby dampen the ability of these cells to repair and maintain the fibrous cap that overlies the necrotic core. Furthermore, activated macrophages show increased production of enzymes of the matrix metalloproteinases (MMPs) family that degrade the interstitial collagen that lends strength to the fibrous cap. Thinning and structural weakening of the fibrous cap increase the susceptibility of the plaque to rupture. As the lesion advances, SMCs and macrophages can undergo cell death including apoptosis. The debris from dead and dying cells accumulates, forming the necrotic, lipid-rich core of the atheroma (Figure 1 - B). Impaired clearance of dead cells (efferocytosis) can contribute to the formation of the necrotic core. Different cycles of inflammatory and muscle cell accumulation, more serum fat entrapment, and the activation of fibrosis by cytokines lead to the formation of a necro-lipidic core and of a fibrous cap, which characterize the so-called advanced lesions. At this point the lesion may protrude in the lumen, leading to stenosis and symptoms.

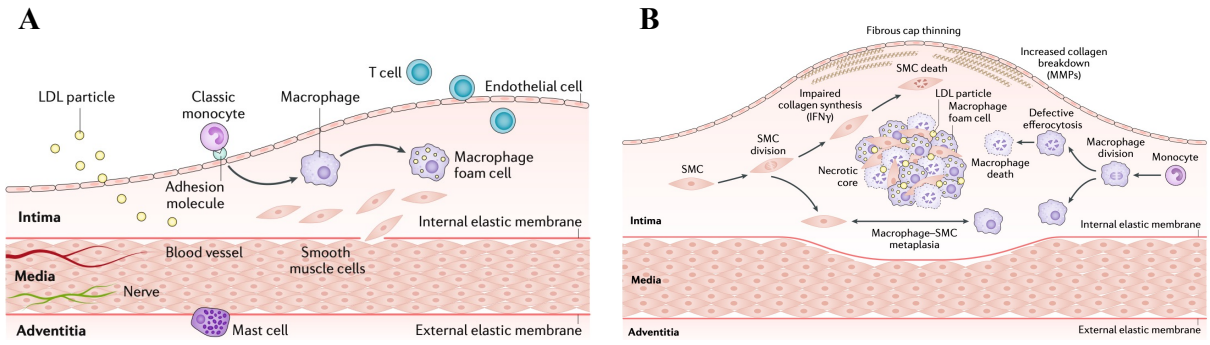

Figure 1. Development and progression of atheromasic lesions (Libby et al., 2019).

A broad spectrum of clinical presentation and various types of atherosclerotic lesions with several degrees of gravity exists. Patients could be asymptomatic, even having atherosclerotic plaques in their vasculature. Usually, they show a slowly growing silent lesions (Edsfeldt et al.,

2014; Libby, 2001; Libby et al., 2019; Naghavi et al., 2003). Patients with ischemic symptoms, such as stroke, usually present one or more unstable plaques with the risk of thrombotic and thromboembolic complications.

1.1.5 Biomarkers and vulnerable plaque

For several decades, lipid biomarkers including total cholesterol and LDL have been considered the predominant circulating biomarkers in the clinical management of CVDs and atherosclerosis. Now, numerous factors that indicate the presence of atheromas in the vascular system, such as cytokines and adhesion molecules, are known and often indicate the patient's risk status, and possible execution of more in-depth clinical examinations (Lubrano and Balzan, 2021).

Atherosclerotic plaque biomarker's studies are based on the concept, that a vulnerable plaque contains predictive information for future cardiovascular events, also in other areas of the vascular tree (W. van Lammeren et al., 2011). The histological evaluation of vulnerable plaques generally is based on five histological features compared to stable plaques: a larger lipid core (>40% of total lesion area), a thinner fibrous cap, inflammatory cells (Bluth et al., 1988; Kern et al., 2004), calcified lesions (Grant et al., 2003) and neoangiogenesis (EI-Barghouty N., 1995). The comprehension of the several cellular processes associated with the development of atherosclerotic plaque and the related molecules released into circulation may provide diagnostic and prognostic indications of plaque vulnerability (Puig et al., 2020). Several soluble biomarkers have been identified and suggested as tools to be used in the diagnosis of plaque vulnerability, but most of them lack sensitivity and their role is not yet well known.

Since atherosclerosis is an everlasting field of research worldwide, there is a multitude of biomarkers whose potential to indicate a vulnerable plaque has been tested.

Owing to the pivotal role of lipids and inflammation in the setting of atherosclerosis, most of the biomarkers originated from one of those processes. Conceptually, it is hypothesized that elevated levels of certain lipids will indicate increased susceptibility to the development of unstable atherosclerotic plaque owing to their role in the origin of plaque destabilization. On the other hand, certain molecules can migrate from vulnerable lesion back to circulation, thus creating an opportunity to serve as a biomarker.

Intravascular accumulation of several lipids is considered to play a crucial role in atherosclerotic plaque. It has been demonstrated by multiple authors that the amount of plaque oxidized LDL (ox-LDL) correlates with plaque instability, especially in symptomatic carotid artery disease (Sigala et al., 2010; Wang et al., 2020, 2017). However, the translation of experimental evidence in humans with aimed at the demonstration of the association between ox-LDL plasma levels with cardiovascular events proved to be difficult as it resulted in contrasting findings (Holvoet et al., 2004; Wilson et al., 2006; Yan et al., 2018; Zhang et al., 2014). Meta-analysis of 22 published studies (30879 subjects) showed a significant association between ApoE and carotid IMT (Paternoster et al., 2008). The association with LDL levels and ischemic stroke was proven in another large meta-analysis (Khan et al., 2013). Moreover, high levels of lipoprotein phospholipase A2 (Lp-LPA2), enzymes which travel along with circulating LDL, were found in patients with high-grade carotid stenosis and unstable plaques in a small series of patients undergoing CEA (Sarlon-Bartoli et al., 2012). Other studies reported that Lp(a), an LDL-like particle, rich in cholesterol and strongly influenced by genetic background, is increased in patients with high-grade unstable carotid stenosis (Sarlon-Bartoli et al., 2012), and with hypoechoic plaques (Iwamoto et al., 2004). In conclusion, all these findings help identify potential plaque lipid species related to plaque instability.

An interesting study on atherosclerotic plaques from 40 patients undergoing CEA showed that adhesion molecules CD146 and their soluble form strongly correlated with the development of inflammation of atherosclerosis and plaque instability, suggesting its promising role in monitoring the development and instability of atherosclerotic plaque in patients with carotid diseases (Qian et al., 2014).

Another study investigated the potential relation between IL-18 expression and signs of plaque instability, collecting clinical data from 22 consecutive patients undergoing the CEA procedure, distinguished by the presence, or absence, of an intraplaque ulcer, upon macroscopic examination by the surgeon. Significantly higher levels of IL-18 mRNA were found in symptomatic (vulnerable) plaques than in asymptomatic (stable) plaques, suggesting the significant role IL-18 performs in atherosclerotic plaque destabilization leading to acute ischemic syndromes (Mallat et al., 2001).

An increase in other cytokines is also at the basis of plaque instability. High plasma levels of inflammatory protein-1β (MIP-1β), TNF-α, and fractalkine predicted future TIA and could be used as markers for the identification of patients with high-risk plaques (Edsfeldt et al., 2015).

Vulnerable plaques have thin, highly inflamed, and collagen-poor fibrous caps that contain elevated levels of MMPs, which might promote plaque rupture (Newby, 2016; Olejarz et al., 2020; Sluijter et al., 2006). Studies on carotid plaques indicate that MMP-9 expression in carotid plaques is higher in vulnerable/symptomatic plaques in comparison to stable ones. In particular, MMP-8 and MMP-9 activity levels were increased in symptomatic plaques (Sluijter et al., 2006). Whereas serum MMP-9 levels are significantly higher in patients with atheromatous plaques in contrast to patients with fibrous plaques (Guo et al., 2018; Langley et al., 2017; Sluijter et al., 2006; Sukhova et al., 1999). In the study of Guo et al. (2018), human carotid plaques obtained from CEA from 64 patients, classified into stable and unstable by ultrasonography and histological analyses, demonstrated that MMP-2, MMP-14, VEGF, and bone sialoprotein 2 were elevated in vulnerable plaques, suggesting their significant role in the rupture of carotid plaques (Guo et al., 2018).

Previous studies demonstrated the correlation between serological and structural markers of inflammation and neoangiogenesis related to the risk of cardiovascular events in the coronary district (Ferri et al., 2006; Hoefer et al., 2015; Upadhyay, 2015).

1.2 MARKERS OF OUR STUDY: MICRORNAs

In addition to structural plaque analysis and inflammation protein, particular attention is given to molecular pathway involved in the atherosclerotic lesions' development.

An increasing number of studies highlight the important role of microRNAs (miRs) in the development and progression of CVDs caused by atherosclerotic lesions of arteries.

These biomarkers can be used to indicate the presence of pathology and even the stage, progression, or genetic link of pathogenesis (Biswas, 2018; Elfimova et al., 2012; Kocerha et al., 2011; Li et al., 2013; Olivieri et al., 2015; Wang et al., 2011; Weir et al., 2011). In certain situations, one miR may be sufficient to identify a health outcome; however, in other cases, a well-defined panel of miRs is necessary for increased diagnostic sensitivity and/or specificity.

1.2.1 MicroRNAs: definition

MiRs are a highly conserved class of small non-coding RNAs single-stranded, approximately 18-25 nt in length. These molecules interact with their mRNA targets by base-pairing with complementary sequences in the 3' UTR region of mRNA molecules. MiRs have emerged as key regulators of gene expression at a post-transcriptional level since their discovery. The first miR, lin-4, was discovered in *C. elegans* by Ambros and his group in 1993 (Lee et al., 1993). Ambros succeeded in cloning lin-4 locus and he identified two small transcripts of approximately 22 and 61 nucleotides, where the latter fragment was the precursor of the shorter one. Sequence alignment analysis confirmed that both of them matched in the 3' UTR region of lin-14 mRNA. These experimental evidences supported the hypothesis of lin-4 being a negative regulator of lin-14, with the imperfect binding of 3' UTR and the formation of multiple RNA duplex that down-regulate lin-14 translation. The conclusion of these discoveries was that lin-4 must have a primary role in *C. elegans* developmental timing during post-embryonic events, controlling lin-14 (Wightman et al., 1993). A second miR, let-7, was identified seven years later in the same nematode model organism. As described for lin-4, let-7 showed an inhibitory effect on various *C. elegans* heterochronic genes (including lin-14) promoting the progression from late-larval to adult life stage by binding to 3' UTR regions of the target mRNA (Reinhart et al., 2000). Because of their role in regulating developmental stages transition, lin-4 and let-7 were named "small temporal RNAs" (stRNAs), guessing they were actually members of a wider class of regulatory small RNAs still unknown at that moment (Pasquinelli et al., 2000). Soon after, many other miRs were isolated in different species and found to be related not only to particular temporal stages, but also to specific cell types. This new class of regulators was subsequently named microRNAs (Lau et al., 2001; Lagos-Quintana et al., 2001). Currently, 48860 mature miR products from 271 different organisms are listed in "miRBase" database. Among them, 1917 are hairpin precursor expressing 2654 human mature miRs (Kozomara et al., 2019). As negative gene regulators, miRs mediate protein synthesis repression in different cell types and they affect various biological processes, such as proliferation, differentiation, ageing and apoptosis. MiRs have a critical role in animal development and are involved in a variety of fundamental biological processes (Fu et al., 2013). Aberrant expression of miRs is associated with many human diseases (Paul et al., 2018; Tüfekci et al., 2014).

1.2.2 MiRs: biogenesis

Most of miRs generate in the nucleus from RNA polymerase II transcription of miR genes. Only a small class of miR-genes, that are interspersed among Alu repetitive elements, are transcribed by RNA polymerase III (Ha and Kim, 2014). MiR loci are widespread throughout the genome and can be found either inside intronic or exonic regions of non-coding genes, intronic sequences of protein-coding genes, or between independent transcription units (intergenic). About 50% of all currently identified miRs are intragenic and processed mostly from introns and relatively few exons of protein coding genes, while the remaining are intergenic, being transcribed independently of a host gene and regulated through their own promoters (de Rie et al., 2017; Kim and Kim, 2007). The majority of intronic miRs share the same promoter of the host gene, while a smaller number is transcribed from its own promoter enabling separate control of transcription (Monteys et al., 2010). Sometimes miRs are transcribed as one long transcript (cluster) and may have similar seed regions, therefore they are considered as part of the same family (Tanzer and Stadler, 2004).

The biogenesis of miR is classified into canonical and non-canonical pathways. The canonical biogenesis pathway is the main pathway used for miR processing, as shown in Figure 2.

MiRs biogenesis starts with RNA polymerase II or III transcription of primary miR (pri-miR), a stem-loop sequence which may encode for more than one mature miR. The sequence of pri-miR can be altered by A- to -I editing by double-stranded RNA-specific adenosine deaminase (ADAR) proteins, which may affect further biogenesis and the sequence of the mature miR or promote degradation of the pri-miR (Nishikura, 2016).

Like mRNAs, pri-miRs are spliced, 7-methylguanosine capped at the 5’ terminus and polyadenylated at the 3’ terminus. The first step of miR maturation is mediated by a protein complex consisting of the RNase-III- type enzyme Drosha and its essential cofactor DGCR8 (DiGeorge critical region 8), a dsRNA binding protein also known as Pasha. Besides the core proteins, the microprocessor requires in vivo other accessory proteins, such as the helicases p68 and p72, and the RNA binding protein hnRNP A1 (Liu et al., 2004). DGCR8 anchors pri-miR at the junction point between single and double strand RNA driving the correct positioning of Drosha, which cleaves approximately 11 nucleotides downstream into the stem region. Experimental evidences show that Drosha-mediated cleavage occurs in a co-transcriptionally fashion along with splicing. Pri-miR processing generates a 70-nucleotide product called

precursor-miR (pre-miR), whose peculiar structure consists of a 5' phosphate, a hairpin region and a 2-nucleotides hydroxyl overhang at the 3' which is essential for nuclear export factor Exportin 5 (EXP5) recognition. The EXP5-Ran GTP complex recognizes the double-stranded stem as well as the 3’ overhang, stabilizes pre-miR preventing its degradation and, finally, actively transports pre-miR intermediate from the nucleus to the cytoplasm. Ran-GTP is then hydrolyzed in Ran-GDP by RanGAP (Denli et al., 2004; Okada et al., 2009), causing EXP5 to release pre-miR molecule. Dicer is a “molecular ruler” (MacRae et al., 2007) which performs a cut on the pre-miR duplex and removes the terminal loop, generating a mature miR (Zhang et al., 2004).

