CIRCULATING MICRO-RNA PROFILES ACCORDING TO ATHEROSCLEROTIC DISEASE EXPRESSION. A CONTRIBUTE TO PHENOTYPE CHARACTERIZATION AND INSIGHTS INTO PATHOPHYSIOLOGY.

TIAGO LUÍS PINTO PEREIRA DA SILVA

A thesis submitted in partial fulfillment of the requirements for the Doctoral Degree in Medicine, in the specialty of Clinical Investigation

at Faculdade de Ciências Médicas | NOVA Medical School of NOVA University Lisbon

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August, 2021

To my dearest

Catarina, Teresinha, and Isabelinha

Strength does not come from physical capacity.

It comes from an indomitable will.

Mahatma Gandhi
(1849–1948)

List of publications related with the PhD thesis

To conduct this PhD research project, two systematic reviews were carried out in order to address the state-of-the-art and rationale regarding the matter under investigation:

- Pereira-da-Silva T, Ferreira V, Castelo A, Caldeira D, Napoleão P, Pinheiro T, Cruz Ferreira R, Mota Carmo M. Soluble CD40 ligand expression in stable atherosclerosis: A systematic review and meta-analysis. **Atherosclerosis** 2021;319:86-100. (Pubmed, quartile 1, impact factor 5.162)

- Pereira-da-Silva T, Coutinho Cruz M, Carrusca C, Cruz Ferreira R, Napoleão P, Mota Carmo M. Circulating microRNA profiles in different arterial territories of stable atherosclerotic disease: a systematic review. **Am J Cardiovasc Dis** 2018;8:1-13. (Pubmed, quartile 3)

Partial results of the research project were published in five additional original articles and are cited in appropriate sections of this document:

- Pereira-da-Silva T, Napoleão P, Costa MC, Gabriel AF, Selas M, Silva F, Enguita FJ, Cruz Ferreira R, Mota Carmo M. Cigarette Smoking, miR-27b Downregulation, and Peripheral Artery Disease: Insights into the Mechanisms of Smoking Toxicity. **J Clin Med** 2021;10:890. (Pubmed, quartile 1, impact factor 4.241)

- Pereira-da-Silva T, Napoleão P, Costa MC, Gabriel AF, Selas M, Silva F, Enguita FJ, Cruz Ferreira R, Mota Carmo M. Circulating miRNAs Are Associated with the Systemic Extent of Atherosclerosis: Novel Observations for miR-27b and miR-146. **Diagnostics (Basel)** 2021;11:318. (Pubmed, quartile 1, impact factor 3.706)

- Pereira-da-Silva T, Napoleão P, Pinheiro T, Selas M, Silva F, Cruz Ferreira R, Mota Carmo M. The Proinflammatory Soluble CD40 Ligand Is Associated with the

Systemic Extent of Stable Atherosclerosis. **Medicina (Kaunas)** 2021;57:39. (Pubmed, quartile 2, impact factor 2.430)

- Pereira-da-Silva T, Napoleão P, Costa MC, Gabriel AF, Selas M, Silva F, Enguita FJ, Cruz Ferreira R, Mota Carmo M. Association between miR-146a and Tumor Necrosis Factor Alpha (TNF-α) in Stable Coronary Artery Disease. **Medicina (Kaunas)** 2021;57:575. (Pubmed, quartile 2, impact factor 2.430)

- Pereira-da-Silva T, Napoleão P, Pinheiro T, Selas M, Silva F, Cruz Ferreira R, Mota Carmo M. Inflammation is associated with the presence and severity of chronic coronary syndrome through soluble CD40 ligand. **Am J Cardiovasc Dis** 2020;10:329-39. (Pubmed, quartile 3)

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Abstract

Background: Atherosclerosis involving multiple territories is frequently encountered in clinical practice and is associated with a higher morbidity and risk of mortality compared with a localized, single-territorial atherosclerosis. Multi-territorial atherosclerosis may have a different pathophysiology from that of single-territorial atherosclerosis, although little is known about the mechanisms that regulate the atherosclerosis extent to single or multiple arterial beds. In fact, the heterogeneity in the systemic extent of atherosclerosis is only partially attributable to acquired cardiovascular risk factors and genetic Mendelian inheritance, and post-transcriptional factors (microRNAs) and inflammatory mediators may contribute independently to such heterogeneity. MicroRNAs are small non-coding molecules of ribonucleic acid that regulate the gene expression at the post-transcriptional level and participate in different pathways associated with atherogenesis and atherosclerotic disease expression. Regarding the inflammatory mediators, soluble CD40 ligand (sCD40L) and tumor necrosis factor alpha (TNF-α) are two proinflammatory and proatherogenic biomarkers with distinct mechanisms of action and both are regulated *in vitro* by microRNAs. Knowledge on the signature of circulating microRNAs and inflammatory markers in single- and multi-territorial atherosclerosis may contribute to not only a better understanding of pathophysiology but also clinical care, since such mediators may potentially be used as diagnostic biomarkers and therapeutic targets.