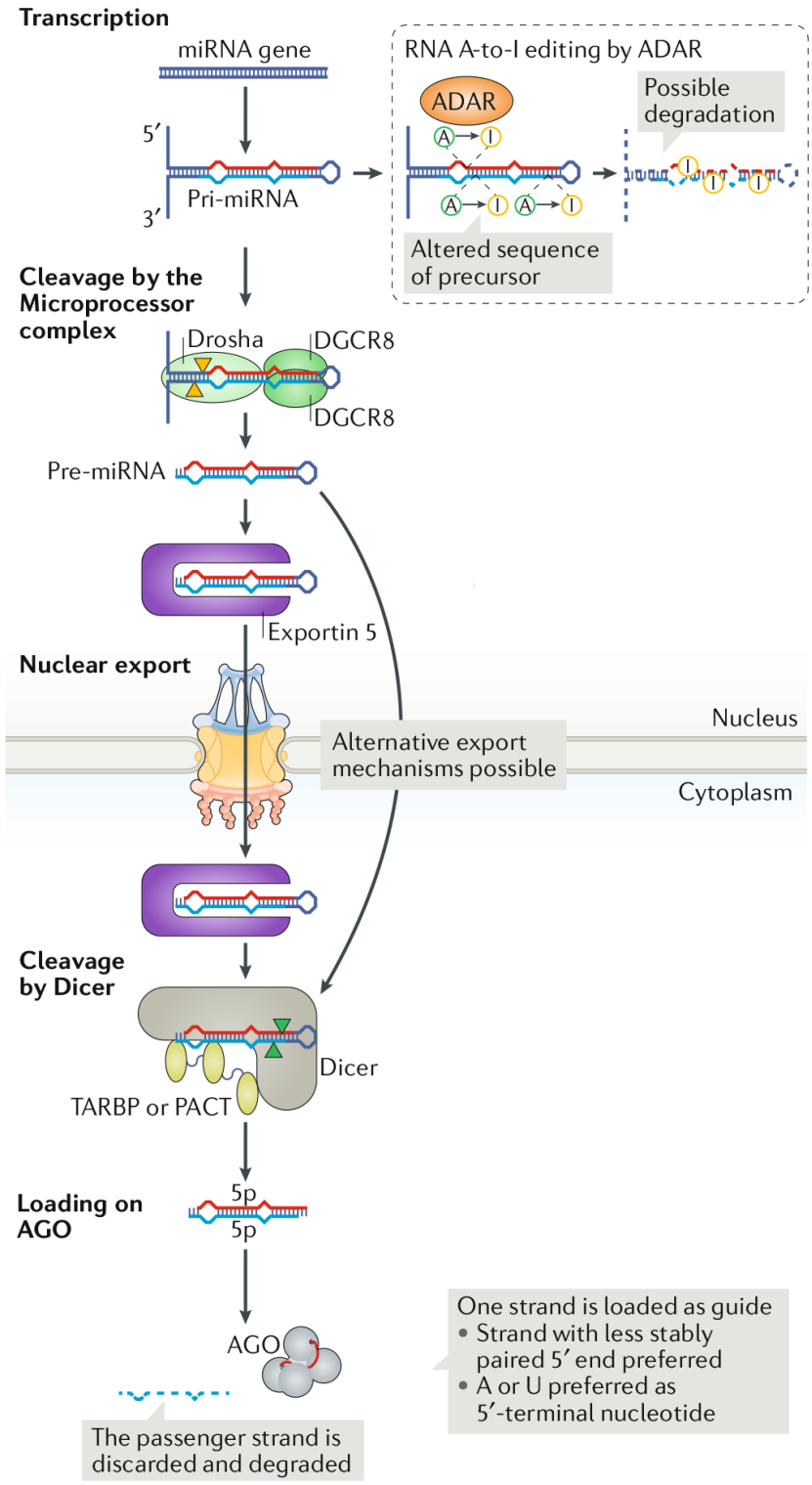

***Figure* 2.** MiR canonical biogenesis pathway and mechanism of action (modified from Gebert and MacRae, 2019).

In vertebrates, cleavage by Dicer is modulated by TAR RNA-binding protein (TARBP) and by protein activator of the interferon-induced protein kinase (PACT) (Lee and Doudna, 2012; Wilson et al., 2015). The directionality of the miR strand determines the name of the mature miR form. The 5p strand arises from the 5′ end of the pre-miR hairpin, while the 3p strand originates from the 3′ end (O'Brien et al., 2018). Both strands derived from the mature miR duplex can be loaded into the proteins belonging to the Argonaute (AGO) family (AGO1-4 in humans) through an ATP-dependent process (Yoda et al., 2010). For each specific miR, the proportion of 5p or 3p strand which are loaded into AGO proteins varies depending on the cell type or cellular environment, ranging from closely equal proportions to predominantly one or the other (Meijer et al., 2014). The AGO-loaded strand (5p or 3p strand) is selected depending on the thermodynamic stability at the 5′ end of the miR duplex or on the presence of a 5′ U or A as the 5-terminal nucleotide (Khvorova et al., 2003). Generally, the strand with lower stability at 5′ end or the one presenting a 5′ U/A is preferentially loaded into AGO (Frank et al., 2010) and is considered the ‘guide strand’. The unloaded strand is called the ‘passenger strand’ and it will be unwound from the guide strand through various mechanisms based on the degree of complementarity. The passenger strands with no mismatches are cleaved by AGO2 and degraded by the cellular machinery, which can generate a significant strand bias. Otherwise, miR duplexes with central mismatches and miRs that are not loaded into AGO2 are passively unwound and degraded (Ha and Kim, 2014).

Regarding the non-canonical miR biogenesis, multiple pathways have been reported so far. Different combinations of the proteins involved in the canonical pathway (mainly Drosha, Dicer, exportin 5, and AGO2) also take part in the non-canonical pathways. The non-canonical miR biogenesis can be divided into two main mechanisms: Drosha/DGCR8-independent and Dicer-independent pathways (Figure 3).

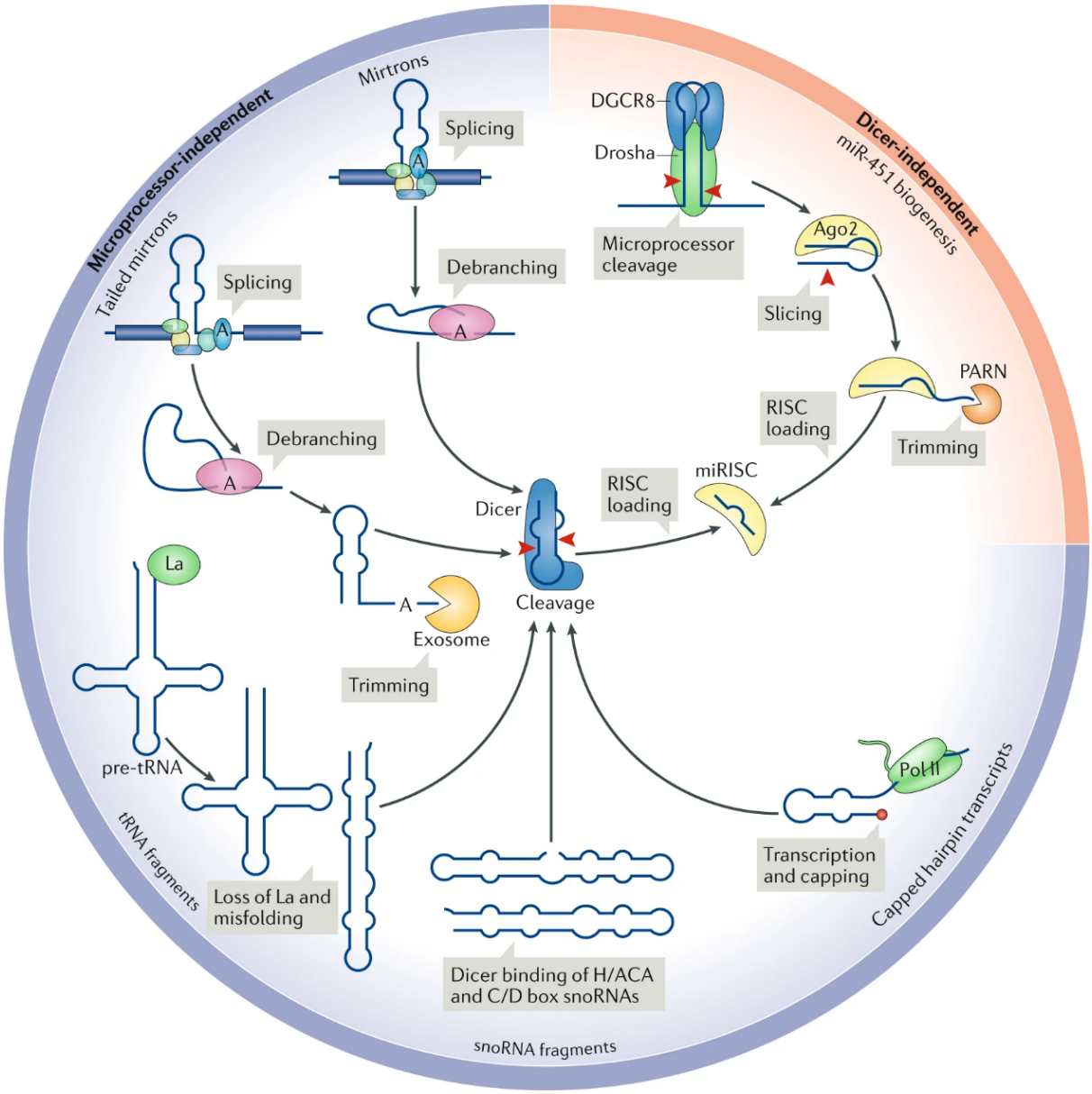

Figure 3. MiR non-canonical biogenesis pathways (Treiber et al., 2019).

The Drosha/DGCR8-independent pathway generates pre-miRs that resemble Dicer substrates. An example of such pre-miRs is mirtrons, which are produced from the introns of the mRNA encoding host genes during splicing (Babiarz et al., 2008; Ruby et al., 2007). The 7-methylguanosine (m7G)-capped pre-miR is another effective example; this kind of nascent RNA is directly exported to the cytoplasm through exportin 1, without the need for Drosha cleavage. There is a strong 3p strand bias, most likely due to the m7G cap which prevents 5p strand from being loaded into AGO (Xie et al., 2013). Conversely, miRs generated via Dicer-

independent pathways are processed by Drosha from endogenous short hairpin RNA (shRNA) transcripts (Yang et al., 2010). These pre-miRs require AGO2 to complete their maturation in the cytoplasm, since their short length prevents them from being fitting Dicer substrates (Yang et al., 2010). Therefore, loading of the entire pre-miR into AGO2 and AGO2-dependent slicing of the 3p strand are promoted. The maturation of miRs is completed with 3′-5′ trimming of the 5p strand (Cheloufi et al., 2010).

1.2.3 MiRs-mediated down-regulation of mRNA

In order to perform their gene silencing activity, miRs usually bind to a specific sequence at the 3′ UTR of their target mRNAs to induce translational repression and mRNA deadenylation and decapping (Huntzinger and Izaurralde, 2011; Ipsaro and Joshua-Tor, 2015). However, miR binding sites have also been detected in other mRNA regions, including the 5′ UTR, the coding sequence, and the promoter regions (Xu et al., 2014). MiR binding to 5′ UTR and coding regions promotes the silencing of gene expression (Forman et al., 2008; Zhang et al., 2018), while it has been reported that miR interaction with promoter regions can induce transcription (Dharap et al., 2013).

AGO proteins are 95 kDa proteins containing a PAZ and a PIWI domain (Müller et al., 2019). According to aminoacid sequence alignments, the AGO family can be clustered in the Ago and Piwi subfamilies: while the former is expressed in all cell types, the latter can be found almost exclusively in stem cells and the germline. Humans have 4 different Ago proteins (Ago 1-4) that bind the 3’ 2 nucleotides overhang through their PAZ domain. Archeal Ago protein analysis showed that PIWI domain is structured as a RNaseH-like catalytic domain, which is characterized by the catalytic triad Asp-Asp-Glu (DDE) coordinating one or two Mg2+ ions. AGO proteins possess a peculiar catalytic triad that comprises two aspartates and one histidine residue (DDH motif), which are responsible for Ago slicer activity. Among human Ago proteins, Ago2 is the only one competent for mRNA cleaving activity due to its unique structural characteristics (Liu et al., 2004; Meister et al., 2004). Nevertheless, AGO3 has recently been shown to slice target RNAs, however, only when loaded with certain miRs (Park et al., 2017). In these cases compared to AGO2, the slicing activity depended strongly on the pairing of the postseed region of the guide RNA as well as on the 5′ and 3′ flanking regions of the target RNA (Park et al., 2017).

The complex generated by the interaction between the guide strand and AGO is called miRISC (minimal miR-induced silencing complex).

Through RISC complex, miRs mediate gene silencing via base-pairing between miR recognition element (MRE, located in the 3' UTR of target mRNA) and miR 5' "seed" region (2 to 8 nucleotides) (Figure 4).

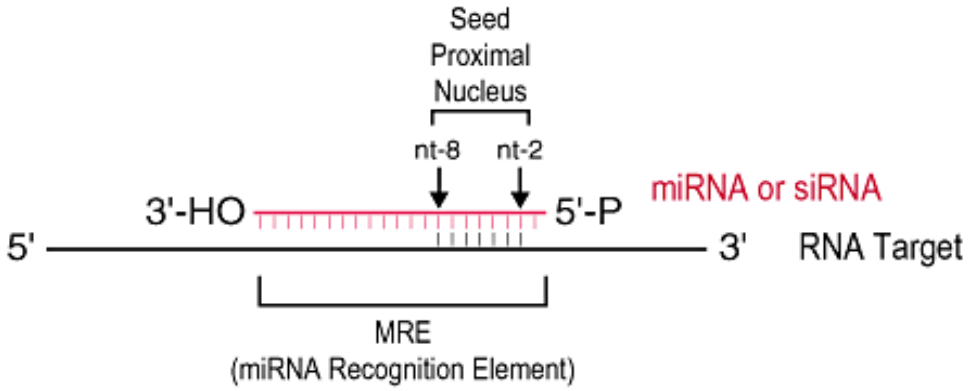

Figure 4. MiR binding to target mRNA (Liu et al., 2008).

Recent findings, however, confirmed that some miRs actually possess a 3' end interaction site specific to the 5' UTR of the cognate mRNA, and that the most of translational repression was achieved when both 3' UTR and 5' UTR interaction sites were present in miR sequence (Lee et al., 2009).

The biological outcome of Ago-miR interaction strictly depends on the extension of base complementarity and on which Ago protein is deposited on the target mRNA. A non-perfect match between miRs and MRE region results in target mRNA destabilization and translational repression through different mechanisms and at different stages of protein synthesis.

First of all, miRs may impair translation initiation by chromatin remodeling or recruiting decapping and deadenylating enzymes as well as exonucleases and endonucleases. Additionally, Ago proteins compete with CBP (cap binding protein) and initiation factors, such as elF4E, in binding the 5' methylguanosine cap, thus preventing mRNA circularization and translation initiation.

At a co-transcriptional stage, miRNPs may interfere with ribosome assembly or induce its dissociation due to steric hindrance. Besides, premature termination and cotranslational

degradation of the nascent protein may occur, owed respectively to miRNPs competition with elongation factors and specific proteases enzymatic activity.

Apart from translational repression and mRNA destabilization, when a miR bound to Ago2 matches perfectly with the cognate mRNA, the target undergoes enzymatic degradation. As happens with other RNase H-like enzymes, Ago2 slicer activity cuts target mRNA near miR's nucleotides 10-11, producing 5′ phosphate and 3′ hydroxyl ends. While the mature miR remains intact, target mRNA is subsequently degraded via routine exonucleolytic digestion. Target mRNA cleavage by miRs is the major mechanism of regulation by plant miRs. In animals, however, there are very few examples of miRs that regulate their mRNA targets by cleavage; rather, the predominant silencing mode of animal miRs is to repress the translation of their mRNA targets and/or to destabilize them without endonucleolytic cleavage.

All mechanisms of miRs interaction with mRNA target are summarized in Figure 5.

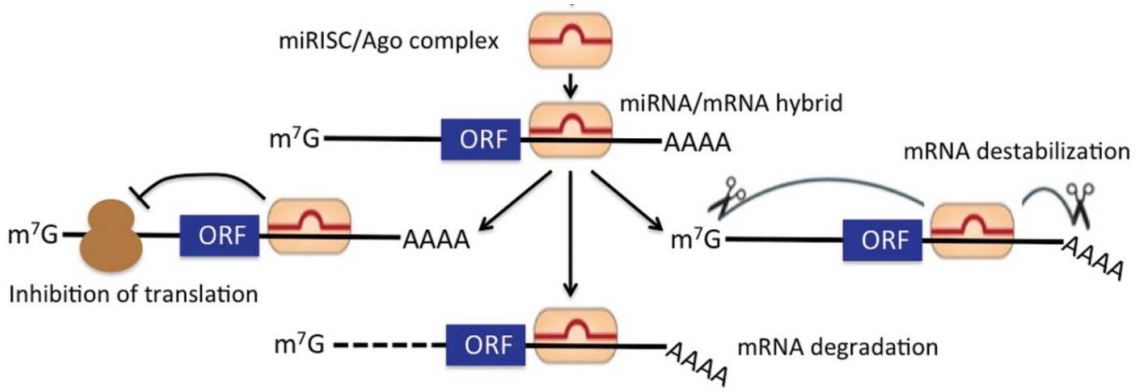

Figure 5. Mechanism of action of miRs (modified from Piva et al., 2013).

1.2.4 MiRs-mediated up-regulation of mRNA

Even though most studies focus on the gene silencing activity of miRs, some have also reported situations in which miRs are capable of up-regulating gene expression.

In most cases, miRs mediate a process of gene silencing by guiding AGO proteins to specific target sites situated in the 3′ untranslated region (UTR) of mRNAs. However, interaction of miRs with other regions, including the 5′ UTR, coding sequence, and gene promoters, have also been reported (Broughton et al., 2016).

Translational enhancement may derive from a direct activation by miRNPs or from the relieving of a preexisting translational repression state.

One of the first cases reported in literature is miR-122, a liver-specific miR that binds either to the 5' UTR or the 3' UTR of Hepatitis C virus (HCV) genome. Recent studies have proved that the sequestration of miR-122 by miR-122 ASO (antisense oligonucleotide) led to an 80% decrease of HCV RNA and viral proteins. Indeed, when the binding site resided in the 5' NCR region, both viral gene replication and translation resulted up-regulated (Jopling et al., 2005). Since miR-122 binds two adjacent sites (seed-match-site 1 and 2) located upstream the internal ribosome entry site, the subsequent recruitment of miRNPs on 5' NCR could scaffold many other proteins required for genome replication and translation, e.g., RNA polymerase. In addition, these multiprotein complexes could conceal the uncapped 5' NRC of viral RNA from cytoplasmic exonucleases as well as mimic a cellular mRNA cap structure, therefore enhancing ribosome association and viral mRNA translation (Valinezhad Orang et al., 2014).

Another example of miRs targeting the 5' UTR is miR-10a, which positively regulates ribosomal proteins (RP) translation. A peculiar characteristic of several mRNAs encoding for RP, elongation factors and poly-A binding proteins, is the 5' terminal oligopyrimidine tract (5' TOP). This highly conserved motif acts as a regulatory sequence which suppresses RP synthesis in response to cellular stress or nutrient deprivation. miR-10a was found to bind to a sequence immediately downstream the 5' TOP motif, thus counteracting 5' TOP-induced down-regulation and leading to an increase in RP mRNA translation and protein synthesis (Ørom et al., 2008).

Recent studies revealed that miR-346 is produced from the second intron of glutamate receptor ionotropic delta 1 (GRID1) mostly in brain tissues and is capable of upregulating RIP140 (receptor- interacting protein 140) gene. RIP140 is a transcription coregulator and modulates many metabolism-related pathways; miR-364 accelerate mRNA interaction with polysomes via binding to 5′ UTR of the target RIP140 mRNA. Nevertheless, miR-346 does not require AGO2 for its activity; therefore, it possibly applies an AGO-independent pathway to control the protein yield of RIP140 without altering its mRNA levels (Tsai et al., 2009).

Translational activation can be also related to cell cycle phase. AU-rich elements (ARE) are cis- acting regulatory sequences of gene expression located in the 3' UTR of several mRNAs, encoding for oncogenes, growth factors, cytokines, etc. AREs are recognized by specific ARE-

binding proteins (ABPs), which are generally mediate mRNA decay via interaction with exosomes, exonucleases and decapping enzymes. However, different ABPs can either perturb mRNA stability or mediate its translational activation through specific interactions with miRNP effector complexes, which are deposited on target mRNA by miR-mRNA matching.

An excellent example is FXR1 (Fragile X mental retardation syndrome-related protein 1), one of the ARE-binding proteins involved in mRNA translational up-regulation. FXR1 is generally associated with Ago2 in RNA-induced silencing complexes. This association was deeply investigated in a 2007 work in which it was reported that, even though FXR-1 and Ago2 are generally thought to switch off mRNA translation, FXR1-Ago2 complex promoted TNF-α upregulation after cell cycle arrest induced by serum starving, hence proving that AU-rich elements can act as translation activation signals in case of cell cycle arrest (Vasudevan and Steitz, 2007).

Furthermore, Zhang and colleagues published a 2014 study where they reported the ability of many cellular miRs to up-regulate gene promoters' activity by targeting the TATA-box region. TATA-box is a cis-regulating element located approximately 25 bp upstream the TSS (transcription start site) within the core promoter. The first step of DNA transcription requires the binding of TBP (TATA- box binding protein) and the pre-initiation complex (PIC) assembly on TATA-box consensus sequence. Many endogenous miRs, complexed with Ago proteins, were found to bind within 50 bp upstream the TSS in a sequence-specific fashion. The direct miR-TATA box interaction possibly facilitates TBP recruitment and PICs assembly on the core promoter (Zhang et al., 2014).

Finally, the same miR can mediate both down- and up-regulation. One of them is miR-145, which on one hand positively regulates the expression of myocardin and other co-activators of vascular smooth muscle cells differentiation, on the other hand inhibits ROCK1 expression in osteosarcoma cells, therefore acting as a tumor suppressor (Cordes et al., 2009; Li et al., 2014). As another example, KLF-4 is upregulated by miR-206 in confluent and nontumor cells, while it is down-regulated by miR-344 in proliferating and normal cells (Lin et al., 2011).

1.2.5 Tissue-associated and circulating miRs

MiRs can be divided into two categories: tissue specific and circulating miRs (c-miRs). Some miRs show restricted tissue distribution, for example, miR-122 is highly enriched in liver, whereas miR-124 is preferentially expressed in neurological tissues. It has been shown that changes in the spectrum of cellular miRs correlate with various physiopathological conditions, including differentiation, inflammation, diabetes, and several types of cancers. Recently, some of the miRs previously identified in cells and tissues have also been found in extracellular fluids, show that miRs mediate long- and short-range cell communications through their secretion in the systemic circulation.

The changes of several miR levels in plasma, serum, urine, and saliva have already been associated with different diseases. Numerous studies reported the presence of miRs in extracellular fluids, such as plasma and serum (Chen et al., 2008; Arroyo et al., 2011), cerebrospinal fluid (Cogswell et al., 2008), saliva (Gallo et al., 2012), breast milk (Zhou et al., 2012), urine, tears, colostrum, peritoneal fluid, bronchial lavage, seminal fluid (Weber et al., 2010), and ovarian follicular fluid (da Silveira et al., 2018).

As opposed to cellular RNA species, extracellular miRs are highly stable; they can resist degradation at room temperature for up to 4 days and in deleterious conditions such as boiling, multiple freeze-thaw cycles and high or low pH (Chen et al., 2008; Mitchell et al., 2008). In contrast to the stability of c-miRs endogenous, when synthetic miRs were added exogenously, they were quickly degraded by the high level of RNase activity in plasma (Mitchell et al., 2008). This suggests that endogenous plasma miRs are protected in some manner to prevent their degradation.

As shown in Figure 6, c-miRs are protected by plasma/serum RNase activity by the formation of RNA-protein complexes with HDL, LDL or Ago2 proteins (Arroyo et al., 2011; Vickers et al., 2011), as well as through their inclusion inside extracellular vesicles. These vesicles are generally referred to as microvesicles, apoptotic bodies, or exosomes. Exosomes are 40–100 nm vesicles released during reticulocyte differentiation as a consequence of multivesicular endosome (MVE) fusion with the plasma membrane and differs from microvesicles that are 50–1.000 nm vesicles and shed directly from the plasma membrane. Apoptotic bodies, the largest vesicles (800–5.000 nm diameter), are released by cells undergoing programmed cell death. It was demonstrated that the cargo of extracellular vesicles included miRs and that

vesicle-associated RNAs could be transfer into target cells (Ratajczak et al., 2006; Turchinovich et al., 2015; Valadi et al., 2007).

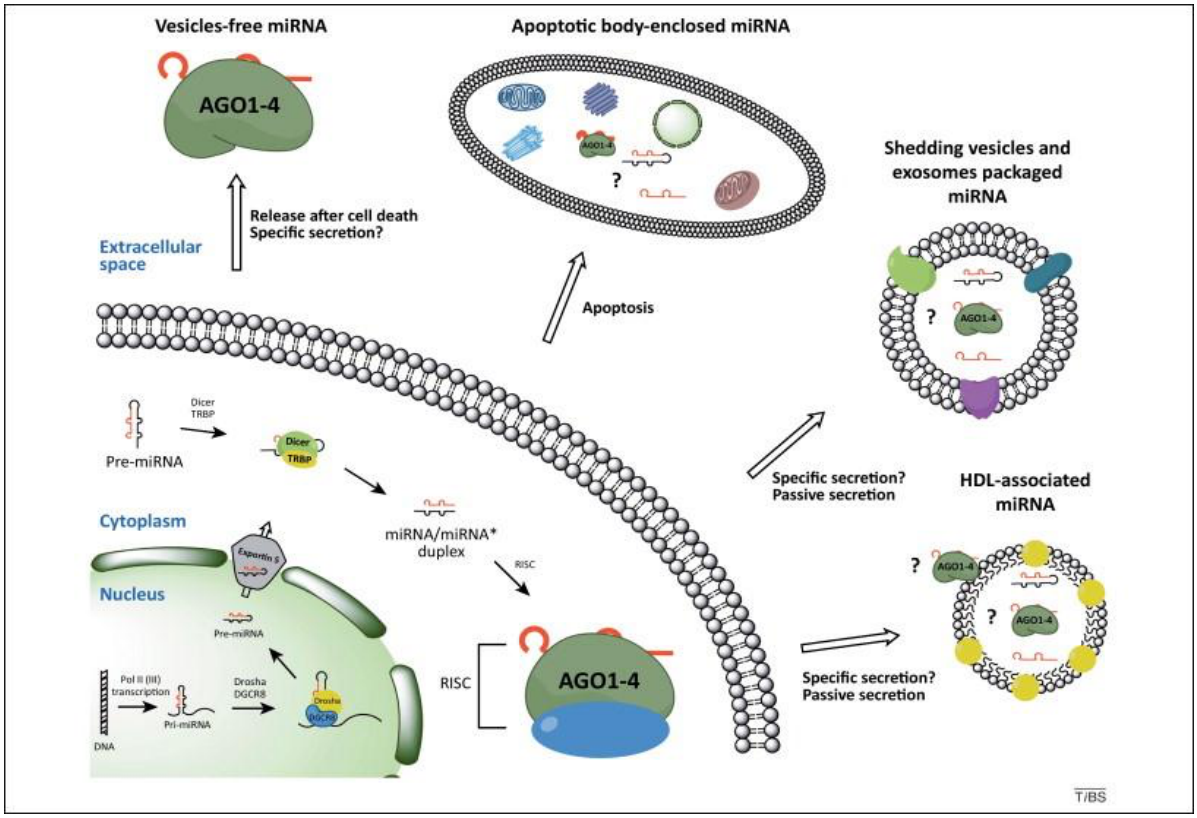

Figure 6. Biogenesis of miR in the cell and the kinds of extracellular miR packaging (Turchinovich et al., 2012).

Several groups investigated the exosome content in cultured cells in greater detail. Valadi and his group revealed that mast cell–derived exosomes carry about 121 different miRs. Some of these were found at relatively higher levels in exosomes than in their donor cells, which implies an active mechanism by which selected miRs are promoted toward exosomes. Other studies confirmed that cells can indeed select some miRs for cellular release while others are retained. Specifically, although 66% of the miRs were released in quantities that reflected their intracellular level, 13% of the miR species were selectively retained by the cell (and thus released at lower levels), whereas on the other hand, 21% of the miRs seemed to be actively released and appeared at disproportionately higher levels (Pigati et al., 2010).

The proportion of miRs in the different cell-derived compartments is not yet settled. Currently, evidence is accumulating that the majority of miRs are not found inside vesicle but rather are bound to RNA-binding proteins. Two populations of c-miRs exist: vesicle-associated and non–

vesicle-associated miRs, and evidences support the hypothesis that vesicle-associated plasma miRs represent the minority, whereas potentially up to 90% of c-miRs are present in a non–membrane-bound form. These non–vesicle-associated miRs were specifically destabilized by proteinase K digestion of plasma, which indicates the existence of an miR-protein complex as a mechanism for their stability in the RNase-rich circulation (Arroyo et al., 2011). Interestingly, some miRs (eg, let- 7a) were exclusively associated with vesicles, whereas others (eg, miR-122) were exclusively present in non-vesicle Ago2 complexes. This may reflect cell type–specific miR release mechanisms. For instance, the liver-specific miR-122 was detected only in protein-associated fractions, which suggests that hepatocytes release this miR through a protein carrier pathway. In contrast, miRs that are mainly associated with vesicles might originate from cell types that are known to generate vesicles, such as reticulocytes or platelets. Secretory miRs act as signalling molecules that modulate gene expression of recipient cell similarly to intra-cellular miRs. It was showed that T cells receive small RNAs from B cells that can affect the expression of target genes in the recipient T cells upon cell contact (Rechavi et al., 2009). Furthermore, it was demonstrated that miRs secreted by Epstein– Barr virus (EBV)-infected cells are transferred to and act in uninfected recipient cells through exosomes (Pegtel et al., 2010). In addition, it was reported that embryonic stem (ES) cell microvesicles contain abundant miRs and that they can transfer a subset of miRs to mouse embryonic fibroblasts in vitro, suggesting that stem cells can affect the expression of genes in neighbouring cells by transferring miRs contained in microvesicles (Yuan et al., 2009).

Though it is well established the existence of c-miRs and their gene silencing activity, it is still to clarify the combination of factors and mechanisms behind their release and uptake. Only few reports show the mechanism of secretion of miRs. Recently, it was proved that miRs are released through a ceramide-dependent secretory machinery and the secretory miRs are transferable and functional in the recipient cells (Kosaka et al., 2010). Ceramide, a bioactive sphingolipid whose biosynthesis is tightly controlled by neutral sphingomyelinase 2, triggers the secretion of exosomes. Blockade of neutral sphingomyelinase 2 by either a chemical inhibitor or by small interfering RNAs reduced secretion of miRs, whereas overexpression of neutral sphingomyelinase 2 increased the amount of extracellular miRs.

Among RNA-binding proteins identified, there were nucleophosmin 1 (NPM1), which has been implicated in the nuclear export of the ribosome, and nucleolin, a known NPM1-interacting protein (Borer et al., 1989). Subsequent experiments showed that NPM1 can fully protect

synthetic miR degradation by RNase A. It has been suggested that NPM1 may be involved in shuttling RNAs from the nucleus to the cytosol, and independent studies have also shown that NPM1 can be released into the extracellular space (Nawa et al., 2009). Together, this suggests that this mechanism may be relevant for miR export and stability.

It has been proposed that the Ago2-miR complexes are passively released by death or apoptotic cells and remain in the extracellular space because of the high stability of the Ago2 protein. It is also possible that cell membrane–associated channels or receptors exist that allow for the specific release of these Ago2-miR complexes.

Concerning how HDL is loaded with miRs is not known exactly; however, biophysical studies suggest that HDL simply binds to extracellular plasma miR through divalent cation bridging. Furthermore, it was reported that ceramide pathway represses miR export to HDL, indicating that the export of specific miRs through the exosomal pathway and the HDL pathway may be distinct mechanisms, possibly opposing, although both pathways are probably regulated by nSMase2 activity and ceramide synthesis (Vickers et al., 2011).

In Figure 7 are summarized the mechanisms for c-miRs transfer in different shuttle and their uptake in the target cells. During “plasma membrane budding,” ectosomes containing cytoplasmic components like miRs are released into the extracellular surrounding (A). The fusion of exocytic multivesicular bodies (MVBs) with the plasma membrane releases miR-containing intraluminal vesicles, exosomes (B). The ABCA1 (ATP-binding cassette transporter A1) mediates the release of HDL-complexed miRs (C). Apoptotic bodies are released from a cell that undergoes apoptosis. These large vesicles contain fragmented DNA and cytoplasmic components including miRs (D).

General mechanisms of vesicle uptake in recipient cells involve endocytosis (E), phagocytosis (F), and fusion (G) with the plasma membrane. The uptake of HDL-complexed miRs in recipient cells is mediated by SR-B1 receptors (H). The uptake mechanisms of extravesicular AGO or NPM-1 complexed miRs are not described yet.

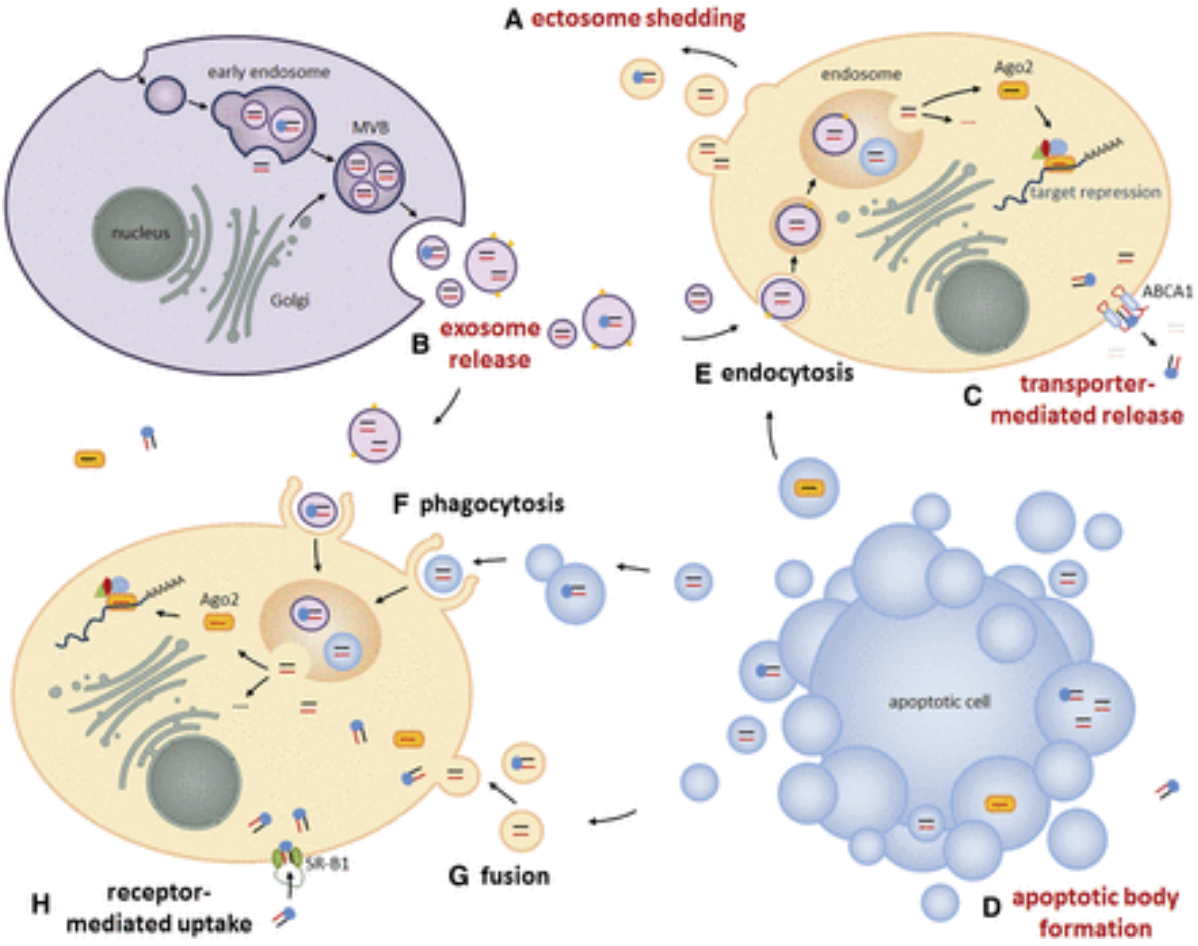

Figure 7. Long-distance intercellular miR transfer (Grasedieck et al., 2013).