Aims: The main objective of this research project was to assess whether the expression of circulating microRNAs is associated with the systemic extent of atherosclerosis to a single (coronary) or multiple (coronary and extra-coronary) arterial territories. In addition, we assessed: whether the expression of circulating microRNAs is associated with the severity of atherosclerosis in different arterial territories; whether the expression of circulating microRNAs is associated with the presence of cardiovascular risk factors, including cigarette smoking; whether the expression of inflammatory biomarkers, specifically sCD40L and TNF-α, is associated with the atherosclerosis extent to a single or multiple arterial territories and with the severity of atherosclerosis in different arterial territories; and whether there is an association between the expression of circulating microRNAs and inflammatory biomarkers in patients with atherosclerosis.

Methods: Participants were prospectively recruited and divided into five age- and sex-matched groups: control, with no coronary, lower extremity (LE), or carotid atherosclerosis; group 1, with isolated coronary atherosclerosis; group 2, with coronary and LE atherosclerosis; group 3, with coronary and carotid atherosclerosis; and group 4, with atherosclerosis of the coronary, LE, and carotid territories. Native obstructive atherosclerosis was the defining criterion for the presence of disease in each territory and group assignment. All the participants were screened for atherosclerotic disease in the three territories. The relative expression levels of six candidate microRNAs (miR-21, miR-27b, miR-29a, miR-126, miR-146a, and miR-218) were assessed. The selection criteria of candidate microRNAs were the distinct biological roles in atherosclerosis regulation, based on experimental studies, and their reported dysregulation in patients with single-territorial atherosclerosis. Serum levels of sCD40L and TNF-α were assessed by an enzyme-linked immunosorbent assay.

Results: A total of 94 participants were included: 26 control participants, 20 with isolated coronary atherosclerosis (group 1), 18 with coronary and LE atherosclerosis (group 2), 12 with coronary and carotid atherosclerosis (group 3), and 18 with atherosclerosis of the coronary, LE, and carotid territories (group 4). The clinical, demographic, and laboratory data and parameters of coronary atherosclerosis severity were well-balanced among groups 1 to 4. Lower expression levels of miR-27b and miR-146a were associated with the presence of multi-territorial atherosclerosis, particularly if involving the coronary, LE, and carotid territories, and with higher severity of atherosclerosis in the three territories. The coexistence of atherosclerosis in the three territories was independently associated with the miR-27b and miR-146a expression levels and both microRNAs presented an area under the receiver operating characteristics curve ≥ 0.75 to predict atherosclerosis of the three territories. Regarding the association between microRNAs expression and cardiovascular risk factors, multivariate models indicated that cigarette smoking was associated with the presence of LE atherosclerosis, cigarette smoking was associated with miR-27b downregulation, and miR-27b downregulation was associated with the presence of LE atherosclerosis. Active smokers, but not prior smokers, presented a downregulation of miR-27b. Regarding the expression of inflammatory biomarkers, higher sCD40L levels were associated with an increased systemic extent of atherosclerosis to multiple territories, specifically the coronary and LE territories, and increased severity of atherosclerosis in those territories. Prior surgical revascularization of the coronary and/or LE arteries was associated with lower sCD40L levels. Regarding TNF-α, metabolic and post-transcriptional (miR-146a) factors were

associated with TNF-α levels in patients with coronary atherosclerosis. miR-146a expression levels were negatively correlated with TNF-α levels and this association was independent of other metabolic and inflammatory parameters.