Extracellular miRs can be delivered to target cells and they may act as autocrine, paracrine, and/or endocrine regulators to modulate cellular activities (Iftikhar and Carney, 2016), showing hormone-like properties.

1.2.6 C-miRs as potential biomarkers

The level and composition of c-miRs show changes that correlate well with diseases or injurious conditions. These observations suggest that extracellular miRs can be used as informative biomarkers to assess and monitor the body's physiopathological status (Etheridge et al., 2011). The ideal biomarker must be accessible using non-invasive protocols, inexpensive to quantify, specific to the disease of interest, translatable from model systems to humans, and a reliable early indication of disease before clinical symptoms appear (early detection). Biomarkers that can be used to stratify disease and assess response to therapeutics are also medically valuable.

Indeed, most current biomarkers are protein based, such as troponin for cardiovascular conditions, carcinoembryonic antigen (CAE) for various cancers, prostate specific antigen (PSA) for prostate cancer, and aminotransferases (alanine aminotransferase, ALT and aspartate aminotransferase, AST) for liver function. Challenges for developing new protein-based biomarkers include the complexity of protein composition in most biological samples (especially blood), the diversity posttranslational modifications of proteins, the low abundance of many proteins of interest in serum and plasma, and the difficulty of reliably developing suitable high-affinity capture agents. These intricacies make the discovery and development of additional protein-based biomarkers with the proper diagnostic specificity and sensitivity an expensive, time-consuming, and difficult task. On the other hand, c-miRs have many requisite features of good biomarkers.

Detecting specific miR species, while not trivial, is generally much easier. Stability in various bodily fluids, lower complexity, no known post- processing modifications, simple detection and amplification methods, tissue-restricted expression profiles, and sequence conservation between humans and model organisms make extracellular miRs ideal candidates for non-invasive biomarkers to reflect and study various physiopathological conditions in the body (Weber et al., 2010).

In recent years, extraordinary progress has been made in terms of finding the origin and functions of miRs and, especially, their potential use in research and clinical practice (for both healthy and diseased patients) is becoming more and more relevant (Condrat et al., 2020). However, the most promising role of miRs is that of potential biomarkers for a variety of diseases and fundamental biological processes.

For example, serum level of miR-141, have been used to discriminate patients with advanced prostate cancer from healthy individuals (Mitchell et al. 2008), the ratio of miR-126 and miR-182 in urine samples can be used to detect bladder cancer (Hanke et al., 2010) and decreased levels of miR-125a and miR-200a in saliva is associated with oral squamous cell carcinoma (Park et al., 2009). In addition to these potential uses in detection of various cancers; another intriguing possibility is the use of levels of organ-specific miRs in body fluids to monitor the physiopathological conditions of specific organs. Other studies have demonstrated that the

plasma level of miR- 499, a heart specific miR, shows a perfect correlation with blood troponin levels in patients with myocardial infarction (Adachi et al., 2010).

They also have high specificity for the tissue or cell type of provenance, and they provide a sensitive system, as they vary according to the disease progression; therefore, they can be used to study the differentiation of cancer stages (Lan et al., 2015) and even for measuring the therapeutical responsiveness to medical treatments (Acunzo et al, 2015). Moreover, many kinds of technologies for the detection of nucleic acids already exist and the development of new assays requires less time and lower costs in comparison with the production of new antibodies for protein biomarkers. Another advantage of miRs lies in their potential as multi-marker models for multi-factorial diseases. While running many protein markers may be both expensive and time-consuming, using multi-marker panels composed of numerous miRs may provide a non-invasive method for diagnosis and prediction of disease progression (Condrat et al., 2020).

All these evidences clearly demonstrate the possibility of using miR in circulation as strong and powerful biomarker to reflect the healthy and unhealthy status.

This is especially important in cancer, a thoroughly heterogenous disease, where a multi-marker approach would be preferable. Moreover, evidence suggests that miRs could play an essential role as biomarkers for the diagnosis and prognosis of Alzheimer's disease (AD) (Wiedrick et al., 2019), for patients with spinal cord injury (Tigchelaar et al., 2019), epilepsy (Raoof et al., 2018) or neurodegenerative pathologies (Sheinerman and Umansky, 2013). These molecules could also be used in other fields like cardiology, as a faster and more accurate means of diagnosis for acute cardiovascular disease or heart failure (Corsten et al., 2010), in atherosclerosis for the monitoring of the carotid plaque (Collura et al., 2020), and for the measurement of biological processes such as inflammation (inflamma-miRs) and ageing (Olivieri et al., 2013).

A mounting body of evidence has been documenting several miRs that are involved in regulating inflammation: their prototypes are miR-155, miR-21, and miR-146a (Quinn and O'Neill, 2011). In physiological conditions, transcription of miR-155, miR-21, and miR-146a is at baseline levels; however, initiation of pro-inflammatory TLR signalling immediately results in strong co-induction of their expression through a mechanism that is largely NF-κB-dependent (Boldin and Baltimore, 2012). A progressive increase in circulating acute-phase

proteins and pro-inflammatory mediators (proteases, cytokines, chemokines, and growth factors), has been described as a general feature of the ageing process and has been denominated inflamm-aging (Franceschi et al., 2000). Age-associated chronic inflammation has mainly been attributed to progressive activation of immune cells over time and to accumulation of senescent cells with a pro-inflammatory secretory phenotype (Olivieri et al., 2013b). The complex inflamm-aging phenotype is the result of age-related cell/tissue adaptation and remodelling interacting with genetic/epigenetic factors. Even though TLR family members do not show consistent age-dependent changes across model systems, there is evidence for impaired downstream signalling events during ageing, including inhibition of positive effectors and activation of negative modulators of TLR signalling (Olivieri et al., 2013). Therefore, during ageing, inflamma-miR levels progressively increase in order to stem the cell and tissue damage induced by the low-level chronic inflammation, also likely sustained by the cell senescence secretome (Murray and Smale, 2012).

Moreover, different strategies for overexpression or down-regulation of specific miRs are being pursued for therapeutic intervention for various diseases. Given the involvement of miRs in gene expression regulation, a peculiar modulation of their expression might contribute to efficient homeostasis in human ageing. It is worth stressing that exceptionally long survival requires dynamic preservation of optimal levels of physiological variables, and that the mean levels of many biomarkers of ageing are not stable but change in the course of life (Spazzafumo et al., 2013). Modulation of miR-21 expression in plasma, circulating cells, and tissues of very old subjects suggests that miR-21 might lie at the intersection of senescence, inflammation, and age-related diseases. It has been reported that positive correlations exist between circulating miR-21 and two important biomarkers of inflammation: C-reactive protein (CRP) and fibrinogen (Olivieri et al., 2012). Notably, circulating miR-146a levels were found to be quite similar to those described for miR-21 (Olivieri et al., 2012, 2013b). Moreover, analysis of global miR expression in the peripheral blood of adult women has shown that miR-155 is one of the most up-regulated miRs among older women (Sredni et al., 2011). Abundant data continue to support the hypothesis that progressive up-regulation of inflammatory gene expression and high levels of inflammatory signalling facilitate the development and progression of the major age-related diseases, such as CVD, type 2 diabetes mellitus (T2DM), AD, rheumatoid arthritis (RA), and cancers. Patients suffering from such diseases show sub-clinical/clinical inflammation and, interestingly, deregulation of most circulating *inflamma-miRs* (Wang et al.,2012). Since multiple co-expressed miRs can cooperatively regulate a given

biological process by targeting common components of that process, the development and progression of human diseases could be associated with abnormal regulation of multiple miRs functioning cooperatively. Some of the most recent data showing that circulating *inflamma-miR* deregulation is shared by the major human age-related diseases, such as type 2 diabetes mellitus (T2DM), cardiovascular diseases (CVD), AD and rheumatoid arthritis (RA) (Olivieri et al., 2013c).

On the one hand, modulation of a single miR offers the opportunity to target multiple genes and regulatory networks simultaneously. However, for the same reason, careful design is needed to prevent unwanted off-target effects. Another reason to pay attention is the amount of delivered miR needed to affect the knockdown efficacy and target selection. MiR-based therapy can be considered as a powerful strategy for therapeutic purpose, but some issues are still to be bring under discussion. Nuclease-mediated degradation before achieving target modulation is a major issue in achieving the desired outcome. Systemic administration of miR using adeno-associated virus or cytoplasmic viruses of negative polarity capable of producing functional miRs have been described. MiR inhibitors termed miR sponges, antagomirs, locked-nucleic-acid-modified oligonucleotides, and reconstituted high-density lipoprotein nanoparticles are some of the approaches that have been pursued. Other strategies are used for the delivery, development of novel nanomaterials to pass barriers and antibodies against various cell surface receptors to be taken up by specific cells via receptor-mediated endocytosis. Vesicular structures that include exosomes and shedding vesicles are also being explored for delivery of exogenous therapeutic cargoes (Ajit, 2012). A better understanding of miR biology and the development of safe and effective delivery strategies can greatly enhance the therapeutic potential of miRs.

2. AIM OF THE STUDY

The present thesis is focused on the study of tissue and circulating miRs as evaluable molecular drivers and markers in carotid pathology.
This study attempted to answer the following questions:

1. May c-miRs profile distinguish between asymptomatic and symptomatic patients? The answer to this question is towering the crucial challenge of decision-making in patients with asymptomatic carotid stenosis, and reliable biomarkers identifying patients at high risk of stroke or cerebrovascular events due to carotid stenosis are still lacking.

2. May the simultaneous evaluation of tissue miRs and c-miRs in two districts (i.e. plaque tissue and blood) give results about a circulating miR-based signature and a specific plaque-phenotype? As miRs are released from the plaque tissue into the circulation, a biomarker panel with these miRs could identify the patient at risk of plaque rupture. This topic is also tightly associated with the plaque phenotype and the possibility to investigate a miR-signature of "carotid plaque instability".

3. May circulating biomarkers (c-miRs signatures) be predictive of the development of carotid disease in outpatients with asymptomatic stenosis? This is a crucial aspect of the research field for new and non-invasive markers for the screening of asymptomatic carotid stenosis. At present, the gold standard method for the diagnosis of carotid asymptomatic stenosis is duplex ultrasound imaging, which is simple and safe, but easily affected by the operator, and it is expensive and not suitable for large-scale screening of the population. Thus, the identification in the preclinical phase of circulating biomarkers that may predict future cerebrovascular events and improve the therapeutic decision-making process becomes of fundamental importance.

Overall, the evaluation of new circulating risk biomarkers, using a non-invasive technique, would allow a possible implication in clinical practice and the management of asymptomatic outpatients.

3. MATERIALS AND METHODS

3.1 Patients' recruitment and study design

A total of 49 participants were included in the present study, divided into two main groups (inpatients or outpatients) as follows:

- **Population 1**: comprising 41 inpatients with carotid stenosis, 26 males and 15 females, mean age 73.8 ± 6.4. Specifically, Population 1 includes symptomatic (N=16) and asymptomatic (N=25) inpatients with 70% carotid stenosis undergoing CEA according to the recommendations of the European Society of Vascular Surgery (ESVS) and the Society of Vascular Surgeons (SVS). Symptomatic carotid stenosis was defined as the occurrence of ipsilateral cerebral ischemic events (major or minor stroke, TIA, or *amaurosis fugax*) within the last 6 months. Endarterectomy and blood samples (before CEA) were collected from each patient.
- **Population 2**: comprising 8 outpatients with carotid stenosis ≤ 60%. Blood samples were collected and carotid stenoses were assessed by Doppler ultrasound (ECST method). Subjects consented to the collection of blood samples at two different time points, T1 and T2 after 1 year, to assess the possible correlation of miR expression levels with changes in carotid stenosis.

Neurological symptoms (*amaurosis fugax*, TIA, minor and major stroke), vascular risk factors (hypertension, coronary artery disease, chronic obstructive pulmonary disease, dyslipidemia, diabetes mellitus, current smoking, chronic renal failure based on a glomerular filtration rate < 60 ml/min) and current therapy were all recorded in a database. Exclusion criteria were current acute illnesses, hepatic, renal, or cardiac insufficiency.

All subjects were also tested for hemato-biochemical analyses, such as white blood cell count (WBC), erythrocytes (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), the average hemoglobin content in erythrocytes (MCH), mean hemoglobin concentration in erythrocytes (MCHC), red blood cell distribution (% RDWCV), neutrophils, lymphocytes, monocytes, eosinophils, basophils, platelets, mean platelet volume (MPV), fibrinogen, urea, creatinine, estimated glomerular filtration rate (egfr), uric acid, sodium, potassium, chloride, calcium, inorganic phosphorus, magnesium, total bilirubin, direct bilirubin; indirect bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transferase (GGT), alkaline phosphatase (ALP), total amylase, creatine kinase (CK), lactate dehydrogenase (LDH), pseudocholinesterase, C-reactive protein, albumin, total proteins

(PROT), iron, glucose, cholesterol, high-density lipoprotein (HDL) cholesterol, LDL and triglycerides (TGC).

Participation in the study was on a voluntary basis, with no reward for the participants or their families. All subjects gave the appropriate informed consent for the study. All samples were collected with the approval of the local ethical committee (88/2019/Sper/AOUBo) and in collaboration with the Vascular Surgery and the Pathology Units of IRCCS, Policlinico S. Orsola Hospital, Bologna, Italy.

The study consisted of two main phases: a preliminary discovery phase and a validation phase, applying different techniques. The first phase allowed the selection of a limited number of miRs which showed significant changes of miRs levels from a few selected inpatients, comparing symptomatic and asymptomatic patients. In the second phase, the differential expression of the selected miRs was analysed through RT-qPCR in an enlarged cohort, in order to confirm the results of miRs expression profiling. The selected miRs were further assessed in Population 2 with carotid stenosis ≤ 60% at two different times (T1 and T2).

3.2 Sample processing

The study is based on two different types of samples for each patient: plasma to assess c-miRs (both Population 1 and Population 2) and carotid plaque biopsy (only Population 1) to assess the miRs present in the tissue.

Blood from inpatients was collected before surgery in vacuum tubes following standard procedures and processed within 1 hour. While blood from outpatients was collected at fasting in the morning. The tubes were centrifuged at 2500 x g for 20 minutes at 4°C, separated plasma was collected and aliquoted in cryotubes for long-term storage at -80°C.

Carotid plaques from inpatients were completely removed during surgery to preserve the plaque structure. After 24 hours of decalcification, samples were cut into serial sections and the area with the highest percentage of stenosis was identified and defined for the analysis. Biopsies were formalin-fixed paraffin-embedded (FFPE) and routinely processed. Carotid atherosclerotic lesions were defined according to the American Heart Association (AHA) classification and grouped as hemorrhagic or non-hemorrhagic plaque. A pathologist performed all histopathological analyses.

3.2.1 RNA extraction from plasma

RNA was extracted from plasma samples using the Total RNA Purification Kit (Norgen Biotek Corporation). As internal control cel-miR-39 were spiked into each sample after lysis. The protocol is reported below:

1) Spin 120 µL of plasma for 5 minutes at 14000 x g (4°C) to clean the sample from big particles, which may clog the column filer and affect the extraction.
2) Add 350 µL of Lysis Buffer (Buffer RL) solution to 100 µL of plasma and mix by vortexing for 15 seconds.
3) Add 1 µL of cel-miR-39 spike-in the solution (20 fmol/µL).
4) Add 200 µL of 100% ethanol and vortex for 10 seconds.
5) Assemble a mini filter Spin Column with a provided collection tube.
6) Transfer 600 µL of the lysate into the column and centrifuge for 1 minute at 14000 x g at room temperature.
7) Discard the collected flow-through and reassemble the column on the same collection tube.
8) Apply 400 µL of Wash Solution (Wash solution A) to the column and centrifuge for 1 minute at 14000 x g at room temperature.
9) Discard the collected flow-through and reassemble the column on its collection tube.
10) Repeat the washing step two more times for a total of three washes.
11) Spin the column for 2 minutes at 14000 x g (room temperature) in order to completely dry the resin filter.
12) Discard the collection tube and place the column onto a fresh 1.5 mL RNase-free tube.
13) Apply 50 µL of Elution Buffer solution to the column and centrifuge for 2 minutes at 200 x g at room temperature in order to elute the RNA.
14) Centrifuge again at 14000 x g at room temperature for 1 additional minute to make sure the filter is completely dry.
15) Total RNA was stored at -80°C.

3.2.2 RNA extraction from FFPE tissue

Total RNA was extracted from FFPE sections. Briefly, 4 slices of 20 µm thickness from each selected biopsy block were deparaffinized (~20 min) and digested with protease (overnight at 50 °C and 15 min at 80 °C). RNA extraction was obtained using a commercial kit (RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE, Thermo Fisher Scientific, Waltham, MA, USA) which allows isolation of RNA including miRs, following the manufacturer's

instructions. Extracted RNA was quantified using Nanodrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA).

Specifically, here is reported the protocol used:

1. Deparaffinization:
    - Assemble FFPE sections equivalent to ≤ 80 µm.
    - Add 1 mL 100% xylene, mix, and incubate for 3 min at 50°C.
    - Centrifuge for 2 min at maximum speed, and discard the xylene.
    - Wash the pellet twice with 1 mL 100% ethanol.
    - Vacuum or air dry the pellet to remove residual ethanol.
2. Protease digestion
    - Add Digestion Buffer and Protease.
    - Incubate overnight at 50°C and then 15 min at 80°C.
3. Nucleic acid isolation
    - Prepare Isolation Additive/ethanol mixture.
    - Add Isolation Additive/ethanol and mix.
    - Pass the mixture through a Filter Cartridge.
    - Wash with 700 µL of Wash 1.
    - Wash with 500 µL of Wash 2/3, and then centrifuge to remove residual fluid.
4. Nuclease digestion and final purification
    - Add DNase mix to each Filter Cartridge and incubate for 30 min.
    - Wash with 700 µL of Wash 1.
    - Wash twice with 500 µL of Wash 2/3, then centrifuge to remove residual fluid.
    - Elute with 60 µL Elution Solution or nuclease-free water at room temperature.
    - RNA was stored at -80°C.

3.3 Discovery phase

To assess global miR expression, 8 selected inpatients (4 symptomatic and 4 asymptomatic) were included in the discovery phase (miRs expression profiling). Patients were selected for advanced age and maximum degree of occlusion of both carotid arteries, main characteristics are reported in Table 1. Carotid plaque specimens and plasma samples from the same patients

were analysed using TaqMan® Array Human MicroRNA A+B Cards Set v3.0 (Applied Biosystems by Life Technologies), for a total of 16 cards.

	Code	Age	Sex	% Stenosis	%Contralateral stenosis
Symptomatic	CM1024	63	F	75%	75%
	CM1002	72	M	80%	55%
	CM1021	82	F	80%	80%
	CM1001	83	M	70%	70%
Asymptomatic	CM1004	71	F	90%	60%
	CM1023	75	M	80%	60%
	CM1003	79	F	95%	80%
	CM1009	80	M	90%	50%

Table 1. Age, gender, and stenosis characteristics of the eight patients chosen for the miRs expression profiling**.**

Both cards A and B consist of 384 wells containing miR-specific primers and probes, as shown in Figure 8. With a single reaction, it is possible to amplify up to 754 miRs. Card A mostly contains well characterized miRs frequently found in literature, while card B contains less known and recently discovered miRs. These arrays allow the investigation of miR expression patterns through the assessment of a cycling threshold (Ct) value, which indicates the number of amplification cycles required for the fluorescence signal to cross the threshold line.

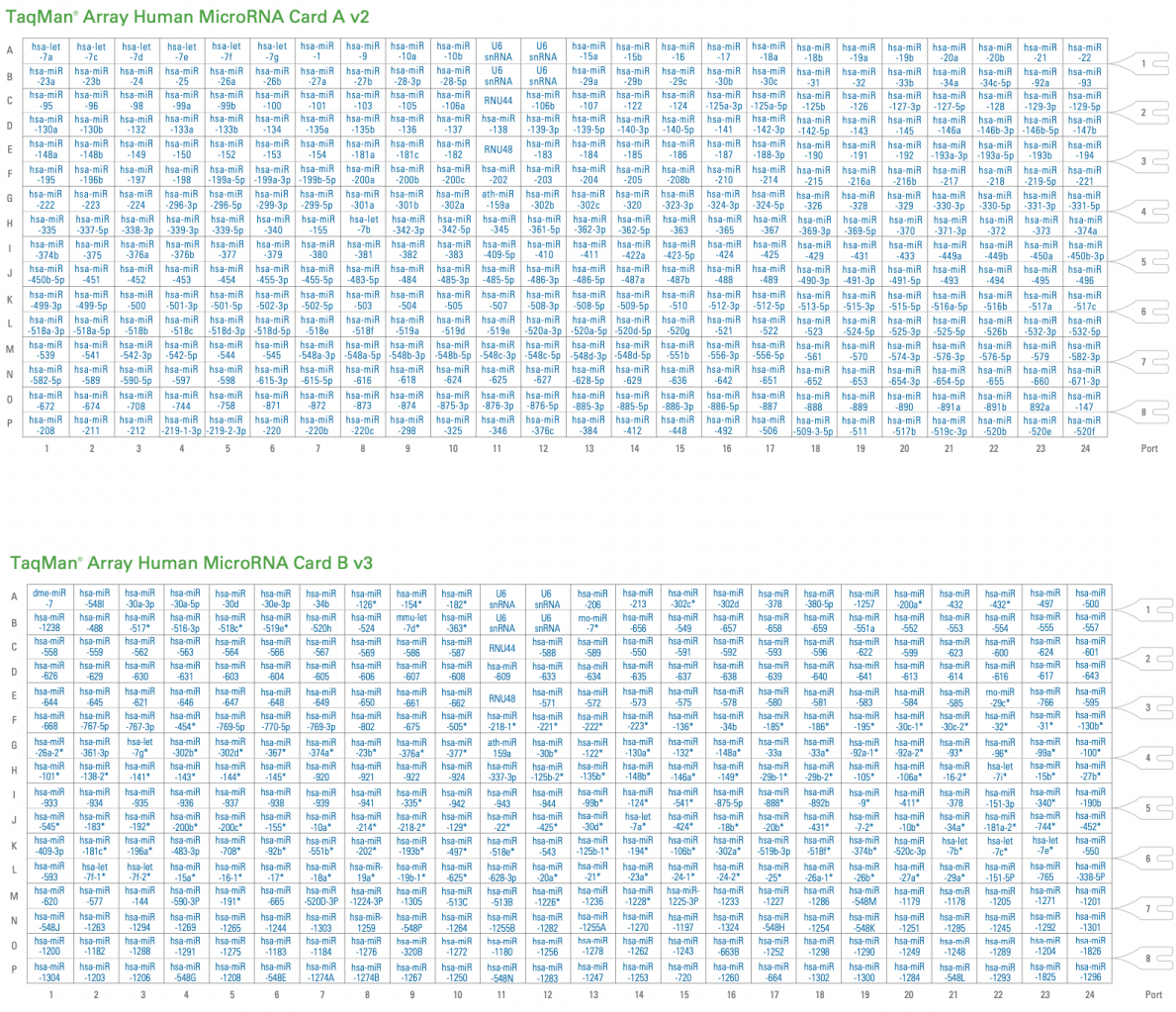

Figure 8. Schematic representation of TaqMan® Array Human MicroRNA Card A and B.

MiR profiling was performed following the Megaplex™ Pools protocol by Applied Biosystems. Firstly, cDNA was obtained through reverse transcription (RT) of total RNA (step 1); then, the amount of cDNA was considerably increased through a pre-amplification phase (step 2).

1. RT reaction:

- place 6 µL of total RNA into the PCR tubes (25 ng for the tissue)
- add 4.5 µL of RT reaction mix.

The RT reaction mix was prepared as shown in the following table:

Component	RT mix volume / 10.5 µL reaction
Megaplex RT Primers (10X)	0.80 µL
dNTPs with dTTP (100 mM)	0.20 µL
Multiscribe Reverse Transcriptase (50 U/µL)	1.50 µL
10X RT Buffer	0.80 µL
MgCl2 (25 mM)	0.90 µL
RNase Inhibitor (20 U/µL)	0.10 µL
Nuclease-free water	0.20 µL
Total	**4.50 µL**

The PCR tubes (total reaction volume = 10.5 µL) were loaded in the Thermal Cycler, setting the instrument as described in the table below:

Stage	Temperature	Time
Cycles (40 cycles)	16°C	2 min
	42°C	1 min
	50°C	1 sec
Stop reaction	85°C	5 min
Hold	4°C	∞

2. Pre-amplification reaction:

A volume of 12.5 µL of TaqMan® PreAmp Master Mix (2X) and 2.5 µL of Megaplex™ PreAmp Primers (10X) were added to the RT reaction product (10.5 µL). The PCR tube was loaded in the Thermal Cycler, which was set as described in the table below:

Step	Temperature	Time
Enzyme activation	95°C	10 min
Anneal	55°C	2 min
Extend	72°C	2 min
Denature Anneal/Extend (12 cycles)	95°C	15 sec
	60°C	4 min
Enzyme inactivation	99.9°C	10 min
Hold	4°C	∞

After the pre-amplification phase, the reaction product was stored at -20°C. Before loading the samples into the card, 24.5 µL of nuclease-free water and 50 µL of TaqMan® Universal Master Mix were added to each tube.

3. The RT-qPCR reaction was performed using 7900 HT Fast Real-Time PCR System (by Applied Biosystems) with the following cycling conditions:

- 50 °C for 2 minutes.
- 94.5 °C for 10 minutes.
- 40 cycles at 97 °C for 30 seconds.
- 59.7 °C for 1 minute.

Data acquired by the instrument were exported to Excel.
MiRs Ct values were normalized to the median Ct value of every sample (ΔCt). Subsequently, ΔΔCt mean value was calculated for every miR with the following formula:

$$\Delta\Delta Ct \text{ mean value} = \Delta Ct \text{ mean value (symptomatic)} - \Delta Ct \text{ mean value (asymptomatic)}$$

The ΔΔCt mean values associated to each miR were used to determine the fold change (FC) value, a measure of the variation in terms of miR expression level between two different conditions (i.e. symptomatic group and asymptomatic group):

$$Fold\ Change = 2^{-\Delta\Delta Ct}$$

A minimum of two-fold difference in miR expression was required to consider the variation as biologically significant: miRs with FC ≥ 2 are considered up-regulated, while miRs with FC ≤ -2 down-regulated. Since RT-qPCR measurement reliability decreases for high Ct values, only miRs with Ct < 32 in all the samples were included in the following analysis.

3.4 Validation phase

In order to validate the results obtained from miR expression profiling, RT-qPCR assay was performed on selected miRs showing a substantial variation in FC values comparing symptomatic and asymptomatic patients. For validation, miRs with significant variation (FC ≥ 2 o ≤ -2) in both tissue and plasma samples were selected from the profiling analysis assuming

a possible tissue cross-talk. These miRs were miR-126-5p, miR-134-5p, miR-145-5p, miR-151a-5p, miR-34b, miR-451a, miR-720 and miR-1271-5p. The analysis was extended to all subjects.

Reverse Transcriptase-quantitative Polymerase Chain Reaction (RT-qPCR) is the most used method to determine miRs expression levels. Five ng of carotid plaque tissue RNA was transcribed to cDNA. C-miRs have extremely low concentrations in plasma samples, thus making the quantitation process difficult to successfully perform with techniques like spectrophotometry (e.g. Nanodrop spectrophotometer) or other traditional RNA quantitation methods.

In this study, RT of total RNA was performed using TaqMan™ MicroRNA Reverse Transcription Kit, while RT-qPCR of the obtained cDNA was carried out using miR-specific TaqMan™ probes (Applied Biosystems by Life Technologies). TaqMan™ probes are 18-22 bp dual-labeled hydrolysis probes that increase the specificity of the assays TaqMan™ probes contain:

- A reporter dye (for example, FAM™ dye) linked to the 5′ end of the probe.
- A nonfluorescent quencher (NFQ) at the 3′ end of the probe.

While the probe is intact, the proximity of the quencher greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer through space (FRET). If the target sequence is present, the probe anneals between primer sites and is cleaved by the 5′ nuclease activity of the Taq DNA polymerase during primer extension. This cleavage of the probe separates the reporter dye from the quencher, increasing the reporter dye signal, and removes the probe from the target strand, allowing primer extension to continue to the end of the template strand. Therefore, inclusion of the probe does not inhibit the overall PCR process. Moreover, additional reporter dye molecules are cleaved from their respective probes with each cycle, resulting in an increase in fluorescence intensity that is proportional to the amount of amplicon produced. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.

In addition, cel-miR-39 was used as spike-in control to determine whether RNA extraction from plasma has been successful, since extracted RNA from plasma cannot be quantified. Spike-in control can be used also to normalize the results obtained from the RT-qPCR analysis. Conversely, miR expression levels analysed in carotid plaque were normalized to miR-16 expression.

RT-qPCR consists of two main steps:

1. RT reaction: protocol was optimized to convert total RNA into cDNA. The final reaction volume is 7.5 µL and each reaction tube contains:

- 2.5 µL of RNA sample.
- 1.5 µL of miR specific primer.
- 3.5 µL of RT Master Mix, prepared as follows:

Component	Master Mix Volume / 7.5 µL reaction
dNTP mix (100 mM total)	0.075 µL
Multiscribe RT enzyme (50 U/µL)	0.5 µL
10 x RT Buffer	0.75 µL
RNase Inhibitor (20 U/µL)	0.095 µL
Nuclease free water	2.08 µL

The Thermal Cycler was set with the following program:

Stage	Temperature	Time
HOLD	16°C	30 min
HOLD	42°C	30 min
HOLD	85°C	5 min
HOLD	4°C	∞

2. RT-qPCR: cDNA is amplified by miR specific probes. The final reaction volume is 15 µL and each reaction tube contains:

Component	Volume / 15 µL reaction
TaqMan® 2X FAST Universal PCR Master Mix	7.5 µL
Nuclease free water	5.75 µL
TaqMan® MicroRNA Assay (20✕)	0.75 µL
Product from RT reaction (Minimum 1:15 Dilution)	1.0 µL

All the samples were analysed in duplicate using the Rotor Gene (Qiagen), following the thermal cycling PCR parameters reported below:

Step	Enzyme Activation	PCR (40 Cycles)	
	HOLD	Denature	Anneal/Extend
Time	20 sec	1 sec	20 sec
Temperature	95°C	95°C	60°C

Data acquired by the instrument were exported to Excel and the Ct values of every miR were normalized to the corresponding Ct value (ΔCt) of normalizer (cel-miR-39 for plasma and miR-16 for tissue). Relative expression values of all miRs were determined for every subject with the following formula:

$$Relative\ expression = 2^{-\Delta Ct}$$

3.5 Statistical analyses

Statistical analyses were performed using the software SPSS v.25 and statistical significance was defined at p-value ≤ 0.05.

Mann-Whitney Nonparametric test was performed to compare relative expression of miRs between symptomatic and asymptomatic groups, in the subsequently amplified cohort. In addition, the Mann-Whitney test was performed to compare the parameters recorded in the database between symptomatic and asymptomatic patients. Finally, the Wilcoxon test for paired samples was performed to compare the two analysis times for the group of asymptomatic subjects with stenosis ≤ 60%.

Correlation analysis between validated miRs expression and hemato-biochemical parameters were investigated using Spearman test.

The model for patient classification was developed from the full dataset, with only the relative percentages of neutrophils, eosinophils and monocytes removed.

To guarantee the model robustness all the analysis were performed using a Leave One Out cross validation approach, where the model was fitted repeatedly on a subset of all the patients except for one, and the prediction was assessed on the left out one, repeating this procedure to obtain

a prediction for every subject. Missing data were estimated using an Iterative Imputation algorithm, then each feature was standardized to mean 0 and standard deviation 1.

The most important features were selected using a Sequential Feature Selection method, based on the usefulness of the features for a random forest classifier, starting with no features and adding them progressively based on the obtained improvement in a secondary Leave One Out cross validation. The actual prediction was performed training a Random Forest Classifier with 100 decision trees.

This prediction method allows to obtain both a binary prediction (symptomatic-asymptomatic) or a continuous one, with a degree of similarity to each class (going from 0, totally different, to 1, completely similar). The quality of the prediction for each set of features was estimated using the Area under the Receiving operator curve (AUC-ROC). For the outpatient subjects, they were classified using the same pipeline, trained on the whole initial dataset.

4. RESULTS

4.1 Differentially expressed miRs from the discovery phase

MiRs profiling was performed through TaqMan® Array Human MicroRNA A+B Cards Set v3.0 analysis, aiming at the identification of miRs changes in plasma and carotid plaque samples obtained from symptomatic and asymptomatic inpatients (Population 1).

The average number of detected miRs in card A was 205 miRs in plasma and 279 miRs in the tissue (mean number of miRs with Ct < 32 was 159 and 250 respectively), while card B detected an average of 124 miRs in plasma and 188 in the tissue (mean number of miRs with Ct < 32 was 71 and 147 respectively).

Differentially expressed miRs were identified according to FC values: FC ≥ 2 indicates an up-regulation of miR expression, while FC ≤ -2 identifies a down-regulation. A total of 63 and 45 miRs were found to satisfy such prerequisites in plasma and the tissue respectively, as shown in Figures 9 and 10. These same miRs are listed in Tables 2 and 3 with their exact FC values.