Conclusions: The expression levels of miR-27b and miR-146a were associated with the presence of multi-territorial atherosclerosis, particularly if involving the coronary, LE, and carotid territories, and with the severity of atherosclerosis in the three territories. Both microRNAs showed reasonable accuracy for predicting multi-territorial atherosclerosis involving the coronary, LE, and carotid territories. Of these microRNAs, miR-27b appeared to be a mediator of cigarette smoking-induced toxicity, specifically LE atherosclerosis. The sCD40L levels were associated with the systemic extent of atherosclerosis to multiple territories, particularly the coronary and LE territories, and atherosclerosis severity in those territories, showing also a distinct expression according to prior arterial revascularization. Finally, TNF-α levels were inversely correlated with miR-146a expression levels. These data provide a post-transcriptional (microRNA) and inflammatory signature of multi-territorial atherosclerosis and point to the relevance of such mediators in the regulation of the systemic extent of atherosclerosis. Moreover, miR-27b and miR-146a are promising noninvasive biomarkers for refining the stratification of systemic atherosclerotic burden, and likely it also applies to sCD40L. These biomarkers may therefore contribute to the tailoring of primary prevention strategies.

Keywords: atherosclerosis; inflammation; microRNA; miR-27b; miR-146a; multi-territorial disease; soluble CD40 ligand; tumor necrosis factor alpha.

Resumo

Introdução: A aterosclerose com envolvimento de múltiplos territórios é frequente na prática clínica e associa-se a maior morbilidade e risco de mortalidade em comparação com a aterosclerose localizada, com envolvimento de um único território. Embora a aterosclerose multiterritorial possa ter uma fisiopatologia diferente da aterosclerose uniterritorial, existem poucos dados sobre os mecanismos que regulam a extensão da aterosclerose a um ou mais territórios arteriais. Efetivamente, a heterogeneidade existente na extensão sistémica da aterosclerose é apenas parcialmente explicada pela presença de fatores de risco cardiovascular adquiridos e pela hereditariedade Mendeliana, podendo haver um papel independente de fatores pós-transcricionais (microRNAs) e de mediadores inflamatórios. Os microRNAs são pequenas moléculas de ácido ribonucleico que regulam a expressão genética a nível pós-transcricional e participam em diferentes vias associadas à regulação da aterogénese e expressão da doença aterosclerótica. Relativamente aos mediadores inflamatórios, o ligando solúvel do CD40 (sCD40L) e o fator de necrose tumoral alfa (TNF-α) são dois biomarcadores proinflamatórios e proaterogénicos com mecanismos de ação distintos e ambos são regulados *in vitro* por microRNAs. O conhecimento sobre perfis de microRNAs circulantes e de marcadores inflamatórios na aterosclerose uni e multiterritorial poderão contribuir para melhor entendimento da fisiopatologia e melhoria na prestação de cuidados de saúde, dado que esses biomarcadores poderão potencialmente ser utilizados com fins de diagnóstico e como alvos terapêuticos.

Objetivos: O objetivo principal deste projeto de investigação foi avaliar se a expressão de microRNAs circulantes está associada à extensão sistémica da aterosclerose a um território isolado (coronário) ou a múltiplos territórios arteriais (coronário e extracoronários). Pretendeu também avaliar-se: se a expressão de microRNAs circulantes está associada à gravidade da aterosclerose em diferentes territórios; se a expressão de microRNAs circulantes está associada à presença de fatores de risco cardiovascular, incluindo o consumo de cigarros inalados; se a expressão de biomarcadores inflamatórios, em particular o sCD40L e o TNF-α, está associada à extensão sistémica da aterosclerose a um território isolado ou a múltiplos territórios arteriais e à gravidade da aterosclerose em diferentes territórios; e se existe associação entre a expressão de microRNAs circulantes e a expressão de biomarcadores inflamatórios em doentes com aterosclerose.

Métodos: Efetuou-se um recrutamento prospetivo de participantes, que foram distribuídos por cinco grupos, emparelhados por idade e sexo: controlo, sem aterosclerose coronária, dos membros inferiores (MIs) ou carotídea; grupo 1, com aterosclerose coronária isolada; grupo 2, com aterosclerose coronária e dos MIs; grupo 3, com aterosclerose coronária e carotídea; e grupo 4, com aterosclerose coronária, dos MIs e carotídea. O critério para definição de doença em cada território e atribuição do grupo de estudo foi a presença de doença aterosclerótica obstrutiva de vaso nativo. A presença de aterosclerose foi avaliada nos três territórios em todos os participantes. Foi avaliada a expressão relativa de seis microRNAs circulantes (miR-21, miR-27b, miR-29a, miR-126, miR-146a e miR-218). Os critérios de pré-seleção destes microRNAs foram apresentar funções biológicas distintas entre si na regulação da aterosclerose, segundo estudos experimentais, e a desregulação reportada em doentes com aterosclerose uniterritorial. Os níveis séricos de sCD40L e TNF-α foram avaliados por um método de ensaio de imunoabsorção enzimática.