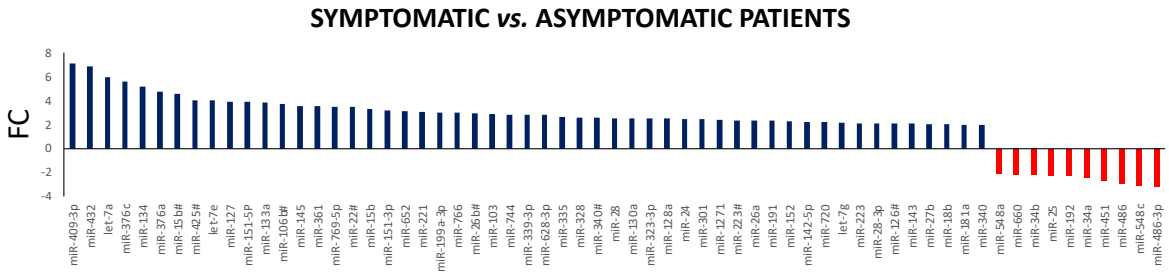

Figure 9. **Discovery phase and plasma miR profiling. Up- and down-regulated miRs (FC ≥ 2, FC ≤ -2) identified in plasma samples comparing 4 symptomatic vs. 4 asymptomatic inpatients**. MiRs showing up-regulation are displayed in blue (N = 53), while miRs with significant down-regulation are displayed in red (N = 10).

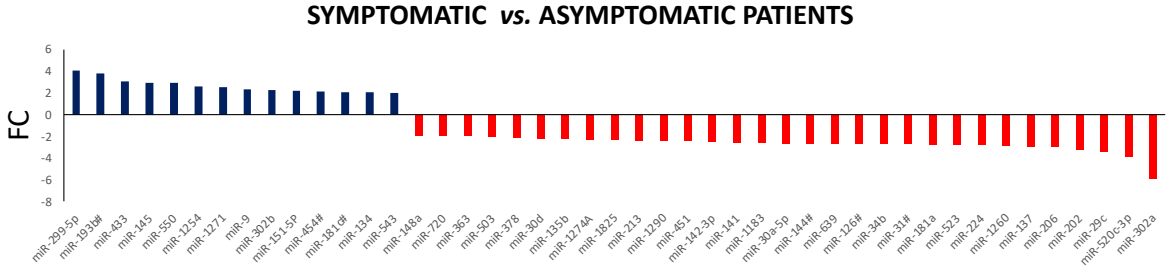

Figure 10. **Discovery phase and carotid plaque miR profiling. Up- and down-regulated miRs (FC ≥ 2, FC ≤ -2) identified in carotid plaque samples comparing 4 symptomatic vs. 4 asymptomatic inpatients.** MiRs showing up-regulation are displayed in blue (N = 14), while miRs with significant down-regulation are displayed in red (N = 31).

Table 2. Fold change values of up- and down-regulated miRs identified in plasma comparing 4 symptomatic vs. 4 asymptomatic inpatients.

	MiR	**FC**		**MiR**	**FC**
Up-regulated	miR-409-3p	7.1	Up-regulated	miR-130a	2.5
	miR-432	6.9		miR-323-3p	2.5
	let-7a	6.0		miR-128a	2.5
	miR-376c	5.6		miR-24	2.5
	miR-134-5p	5.2		miR-301	2.4
	miR-376a	4.8		miR-1271-5p	2.4
	miR-15b#	4.6		miR-26a	2.3
	let-7e	4.0		miR-191	2.3
	miR-425#	4.0		miR-223#	2.3
	miR-127	3.9		miR-152	2.3
	miR-151a-5p	3.9		miR-142-5p	2.2
	miR-133a	3.9		miR-720	2.2
	miR-106b#	3.7		let-7g	2.2
	miR-145-5p	3.5		miR-223	2.1
	miR-361	3.5		miR-28-3p	2.1
	miR-769-5p	3.5		miR-126-5p	2.1
	miR-22#	3.5		miR-143	2.1
	miR-15b	3.3		miR-27b	2.1
	miR-151-3p	3.2		miR-18b	2.1
	miR-652	3.1		miR-181a	2.0
	miR-221	3.1		miR-340	2.0
	miR-766	3.0	Down-regulated	miR-548a	-2.1
	miR-199a-3p	3.0		miR-34b	-2.2
	miR-26b#	2.9		miR-660	-2.2
	miR-103	2.9		miR-25	-2.3
	miR-744	2.8		miR-192	-2.3
	miR-339-3p	2.8		miR-34a	-2.5
	miR-628-3p	2.8		miR-451	-2.7
	miR-335	2.6		miR-486	-2.9
	miR-340#	2.6		miR-548c	-3.1
	miR-328	2.6		miR-486-3p	-3.2
	miR-28	2.5			

Table 3. Fold change values of up- and down-regulated miRs identified in carotid plaque comparing 4 symptomatic vs. 4 asymptomatic inpatients.

	MiR	**FC**		**MiR**	**FC**
Up-regulated	miR-299-5p	4.0	Down-regulated	miR-213	-2.4
	miR-193b#	3.8		miR-1290	-2.4
	miR-433	3.1		miR-451	-2.4
	miR-145-5p	2.9		miR-142-3p	-2.5
	miR-550	2.9		miR-141	-2.6
	miR-1254	2.6		miR-1183	-2.6
	miR-1271-5p	2.5		miR-30a-5p	-2.7
	miR-9	2.3		miR-144#	-2.7
	miR-302b	2.3		miR-639	-2.7
	miR-151a-5p	2.2		miR-126-5p	-2.7
	miR-454#	2.1		miR-34b	-2.7
	miR-181c#	2.1		miR-31#	-2.7
	miR-134-5p	2.0		miR-181a	-2.8
	miR-543	2.0		miR-523	-2.8
Down-regulated	miR-148a	-2.0		miR-224	-2.8
	miR-720	-2.0		miR-1260	-2.8
	miR-363	-2.0		miR-137	-2.9
	miR-503	-2.1		miR-206	-3.0
	miR-378	-2.1		miR-202	-3.3
	miR-30d	-2.3		miR-29c	-3.5
	miR-135b	-2.3		miR-520c-3p	-3.9
	miR-1274A	-2.3		miR-302a	-5.9
	miR-1825	-2.4			

4.2 MiRs selection and validation phase

Among the identified miRs whose expression was particularly altered when comparing symptomatic and asymptomatic samples (FC ≥ 2 or ≤ -2) eight miRs were found in common of both tissue and plasma as shown in Figure 11. These miRs have been considered for the subsequent validation analysis and are specifically the following: miR-126-5p, miR-134-5p, miR-145-5p, miR-151a-5p, miR-34b, miR-451a, miR-720 and miR-1271-5p (as reported in Table 4).

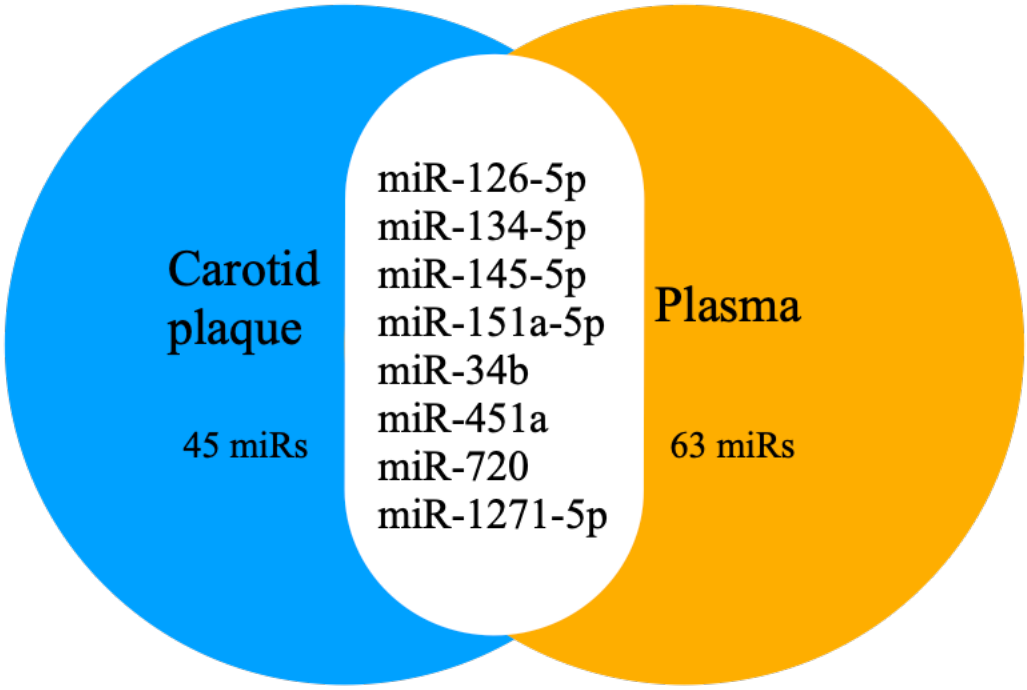

Figure 11. The eight selected miRs showing FC ≥ 2 and FC ≤ -2 in common between plasma and tissue emerged from miR profiling.

Table 4. List of the eight miRs selected for the validation phase with respective FC values in the two tissues.

MiR	**FC Carotid plaque**	**FC Plasma**
miR-126-5p	-2.7	2.1
miR-134-5p	2.0	5.2
miR-145-5p	2.9	3.5
miR-151a-5p	2.2	4.0
miR-1271-5p	2.5	2.4
miR-34b	-2.7	2.2
miR-451a	-2.4	-2.7
miR-720	-2.0	2.2

FC: Fold Change.

RNA isolation efficiency from plasma was evaluated through spike-in cel-miR-39 RT-qPCR analysis, the spike-in recovery showed non-significant variations and consistent Ct values (Ct mean value = 18.2 ± 0.37), confirming that RNA extraction was performed successfully. Cel-miR-39 Ct values were used to normalize miR Ct values in plasma samples. Extracted RNA

from tissue was quantified using Nanodrop and miR-16 Ct values were used to normalize miR Ct values in carotid plaque samples.

MiR-134-5p and miR-34b revealed Ct values ≥ 32 and amplification issues with RT-qPCR analysis, meaning that their expression levels in plasma and tissue were not sufficiently high to provide accurate results; therefore, miR-134-5p and miR-34b were excluded from further investigations.

Validation through RT-qPCR analysis was performed for the selected miRs in the total cohort of donors, i.e. 25 asymptomatic and 16 symptomatic inpatients from Population 1.

Comparing the asymptomatic with the symptomatic group, significant differences in c-miRs expression levels were reported for miR-126-5p and miR-1271-5p, being more expressed in symptomatic inpatients. Conversely miR-145-5p, miR-151a-5p, miR-451a, and miR-720 showed no significant difference in expression levels between asymptomatic and symptomatic inpatients, as presented in Figure 12.

In tissue samples of the same inpatients, single miR analyses of miR-126-5p, miR-1271-5p, miR-145-5p, miR-151a-5p, miR-451a, and miR-720 by RT-qPCR did not confirm data obtained by the miR profiling. No significant differences were observed, as presented in Figure 13.

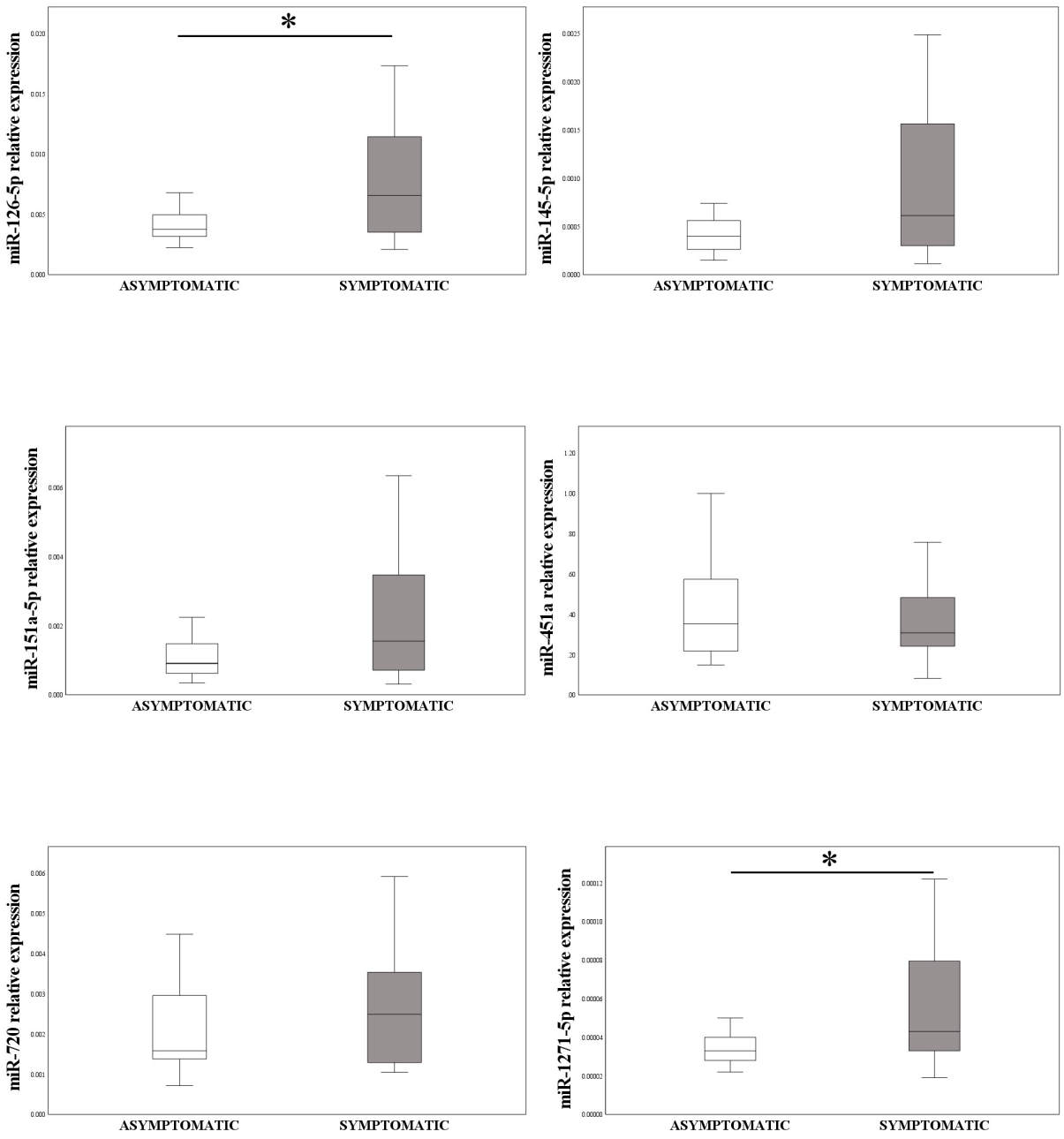

Figure 12. **C-miR expression levels comparing asymptomatic and symptomatic inpatients.** MiR relative expression was evaluated in plasma samples from asymptomatic inpatients (N = 25) and compared to symptomatic inpatients (N = 16). Data are reported as box plot of data distribution (median value and quartile ranges). Statistical analysis was performed with Mann-Whitney Nonparametric U test. * = $p \leq 0.05$

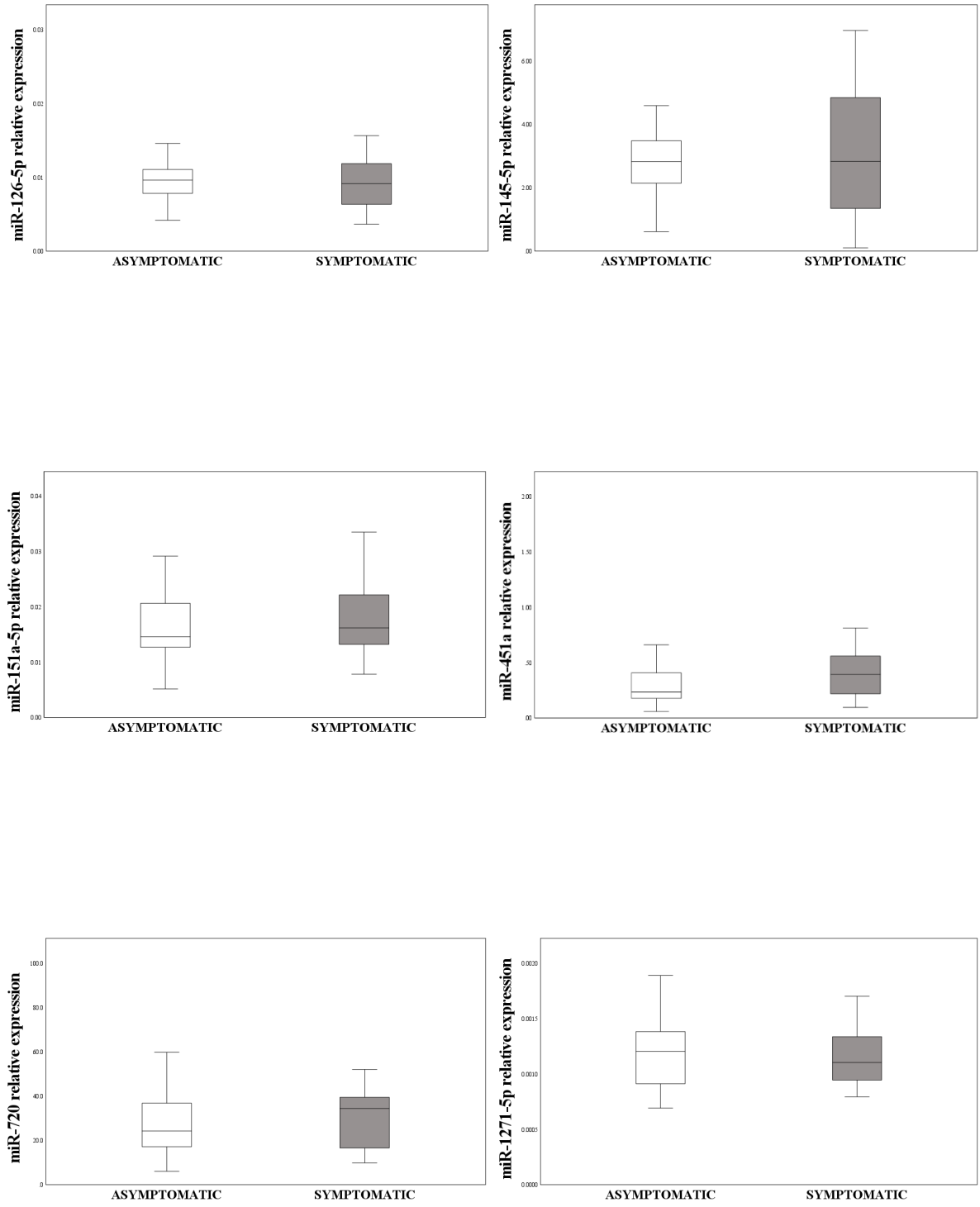

Figure 13. **Tissue miR expression levels comparing asymptomatic and symptomatic inpatients**. MiR relative expression was evaluated in carotid plaque samples from asymptomatic inpatients (N= 25) and compared to symptomatic inpatients (N = 16). Data are reported as box plot of data distribution (median value and quartile ranges). Statistical analysis was performed with Mann-Whitney Nonparametric U test. * = $p \leq 0.05$

Moreover, the differences between asymptomatic and symptomatic phenotype were also evaluated by analyzing hemato-biochemical parameters. Comparing asymptomatic and symptomatic patients, only two hemato-biochemical parameters resulted significant, i.e. monocytes and albumin $p \leq 0.05$, and reported in Figure 14.