Resultados: Foram recrutados 94 participantes: 26 controlos, 20 com aterosclerose coronária isolada (grupo 1), 18 com aterosclerose coronária e dos MIs (grupo 2), 12 com aterosclerose coronária e carotídea (grupo 3) e 18 com aterosclerose coronária, dos MIs e carotídea (grupo 4). As características clínicas, demográficas e laboratoriais, assim como os parâmetros de gravidade da doença coronária estavam equilibrados entre os grupos 1 ao 4. A sub-regulação dos miR-27b e miR-146a associou-se a um aumento da extensão sistémica da aterosclerose a múltiplos territórios, em particular se envolvesse os territórios coronário, dos MIs e carotídeo, e a maior gravidade da aterosclerose em cada um destes territórios. A coexistência de aterosclerose nos três territórios associou-se de forma independente à expressão dos miR-27b e miR-146a e ambos apresentaram uma área sob a curva da característica de operação do recetor ≥ 0,75 para predizer aterosclerose dos três territórios. Relativamente à associação entre a expressão dos microRNAs e os fatores de risco cardiovascular, os modelos de análise multivariável revelaram que o consumo de cigarros inalados se associou à presença de aterosclerose dos MIs, o consumo de cigarros inalados se associou a sub-regulação do miR-27b e que essa sub-regulação se associou à presença de aterosclerose dos MIs. O tabagismo ativo, mas não o tabagismo prévio, associou-se a sub-regulação do miR-27b. No que diz respeito à expressão dos biomarcadores inflamatórios, níveis mais elevados de sCD40L associaram-se a um aumento da extensão sistémica da aterosclerose a múltiplos territórios, nomeadamente o coronário e dos MIs, e a maior gravidade da aterosclerose nesses territórios. A revascularização cirúrgica prévia dos territórios coronário e/ou dos MIs associou-se a

níveis mais reduzidos de sCD40L. Quanto ao TNF-α, verificou-se que fatores metabólicos e pós-transcricionais (miR-146a) se associaram à expressão do TNF-α em doentes com aterosclerose coronária e que o miR-146a se correlacionou de forma inversa com os níveis de TNF-α, sendo essa associação independente de outros parâmetros metabólicos e inflamatórios.

Conclusões: Os níveis de expressão dos miR-27b e miR-146a associaram-se à presença de doença aterosclerótica multiterritorial, em particular se envolvesse os territórios coronário, dos MIs e carotídeo, e à gravidade da aterosclerose em cada um destes territórios. Os dois microRNAs apresentaram razoável acuidade para a predição de doença aterosclerótica multiterritorial envolvendo os territórios coronário, dos MIs e carotídeo. Destes microRNAs, o miR-27b foi um provável mediador da toxicidade induzida pelo consumo de cigarros inalados, nomeadamente a aterosclerose dos MIs. O sCD40L associou-se à extensão sistémica da aterosclerose a múltiplos territórios, em particular o coronário e o dos MIs, e à gravidade da aterosclerose nestes territórios, apresentando também uma expressão distinta de acordo com a história prévia de revascularização arterial. Por fim, os níveis de TNF-α correlacionaram-se de forma inversa com os níveis de expressão do miR-146a. Estes dados indicam-nos uma assinatura pós-transcricional (microRNAs) e inflamatória da aterosclerose multiterritorial e apontam para a importância destes mediadores na regulação da extensão sistémica da aterosclerose. Por outro lado, os miR-27b e miR-146a são biomarcadores promissores para aprimorar a estratificação da carga de aterosclerose sistémica, de forma não invasiva, e provavelmente o mesmo se aplicará ao sCD40L. Desta forma, os biomarcadores referidos poderão contribuir para guiar as estratégias de prevenção primária.

Palavras-chave: aterosclerose; doença multiterritorial; fator de necrose tumoral alfa; inflamação; ligando solúvel do CD40; microRNA; miR-27b; miR-146a.