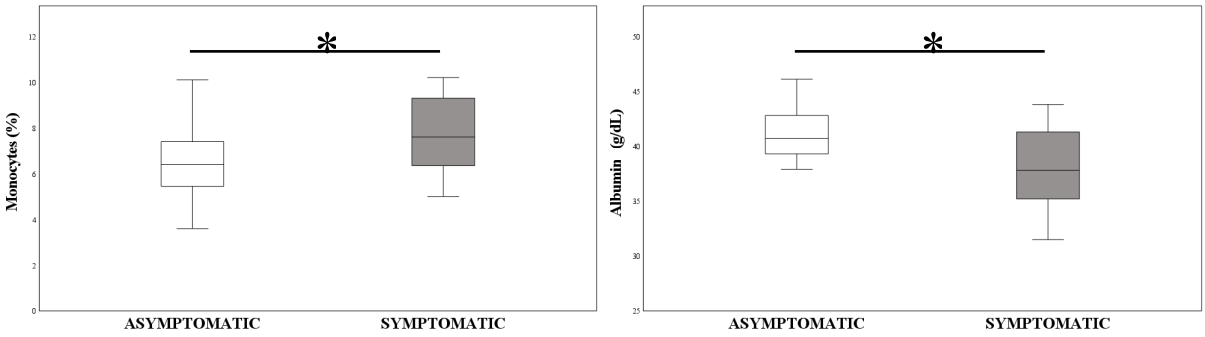

Figure 14. **Hemato-biochemical parameters significant different between asymptomatic and symptomatic inpatients.** Monocytes and albumin are the only two parameters resulted significantly different comparing asymptomatic *vs.* symptomatic inpatients. Data are reported as box plot of data distribution (median value and quartile ranges). Statistical analysis was performed with Mann-Whitney Nonparametric U test. * = $p \leq 0.05$

4.3 Correlation analysis: miRs and hemato-biochemical parameters

Data correlations were performed between significantly different miRs obtained from the validation analysis (i.e. miR-126-5p and miR-1271-5p) and hemato-biochemical parameters (WBC, RBC, HGB, HCT, MCV, MCH, MCHC, % RDWCV, neutrophils, lymphocytes, monocytes, eosinophils, basophils, platelets, MPV, fibrinogen, urea, creatinine, egfr, uric acid, sodium, potassium, chloride, calcium, inorganic phosphorus, magnesium, total bilirubin, direct bilirubin; indirect bilirubin, AST, ALT, GGT, ALP, total amylase, CK, LDH, pseudocholinesterase, C-reactive protein, albumin, PROT, iron, glucose, cholesterol, HDL cholesterol, LDL and TGC) applying Spearman Correlation tests.

Significant correlations, reported in Table 5, were found in asymptomatic group as follows: miR-126-5p positively correlates with eosinophils ($p = 0.001$) and with eosinophils % ($p = 0.001$). While miR-1271-5p positively correlates with eosinophils ($p = 0.009$), with eosinophils % ($p = 0.02$), and negatively correlates with LDH ($p = 0.021$).

Table 5. **Correlation output between miR-126-5p, miR-1271-5p and hemato-biochemical parameters.** Table shows miR-126-5p and miR-1271-5p significant correlation results occur in the asymptomatic group.

Asymptomatic			miR-126-5p	miR-1271-5p
Spearman's Rho	Eosinophils	Correlation coefficient	0.780**	0.518**
		Sig. (2-tailed)	< 0.001	0.009
		N	24	24
	Eosinophils %	Correlation coefficient	0.799**	0.473*
		Sig. (2-tailed)	< 0.001	0.02
		N	24	24
	LDH	Correlation coefficient	-0.0334	-0.512*
		Sig. (2-tailed)	0.15	0.021
		N	20	20

*. Correlation is significant at the 0.05 level (2-tailed) **. Correlation is significant at the 0.01 level (2-tailed)

Significant correlations, as shown in Table 6, were found in symptomatic group as follows: miR-126-5p positively correlates with potassium ($p = 0.008$) and negatively correlates with uric acid ($p = 0.04$). While miR-1271-5p positively correlates with C-reactive protein ($p = 0.007$), and negatively correlates with albumin ($p = 0.035$), with cholesterol HDL ($p = 0.05$) and with uric acid ($p = 0.009$).

Table 6. **Correlation output between miR-126-5p, miR -1271-5p and hemato-biochemical parameters.** Table shows miR-126-5p and miR-1271-5p significant correlation results occur in the symptomatic group.

Symptomatic			miR-126-5p	miR-1271-5p
Spearman's Rho	Albumin	Correlation coefficient	-0.479	-0.669*
		Sig. (2-tailed)	0.162	0.035
		N	10	10
	Cholesterol HDL	Correlation coefficient	-0.2	-0.661
		Sig. (2-tailed)	0.606	0.05
		N	9	9
	C-reactive protein	Correlation coefficient	0.467	0.661**
		Sig. (2-tailed)	0.079	0.007
		N	15	15
	Potassium	Correlation coefficient	0.634**	0.454
		Sig. (2-tailed)	0.008	0.077
		N	16	16
	Uric acid	Correlation coefficient	-0.626*	-0.742**
		Sig. (2-tailed)	0.04	0.009
		N	11	11

*. Correlation is significant at the 0.05 level (2-tailed) **. Correlation is significant at the 0.01 level (2-tailed)

4.4 Histopathological analysis of endarterectomy specimens

Among the histological hallmark that characterize a vulnerable plaque, the presence or absence of intraplaque hemorrhage was analyzed in our study. Following histological analysis, carotid atherosclerotic specimens were grouped as hemorrhagic plaque (N=21) or non-hemorrhagic plaque (N=20).

In particular, the hemorrhagic plaques included 10 plaques from asymptomatic inpatients and 10 plaques from symptomatic inpatients. In contrast, the group of non-hemorrhagic plaques consisted of 15 plaques from inpatients with no symptoms and only 6 plaques from inpatients with symptoms, as shown in Table 7.

Table 7. **Histopathological analysis comparing endarterectomy specimens from asymptomatic and symptomatic inpatients**. The presence of intraplaque hemorrhage was evaluated in carotid plaque samples from asymptomatic inpatients (N= 25) and compared to symptomatic inpatients (N = 16).

	Asymptomatic inpatients	Symptomatic inpatients
Hemorrhagic plaque	N=10	N=10
Non-hemorrhagic plaque	N=15	N=6

Comparing the hemorrhagic with the non-hemorrhagic group, no significant differences in tissue miRs and c-miRs expression levels were reported for miR-126-5p and miR-1271-5p (data not shown).

4.5 ROC curve analysis

The challenge of distinguishing between symptomatic and asymptomatic phenotype was also assessed considering validated miRs (miR-126-5p and miR-1271-5p) combined with the hemato-biochemical parameters significantly different comparing the two groups (i.e. albumin and monocytes) and with significant correlations (i.e. albumin, cholesterol HDL, C-reactive protein, eosinophils, LDH, potassium, uric acid). This analysis by using receiver operating characteristic (ROC) curves, showed that albumin, C-reactive protein, and monocytes jointed with identified and validated miRs are able to distinguish symptomatic and asymptomatic

inpatients (the areas under the curve of the signature were AUC = 0.795) as shown in the Figure 15.

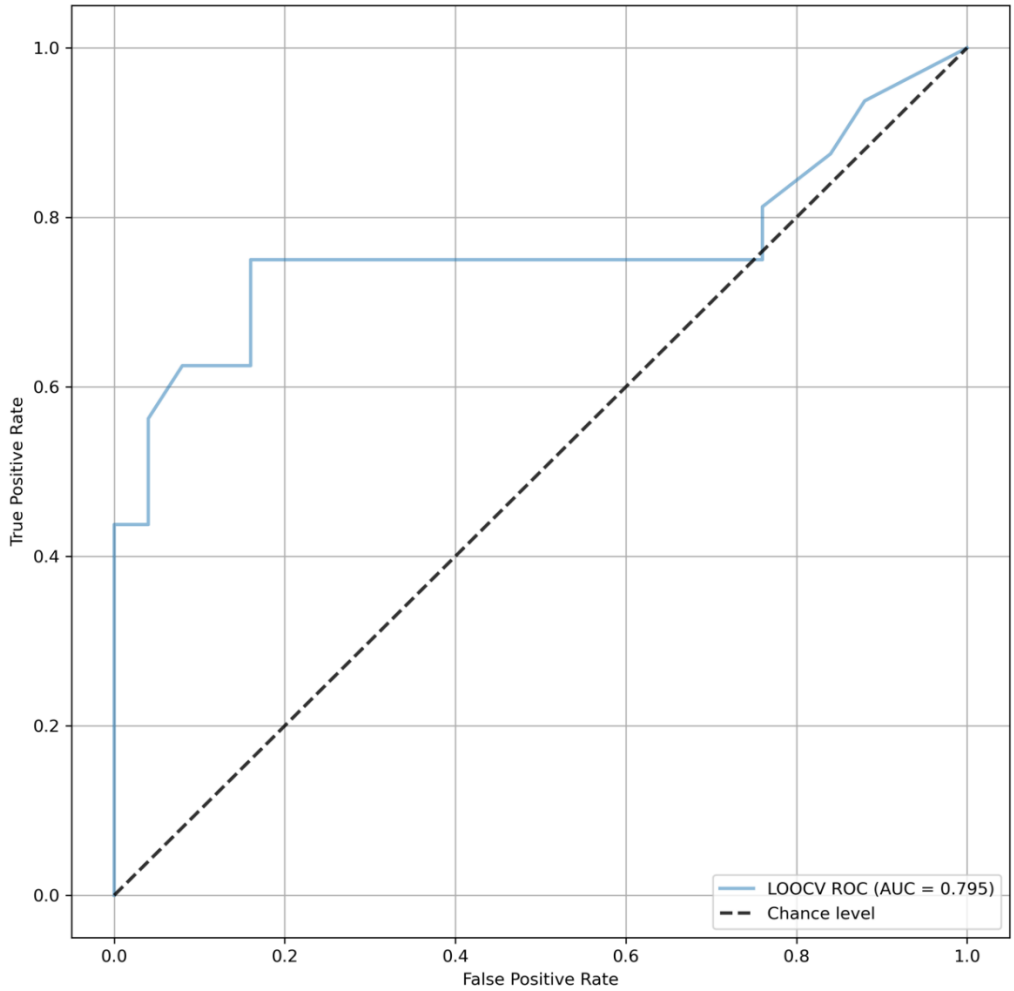

Figure 15. **Receiver operating characteristic (ROC) curves of miRs and hemato-biochemical parameters.** A signature composed by miR-126-5p, miR-1271-5p, albumin, C-reactive protein, and monocytes, is able to distinguish between asymptomatic and symptomatic inpatients with an AUC = 0.795.

4.6 MiRs analysis on outpatients' group (Population 2)

Eight outpatients with carotid stenosis ≤ 60% were recruited in order to evaluate the possible correlation of c-miRs validated in the previous phase with changes in carotid stenosis.

The molecular signature obtained in the first part of this study, and able to distinguish between the symptomatic and the asymptomatic inpatients in Population 1, were evaluated in the two times points collected from each outpatient. Thus c-miRs and hemato-biochemical parameters that compose the identified signature, i.e. miR-126-5p, miR-1271-5p, albumin, C-reactive

protein and monocytes, were analysed. No relevant clinical changes in terms of stenosis, albumin, monocyte, and C-reactive protein values were identified between the first time (T1) and the second time of analysis (T2) as reported in Table 8.

Table 8. Table reports in each outpatient subject the measurements at initial time T1 and at follow-up T2 of the degree of stenosis and of the values of the hemato-biochemical parameters that are part of the molecular signature.

	Right carotid stenosis		**Left carotid stenosis**		**Albumin (g/dL)** Reference range 35 - 50		**Monocyte (10^9/L)** Reference range 0.10 – 0.90		**C-reactive protein(mg/L)** Reference range < 5.0	
Outpatient	**T1**	**T2**	**T1**	**T2**	**T1**	**T2**	**T1**	**T2**	**T1**	**T2**
CM2001	58%	60%	40%	40%	43	44	0.43	0.49	0.5	0.7
CM2002	60%	60%	50%	50%	45	42	0.61	0.53	0.2	0.2
CM2004	0%	20%	0%	0%	45	43	0.75	0.67	3.2	3.7
CM2006	30%	30%	65%	65%	46	46	0.72	0.53	2.2	0.6
CM2008	0%	40%	55%	55%	44	43	0.44	0.36	0.8	0.3
CM2009	25%	25%	0%	25%	42	42	0.92	0.60	8.6	1.1
CM2010	30%	30%	45%	50%	40	42	0.66	0.57	1.5	1.2
CM2011	45%	45%	35%	30%	44	44	0.50	0.50	1.3	0.6

RT-qPCR analysis was performed for the two validated miRs and no significant variations of miR-126-5p and miR-1271-5p levels in plasma have been reported at the follow-up as shown in Figure 16.

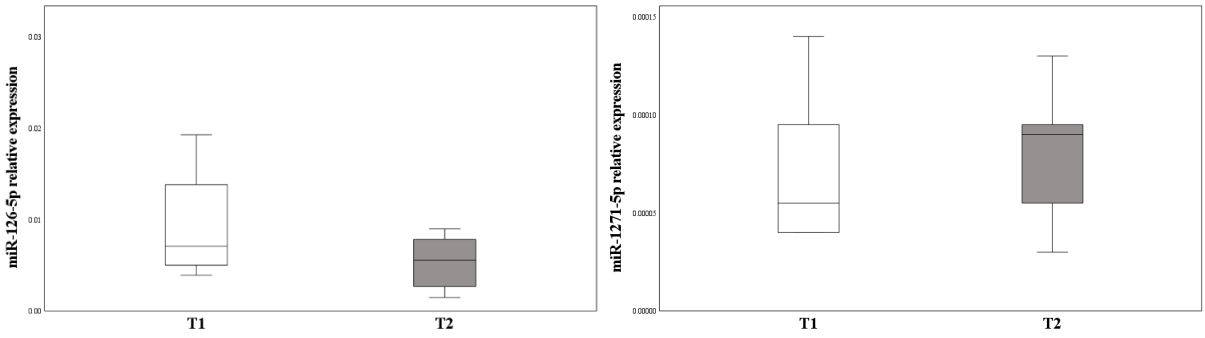

Figure 16. **C-miRs expression levels comparing T1 and T2 in outpatients' group.** MiRs relative expression was evaluated in plasma samples (N = 8).
Data are reported as box plot of data distribution (median value and quartile ranges). Statistical analysis was performed with Wilcoxon test. * = $p \leq 0.05$

The mathematical prediction method developed, allowed us to obtain a degree of similarity with each class of patients belonging to Population 1 (i.e. asymptomatic and symptomatic). In particular, outpatients with a value < 0.5 have a similarity profile to the asymptomatic group, while outpatients with a value > 0.5 have a similarity profile to the symptomatic group as reported in Table 9.

Table 9. Table shows the result of the mathematical model applied to each outpatient in terms of similarity with one of the two groups of the Population 1. Values > 0.5 indicate a similarity profile with the symptomatic group, values < 0.5 indicate a profile of resemblance to the asymptomatic group.

Samples	**T1**	**T2**
CM2001	0.61	0.34
CM2002	0.39	0.29
CM2004	0.61	0.61
CM2006	0.45	0.34
CM2008	0.43	0.28
CM2009	0.69	0.55
CM2010	0.31	0.58
CM2011	0.42	0.19

5. DISCUSSION AND CONCLUSIONS

CVDs have become one of the major social, political, and economic issues of the last few decades and a leading cause of disability and premature mortality globally (Beaglehole and Yach, 2003; Roth et al., 2017). According to the Global Burden of Diseases, Injuries, and Risk Factor Study 2015, CVDs affected an estimated 422.7 million people and caused an estimated 17.9 million deaths worldwide in 2015, comprising 31% of all global deaths (Beaglehole and Yach, 2003; Mozaffarian, 2017; Roth et al., 2017). Worldwide, Song and colleagues found that approximately 21% of people aged 30–79 years had carotid plaque and 1.5% had carotid stenosis, equivalent to approximately 816 million people with carotid plaque and 58 million with carotid stenosis (Song et al., 2020).

Previous studies have suggested that people with carotid plaque or carotid stenosis are at an increased risk of developing CVDs (Inaba et al., 2012; Johnsen et al., 2007; Touboul et al., 2012). The systematic detection of carotid plaque has been recommended in assessing cardiovascular risk (Piepoli et al., 2016).

Carotid stenosis is more common in older people than in younger people, which reinforces the hypothesis that atherosclerosis is a chronic disease process of the artery that manifests more commonly as people age (Hong, 2010; McGill et al., 2000). As shown in our previous review (Collura et al., 2020) carotid plaque lesion can be considered a tissue site-specific case of accelerated aging, i.e., a sort of chronic pro-inflammatory macro-niche. The molecular and cellular events occurring within the niche include the classical plethora of basic aging mechanisms, such as mitochondria dysfunction, defective autophagy, endoplasmic reticulum stress, defective ubiquitin/proteasome system, inflammasome activation, DNA damage response, and cell senescence with its characteristic senescence-associated secretory phenotype (Collura et al., 2020).

In this complex scenario, patients with carotid stenosis may be evaluated for several variables such as type of plaque (symptomatic *vs*. asymptomatic), and for different risk factors that may accelerate plaque instability and stroke risk.

During the last decade, many teams have been tried to identify new crucial biomarkers of plaque vulnerability in patients who are at high risk of atherothrombotic stroke and would benefit from carotid revascularization. Unfortunately, despite the extensive up-to-now search for circulating biomarkers in stroke, no single reliable individual biomarker has demonstrated enough

sensitivity and specificity to be established in the clinical practice yet. The most feasible possibility would be to find a combination of several molecules in order to develop a panel of biomarkers. Among them, lipid-related and inflammatory molecules, which are distinctive features of this etiology, may be released from the carotid plaque into the circulation as a consequence of plaque instability or rupture (Puig et al., 2020).

An extensive literature suggests a strong role of circulating blood c-miRs as potential biomarkers for carotid plaque disease. In this regard, we have recently shown that the epigenetic structure of arteries, focussed on miRs, is a key factor characterizing the various arteries, not only in the normal but also in pathological conditions (Collura et al., 2021).

MiRs are important regulators of gene expression at the post-transcriptional level, and they are clearly involved in many physiological processes (Kloosterman and Plasterk, 2006). Indeed, many studies have shown that miRs could play a role in atherosclerotic plaque formation and stability (Raju et al., 2020), in stroke (Sepramaniam et al., 2014), and can be associated with age-related diseases (Harries, 2014). From the first evidence that miRs are released in the body fluid, such as blood, urine, breast milk, saliva and so on, c-miRs were considered not only epigenetic gene regulators but also non-invasive markers, in different physiologically and pathologically conditions, including CVDs.

Recently, tissue and circulating blood miRs attracted the attention of investigators in the field, given the widespread and pervasive effects of miRs both as modulators of gene expression and biomarkers of health condition, as recently described (Dolz et al., 2017; Maitrias et al., 2017). MiR-profiling could be performed on plaque surgical material and on blood, thus reflecting on one side the local carotid-specific alterations and on the other offering a tool to monitor patients at the systemic level. The simultaneous evaluation of miRs in these two districts (plaque and blood) could give accurate information about a circulating miR-based signature and a specific plaque-phenotype. Therefore, several miRs are described to be differentially found in the plasma from symptomatic and asymptomatic patients, some of them are circulating biomarkers, others can be found both within atherosclerotic plaques and in the blood flow (Magenta et al., 2018; Wei et al., 2019; Zeng et al., 2015). In some cases, miRs levels are dependent on the carotid plaque morphology, but not on stenosis degree (Badacz et al., 2018). The challenge is to find treatments, likely in a precise medicine vision, able to slow down, at an early stage, the onset of atherothrombotic stroke and promote carotid plaque assessment, prognosis, and risk prediction. Overall, the current literature shows a complex framework where many variables,

such as the presence of different patient cohorts, morbidities/comorbidities, and other variables (drug-therapies, gender, body mass index, age and control tissues, technology miRs detection) make difficult to identify a single/clear circulating miR-signature associated with carotid plaque phenotypes (Puig et al., 2020).

The main purpose of the current thesis was to investigate the role of tissue and circulating blood miRs and their expression profile in the context of carotid disease development. To achieve this goal, a robust human model with definite assumptions was conceptualized, accordingly.
The model consists of the comparison of three different populations as follows:

1. Subjects with symptomatic carotid stenosis ≥ 70%, i.e. inpatients who have manifested signs of prior cerebrovascular events, such as TIA, ischemic stroke and *amaurosis fugax*, and urgently need of a surgical intervention at the carotid artery.
2. Subjects with asymptomatic carotid stenosis ≥ 70%, i.e. inpatients who submitted to CEA without clinical signs or symptoms.
3. Subjects with asymptomatic carotid stenosis ≤ 60%., i.e. outpatients that consented to the collection of blood samples at two different time points (T1 and T2 follow-up after 1 year).

As the first approach, the differences between the two groups of symptomatic and asymptomatic patients were considered with the aim to study the high-risk plaque profile and likely to identify a signature for the identifying subjects with a high likelihood of developing cerebrovascular events in the near future. The simultaneous evaluation of miRs in these two districts (plaque and blood) could give accurate information about a circulating miR-based signature and a specific plaque-phenotype.

MiR profiling was conducted in two different phases, i.e. discovery and validation. The former was performed on a few samples but at the highest resolution using TaqMan Array Human MicroRNA Cards A + B to identify differently expressed miRs. Plasma samples were used for the analysis of c-miRs while carotid plaque specimens were used to assess the miRs present in the tissue. MiR expression profiling was performed on 4 symptomatic *vs.* 4 asymptomatic inpatients. As the first analysis, differentially expressed miRs between symptomatic and asymptomatic inpatients were considered. Interestingly, a total of eight miRs were found in common of both tissue and plasma i.e. miR-126-5p, miR-134-5p, miR-145-5p, miR-151a-5p,

miR-34b, miR-451a, miR-720 and miR-1271-5p, and were selected through miR profiling for the subsequent RT-qPCR validation analysis.
RT-qPCR analysis was only performed on 6 out of the 8 selected miRs, since miR-134-5p and miR-34b revealed insufficient expression levels in plasma, leading to inaccurate results. Therefore, miR-134-5p and miR-34b were excluded from further investigations.

The latter phase was spent to validate data on the selected miRs through RT-qPCR analysis in the total cohort of Population 1, i.e. 25 asymptomatic and 16 symptomatic inpatients. Comparing the two groups, significant differences in c-miRs expression levels were reported for miR-126-5p and miR-1271-5p, being more expressed in symptomatic inpatients. Conversely, miR-145-5p, miR-151a-5p, miR-451a, and miR-720 showed no significant difference in expression levels between asymptomatic and symptomatic inpatients. In tissue samples of the same Population, single miR analyses of miR-126-5p, miR-1271-5p, miR-145-5p, miR-151a-5p, miR-451a, and miR-720 by RT-qPCR did not confirm data obtained by the miR profiling. No significant differences were observed.

As far as the identified miRs are concerned, a study by Mocharla et al. reported that miR-126-5p plays a crucial anti-atherogenic role by regulating the function of endothelial cells and enhancing endothelial repair (Mocharla et al., 2013). It has been established that miR-126-5p is significantly down-regulated in human atherosclerotic lesions (Schober et al., 2014). A study by Gao et al. reported that the plasma levels of miR-126 might be of great worth in predicting cerebral atherosclerosis. Meanwhile, c-miR-126-5p also is down-regulated in patients with acute coronary syndrome (Long et al., 2012; Ren et al., 2013). However, Sun et al. found that plasma was not down-regulated in patients with coronary artery disease (Sun et al., 2012). We can assume that the different plaque burden of the diseased vessels in the enrolled participants might be responsible for the discrepancy findings among studies.

MiR-1271-5p is another miR that showed significant differences in c-miRs expression levels in symptomatic compared to asymptomatic patients. This miR has been reported to be involved in numerous kinds of cancer (Zhou et al., 2021), but its role in atherosclerosis and carotid artery disease has yet to be investigated. Currently, the possible role of this c-miR in the atherosclerosis process is poorly understood and needs to be further explored.

Thus, the results obtained in the current thesis may reveal a possible involvement of miR-1271-5p in the atherosclerotic process and its potential function as a biomarker of carotid plaque progression.

The current work answered three main questions:

1. May c-miRs profile distinguish between asymptomatic and symptomatic patients?

Looking at miRs validated profiles exclusively, no clear evidence of a signature between asymptomatic and symptomatic patients was found. On the other hand, the validated c-miRs (i.e. miR-126-5p, miR-1271-5p) combined with selected hemato-biochemical parameters that emerged from the comparison between the two groups (i.e. albumin, monocytes) and correlation analysis (i.e. C-reactive protein) produced a good "signature" to distinguish the two groups of patients. In fact, a specific analysis, i.e. Random Forest Classifier with 100 decision trees, can distinguish asymptomatic and symptomatic phenotype in Population 1. One important parameter among those used is the C-reactive protein, normally found in the blood, whose levels rise in response to inflammation. C-reactive protein was already described as correlated to inflamma-miR, i.e. circulating miR-21, (Olivieri et al., 2012) and importantly is confirmed to be a good marker for cardiovascular risk. Many studies have demonstrated the predictive role of C-reactive protein for cardiovascular events such as myocardial infarction, coronary heart diseases, sudden death, peripheral artery disease, and stroke in apparently healthy subjects (Jialal and Devaraj, 2003; Pearson et al., 2003; Ridker, 2003; Ridker et al., 2000). In addition, C-reactive protein levels are particularly high in symptomatic atherosclerosis (Chaudhuri et al., 2013), and it is associated with risk of ischemic stroke (Zhou et al., 2016), poor outcome (Zheng et al., 2018) and future recurrence of stroke (Zhang et al., 2017). The parameters used to discriminate the two different groups of patients also suggest the role of immune system cells and in particular the number of monocytes that is altered in patients with atherosclerosis (Jaipersad et al., 2014). Monocytes from recently symptomatic patients have already been described as bearing signs of activation: in particular, they were found to express high concentrations of the adhesion molecules CD11b and thrombospondin 1 (Jurk et al., 2010), thus further confirming the association between circulating immune cells and advanced carotid atherosclerotic disease. Furthermore, cumulative evidence has shown the protective effects of serum albumin in carotid atherosclerosis (Ishizaka et al., 2007) and it was already described that a reduction in albumin level is negatively associated with ischemic stroke severity (Babu et al., 2013; Chakraborty et al., 2013; Gao et al., 2021).

2. May the simultaneous evaluation of tissue miRs and c-miRs in two districts (i.e. plaque tissue and blood) give results about a circulating miR-based signature and a specific plaque-phenotype?

The strategy adopted in the current thesis to validate the miRs shared between blood and plaque in the discovery phase did not reveal significant difference in the miRs expression levels between asymptomatic and symptomatic inpatients.

Actually, miR analysis of carotid plaque tissue is limited to inpatients undergoing vascular surgery, thus making the results tightly related to the evolution of the plaque and likely, making difficult to obtain homogeneous results.

In addition, intraplaque hemorrhage is one of the main factors involved in atherosclerotic plaque instability. Its recognition is crucial for the correct staging and management of patients with carotid artery plaques to limit ischemic stroke. Imaging can play a crucial role in identifying intraplaque hemorrhage, even if the great variability of intraplaque vascularization and the limitations of our current imaging technologies make it difficult. Several papers demonstrated the association between intraplaque hemorrhage and cerebral ischemic events (Gao et al., 2007; Isabel et al., 2014; Park et al., 2015; Singh et al., 2009). This trend was only partially confirmed in our study, in fact, in the group of symptomatic inpatients (N=16) only 11 plaques were classified as hemorrhagic after histological analysis. Therefore, it is critical to clarify the molecular mechanism behind the development and progression of intraplaque hemorrhage, as well as to identify possible new molecular biomarkers or new targets for pharmacological therapy to decrease plaque instability and avoid ischemia events.

Comparing the hemorrhagic with the non-hemorrhagic group, no significant differences in tissue and circulating miRs expression levels were reported for miR-126-5p and miR-1271-5p. Furthermore, it must be considered that analyses performed on c-miRs in blood do not necessarily reflect the expression levels of the same miRs within the plaque since different mechanism can influence their expression.

3. May circulating biomarkers (c-miRs signatures) be predictive of the development of carotid disease in outpatients with asymptomatic stenosis?

No relevant clinical changes in terms of stenosis, albumin, monocyte, and C-reactive protein values were identified between the first time (T1) and the second time of analysis (T2) in

outpatient subjects. Similarly, no significant changes in plasma levels of miR-126-5p and miR-1271-5p were reported at follow-up. However, this result may be affected by the low number of recruited subjects. In fact, due to the health emergency related to COVID-19, it was not possible to complete the recruitment of subjects. For this reason, it was not possible to develop a predictive model as the main proposed aim of the study. Likely, an increased number of enrolled subjects and possibly, more time points for Population 2 would have permitted to achieve the task.

Therefore, to further investigate the molecular signature identified in the previous step, an additional question is included in the study: may the molecular signature characterize the trajectories of outpatients by indicating a similarity to the symptomatic or asymptomatic phenotype?

The mathematical prediction method developed allowed us to give a level of similarity to one of the two phenotypes in Population 1 for each outpatient. The model mathematically provides a similarity value towards one group than the other. The results obtained are in most cases consistent with the fact that no case was observed to have significantly changed its clinical condition at follow-up. It is interesting to note that almost all the subjects maintain their similarity profile at both the first and second times. While it would be interesting to investigate further in more detail those subjects who changed their profile from T1 to T2 or vice versa. In this regard, pharmacological interventions and lifestyle modification may have influenced some of the results.

Our study has several limitations, among which the most prominent is the small cohort size. Due to the health emergency related to COVID-19, it was not possible to complete the recruitment of subjects. The initial idea of the study was to have a much larger number of inpatients and outpatients. This would have allowed testing the molecular signature of circulating miRs obtained from the early stages of the study in an enlarged cohort with more statistical power. Nevertheless, a common miR profile for advanced carotid atherosclerosis has been identified, which may supplement existing screening opportunities and preventive measures in the setting of cerebrovascular disease.

Potentially, future translational research comprising circulating biomarkers and carotid atherosclerosis should be focused on three main frameworks:

1. Testing the association between circulating biomarkers and clinical outcomes in big cohorts. Although little observational studies are useful to drive hypothesis, they have to be tested ultimately in big cohorts, in order to increase the sample size. Biomarkers substudies within big cohort studies or trials are warranted to increase the evidence of the predictive value of circulating biomarkers.
2. Studying the association between circulating and imaging biomarkers. There are many validated imaging biomarkers that are currently used to predict carotid plaque instability and the onset of thromboembolic sequelae. Studying the association between these validated imaging biomarkers and circulating molecules can be a useful intermediate step for selecting good candidates before testing their predictive value in big cohorts.
3. Including blood circulating biomarkers in predictive scores. In the field of stroke, several examples demonstrate that merging information from clinical, imaging, and laboratory variables increases the predictive power of some scales created to predict stroke recurrences. An ideal biomarker needs to be easily accessible, a condition that applies to c-miRs since they can be easily extracted through liquid biopsies. Moreover, many kinds of technologies for the detection of nucleic acids already exist and the development of new assays requires less time and lower costs in comparison with the production of new antibodies for protein biomarkers. We believe that this approach should be taken into account in future research as it may increase the chances of a hypothetical implementation of circulating biomarkers in clinical practice.

Data emerging from the current thesis suggest that miR-126-5p, miR-1271-5p, albumin, C-reactive protein, and monocytes may represent a circulating molecular signature for the characterization of the carotid disease phenotype. Future perspectives will involve a larger panel of miRs in an enlarged cohort of patients and their combination with other factors in a more sensitive signature will attempt to disentangle the complexity of carotid atherosclerotic development process. In this regard, system medicine combines data from different levels of analysis, such as transcriptomics, proteomics, metabolomics using ad hoc experimental design (cross-sectional and longitudinal cohorts), thus providing new multidimensional biomarkers capable of distinguishing among different pathological conditions.

In conclusion, the topic of the current thesis is inserted in the complex scenario of carotid disease markers in order to identify new anti-atherosclerosis interventions. Data obtained suggest that the use of many parameters, including miRs, permit to distinguish different phenotypes, such as symptomatic and asymptomatic inpatients, and further data could be used in a more complex analysis, to create algorithms for modeling in the context of personalized and precise medicine.

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