List of abbreviations

ANOVA, analysis of variance

CABG, coronary artery bypass grafting

CAD, coronary artery disease

cDNA, complementary deoxyribonucleic acid

CI, confidence interval

C_t, cycle threshold

DUS, Doppler ultrasound

ELISA, enzyme-linked immunosorbent assay

HDL, high-density lipoprotein

IL, interleukin

IMT, intima–media thickness

IQR, interquartile range

LDL, low-density lipoprotein

LE, lower extremity

QUADAS-2, Quality Assessment of Diagnostic Accuracy Studies 2

RNA, ribonucleic acid

ROC, receiver operating characteristics

sCD40L, soluble CD40 ligand

SMD, standardized mean difference

SYNTAX, SYNergy between percutaneous coronary intervention with TAXus and cardiac surgery

TNF-α, tumor necrosis factor alfa

VEGF, vascular endothelial growth factor

ΔC_t, delta cycle threshold

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1. Background

This investigation is based on a central clinical question: “Why do some patients develop a localized, single-territorial atherosclerosis, while others develop a systemic, multi-territorial atherosclerosis?”

In order to conduct a clinical research focused on the systemic extent of stable atherosclerosis to single or multiple territories based on the associated molecular signatures, it is important to address published data regarding the different presentations of atherosclerosis in clinical practice, including the systemic extent of stable atherosclerosis to multiple territories, the multifactorial regulation of atherosclerosis, and the role of post-transcriptional (microRNA), inflammatory, and vascular mediators in the regulation of atherosclerosis.

1.1. Stable atherosclerosis and its systemic extent

Atherosclerosis is highly prevalent and, despite the recent progresses in the diagnosis and treatment of atherosclerosis, it is still accountable for substantial cardiovascular morbidity and mortality worldwide [1,2]. It is a highly heterogeneous condition regarding its clinical expression and, in some patients, it may manifest as an acute event, while in others it presents as a chronic disease [2,3]. Although there is a great overlap between acute and chronic presentations of atherosclerosis, the pathophysiology of the later is more related with the initiation and expression of stable lesions rather than plaque instability and activating of the thrombotic cascade [2-4]. This research project was focused on stable atherosclerotic disease, considering the importance of understanding the pathophysiology of atherogenesis and the molecular phenotypes associated with stable atherosclerosis expression [2-4]. Specifically, the investigation was focused on stable obstructive atherosclerosis, considering its higher relevance for clinical practice compared with subclinical, non-obstructive atherosclerosis, due to the symptomatic, therapeutic, and prognostic implications [2,3].

Stable atherosclerosis may develop in different territories, including the coronary, lower extremity (LE), and carotid arteries [2,3]. Of these, coronary artery disease (CAD) is the leading cause of mortality [1]. Importantly, atherosclerosis may present as a localized, single-territorial disease, while in other patients it presents as a systemic,

multi-territorial disease [2-4]. Multi-territorial atherosclerosis warrants special attention not only because it may have a pathophysiology different from that of single vascular atherosclerosis, but also because it is frequently encountered in clinical practice and is associated with a higher morbidity and risk of mortality compared with single-territorial atherosclerosis [5,6]. Little is known on the mechanisms that regulate the atherosclerosis extent to single or multiple arterial beds [7,8]. Specifically, it is not completely understood why some patients develop isolated coronary artery disease (CAD), while others develop a more systemic disease involving coronary and extra-coronary lesions [7,8]. For this project, single-territorial atherosclerosis was based on CAD due to the wider prognostic implications of coronary atherosclerosis compared with atherosclerosis in other territories [1,2].

1.2. Multifactorial regulation of atherosclerosis

Acquired cardiovascular risk factors and genetic Mendelian inheritance contribute to the development of atherosclerosis and its severity [8,9]. These proatherogenic risk factors explain partially the heterogeneity of atherosclerosis presentation in clinical practice [8,9]. For instance, acquired cardiovascular risk factors may promote atherosclerosis preferentially in specific arterial territories, as is the case with cigarette smoking, which is a more influential risk factor for LE atherosclerosis than for atherosclerosis of other territories, including the coronary arteries [10]. Nevertheless, much of the heterogeneity of atherosclerosis expression is not attributable to acquired cardiovascular risk factors or genetic Mendelian inheritance, and the underlying pathophysiology is not fully understood [8,9]. Post-transcriptional regulators and mediators of inflammation and vascular function may contribute to such heterogeneity by mechanisms associated with, or independent of, acquired cardiovascular risk factors and genetic Mendelian inheritance [4,11]. Of note, post-transcriptional regulators, including microRNAs, and mediators of inflammation and vascular function are closely related and interact among each other, as described in experimental models [12].

Knowledge on the molecular mediators of development and progression of stable atherosclerosis may contribute to not only a better understanding of pathophysiology but also clinical care, since such mediators may potentially be used as diagnostic biomarkers and therapeutic targets [13,14].

1.3. MicroRNAs and atherosclerosis

MicroRNAs are non-coding molecules of ribonucleic acid (RNA) of about 20 nucleotides [15]. They regulate the gene expression at the post-transcriptional level by binding to the target messenger RNA, preventing its expression or promoting its elimination [15]. The biological roles of many microRNAs have been identified in experimental models, including the regulation of cardiac and non-cardiac diseases [15,16]. As described in section 1.3.1., microRNAs participate in different pathways associated with the regulation of atherogenesis, according to experimental studies. Moreover, microRNAs are present in serum (circulating microRNAs) and have high stability, which allows for their use as potential noninvasive biomarkers of cardiovascular diseases [15]. In that context, circulating microRNA profiles have been investigated in patients with stable atherosclerosis, as detailed in section 1.3.2., although data were almost entirely reported in single-territorial atherosclerosis. The potential usefulness of investigating the expression levels of circulating microRNAs in single- and multi-territorial atherosclerosis is put into perspective in section 1.3.3. Based on the reported roles of microRNAs in the regulation of atherosclerosis (section 1.3.1.) and the altered circulating microRNA profiles in patients with stable atherosclerosis (section 1.3.2.), six candidate microRNAs were selected for study in single- and multi-territorial atherosclerosis (section 1.3.4.).

1.3.1. Biological roles of microRNAs in the regulation of atherosclerosis

Different microRNAs are associated with the regulation of atherosclerotic plaque development (Figure 1). An important concept is that some microRNAs participate in multiple pathways and in different steps of a specific pathway, acting as post-transcriptional hubs [17-21]. Furthermore, each microRNA may exert atheroprotective, proatherogenic, or both atheroprotective and proatherogenic effects, which highlights the complexity of atherosclerosis regulation by microRNAs [17-21].

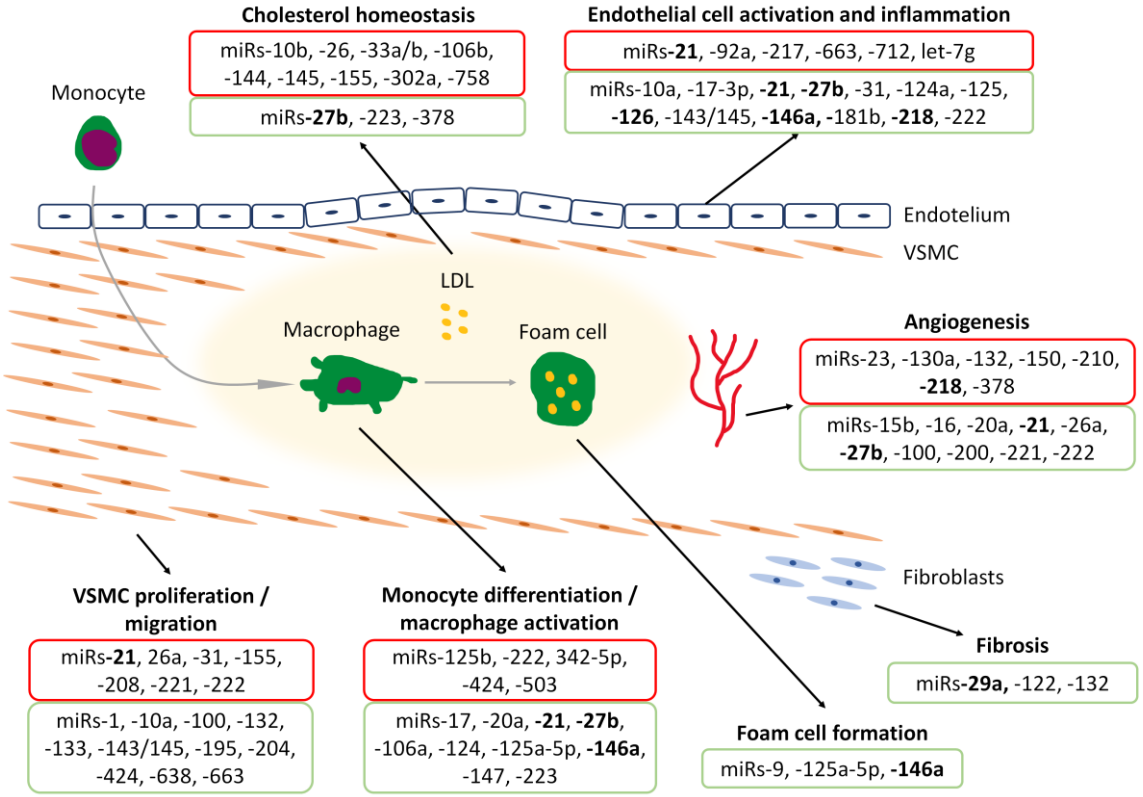


Figure 1. Biological roles of microRNAs in different steps of atherosclerotic plaque development. MicroRNAs with proatherogenic and atheroprotective effects are highlighted in red and green boxes, respectively [17-21]. VSMC, vascular smooth muscle cell.

Endothelial cell function is a main target of several microRNAs [19]. Endothelial cells play an essential role in the occurrence of vascular diseases, particularly atherosclerosis, as they regulate the synthesis and secretion of vasoactive substances, platelet aggregation, leukocyte adhesion, and thrombosis, among other functions [19]. In fact, endothelial dysfunction is considered an early marker of atherosclerosis development [19]. Different microRNAs, such as miR-21, miR-126, and miR-146a, regulate endothelial cell senescence, proliferation, and migration and influence inflammatory pathways initiated in endothelial cells [17-19]. Of note, some microRNAs, including miR-21, miR-27b, and miR-218, specifically modulate angiogenesis by targeting endothelial cells [17,18]. Monocyte–macrophage activity is also regulated by microRNAs [19]. In the early stages of atherogenesis, monocytes infiltrate into the sub-endothelium and differentiate into macrophages, which not only uptake low-density lipoprotein (LDL) cholesterol and transform into foam cells but also release a large number of inflammatory cytokines that induce acute inflammatory reaction and thereby promote atherosclerosis progression [19]. Among other microRNAs, miR-21

and miR-146a modulate monocyte–macrophage activity, including monocyte differentiation, macrophage phenotype, and macrophage-mediated inflammation, which affect the atherogenic process [18,19]. Vascular smooth muscle cell proliferation, migration, and phenotype influence the composition of the atherosclerotic plaque and are also highly regulated by microRNAs, including miR-21 [18,19]. Importantly, systemic cholesterol homeostasis and the LDL inflow into the arterial wall affect the atherosclerotic plaque development and are regulated by different microRNAs, particularly miR-27b [18]. Finally, the extracellular matrix composition of the atherosclerotic plaque, including the fibrotic tissue, is associated with the atherosclerotic plaque size and its stability; miR-29a is one of the microRNAs that modulate the fibrotic content of the atherosclerotic plaque [18]. Therefore, microRNAs participate in different processes associated with the regulation of atherosclerotic plaque development.

1.3.2. Circulating microRNAs as biomarkers of stable atherosclerosis: review of the literature

Circulating microRNAs are more easily obtainable for use in clinical practice compared with microRNAs isolated from tissues or cells [15,18], which indicates that the former are more likely to be used as biomarkers of cardiovascular diseases compared with the latter. In fact, circulating microRNA profiles have been investigated in patients with stable atherosclerosis, as discussed below.

In humans, the presence of atherosclerotic disease in different locations, including the coronary, LE, and carotid arteries, is associated with altered circulating microRNA profiles [22,23]. For CAD, a systematic review has reported a microRNA signature, for diagnostic purposes [22]. The identified microRNAs were found to regulate endothelial function and angiogenesis (miR-1 and miR-133), vascular smooth muscle cell differentiation (miR-133 and miR-145), communication between vascular smooth muscle cells and endothelial cells to stabilize plaques (miR-145), apoptosis (miR-1, miR-133, and miR-499), cardiac myocyte differentiation (miR-1, miR-133, miR-145, miR-208, and miR-499), and cardiac hypertrophy (miR-133) [22]. For other locations of atherosclerotic disease, no systematic data were available and there was a substantial heterogeneity among studies regarding the selection criteria of participants and methods for quantification of microRNAs [23]. This limited the selection of the most appropriate candidate microRNAs for research or clinical purposes [23]. In order to assess whether the expression profile of circulating microRNAs differed according to

the location of atherosclerosis, we conducted a systematic review [23]. The review protocol was registered in PROSPERO, an international database of prospectively registered systematic reviews in health and social care, under the reference number CRD42017073846. Briefly, major public databases were searched for studies comparing circulating microRNA profiles between individuals with and without native stable atherosclerotic disease of the aorta or aortic branches with large or medium size diameter, in humans, in serum. Seventeen articles were included, which were all case-control studies, including six focused on LE arteries, nine on carotid arteries, and two on renal arteries. Concomitant atherosclerotic disease in other major arterial territories was excluded systematically in only two studies. The median score of Quality Assessment of Diagnostic Accuracy Studies 2 (QUADAS-2) was 4 (interquartile range, [IQR] 3–4). There was a common microRNA expression profile in the presence of atherosclerosis, irrespective of the involved territory, including the altered expression of miR-21, miR-30, miR-126, and miR-221-3p. Unique microRNA expression profiles were also identified in the presence of atherosclerosis of specific territories, with consistent results across studies, including the dysregulation of let 7e, miR-130a, and miR-210 in LE atherosclerosis and the dysregulation of miR-29 in carotid atherosclerosis. Of note, the altered expression profile of some microRNAs remained significant after adjusting for baseline characteristics, including miR-29 in carotid atherosclerosis.

For multi-territorial atherosclerosis, one study reported a trend for lower levels of miR-18a-5p, miR-27a-3p, miR-199a-3p, miR-223-3p, and miR-652-3p in association with an increasing number of manifestations of atherosclerosis in different territories [24]. However, the expression levels of the microRNAs differed significantly only when comparing patients with and without LE atherosclerosis and, therefore, a microRNA signature was not identified in multi-territorial atherosclerosis [24]. In addition, all patients had heart failure, which may have altered the expression levels of several microRNAs [24,25], and the definition of atherosclerotic manifestations included prior acute ischemic events or revascularization procedures, without a systematic screening of atherosclerotic lesions in the corresponding arterial territory [24]. In fact, no previous studies reported data on circulating microRNAs in both single- and multi-territorial atherosclerosis with the systematic screening of atherosclerotic lesions in different territories.

1.3.3. Investigating circulating microRNA profiles in single- and multi-territorial atherosclerosis

Investigating the expression of circulating microRNAs in single- and multi-territorial atherosclerosis may contribute to a better phenotypic characterization of these two potentially distinct entities, which may be useful for understanding pathophysiology and clinical practice.

As previously mentioned, the role of some microRNAs in the development of atherosclerosis has been described in experimental models. Not only are circulating microRNAs biological active but also the circulating levels are correlated with their expression in human tissues [26,27]. Therefore, the identification of an altered circulating microRNA profile in single- and multi-territorial atherosclerosis may provide insights into the mechanisms that regulate the systemic extent of atherosclerosis to multiple territories.

One potential clinical application of circulating microRNAs in patients with suspected atherosclerosis is their use as diagnostic biomarkers [28]. In fact, screening of multi-territorial atherosclerosis in daily clinical practice by assessing simultaneously different arterial territories, using currently available methods, is potentially laborious [2,3]. Investigating a potential signature of circulating microRNAs in single- and multi-territorial atherosclerosis may contribute to identify simple noninvasive diagnostic biomarkers for the stratification of systemic atherosclerotic burden [28]. Such a simple method may facilitate an early tailoring of the atheroprotective strategies, specifically the intensification of treatment regimens in high-risk patients [29-33].

MicroRNAs have also been studied as prognostic markers in patients with atherosclerosis, being associated with the incidence of acute ischemic events [34]. Furthermore, the modulation of microRNAs expression using microRNA antagonists (antagomiRs) and agonists (microRNA mimetics) have been studied with therapeutic purposes and showed promising results, although such therapies are not yet ready for routine use in clinical practice [35]. The assessment of prognostic markers and therapeutic targets is out of the scope of this research project. However, the identification of a circulating microRNA signature in single- and multi-territorial atherosclerosis may contribute to select specific microRNAs that, according to their biological roles reported in experimental studies, may deserve further research in the prognostic and therapeutic fields in patients with atherosclerosis.

1.3.4. Six candidate microRNAs for study in single- and multi-territorial atherosclerosis

Of the diversity of microRNAs associated with the regulation of atherosclerosis, miR-21, miR-27b, miR-29a, miR-126, miR-146a, and miR-218 participate in distinct pathways and/or have distinct mechanisms of action, as described in experimental models, and were also reported to be dysregulated in patients with stable atherosclerosis [11,18,22,23,36-41]. Table 1 summarizes the main targets of the six candidate microRNAs and the territories of atherosclerosis with a reported dysregulation of these circulating microRNAs.

Table 1. Targets of candidate microRNAs and territories of atherosclerosis with a reported dysregulation of circulating microRNAs.

MicroRNAs	Targets of microRNAs	Territories of atherosclerosis with dysregulated circulating microRNAs
miR-21	Endothelial cell and vascular smooth muscle cell functions, macrophage activity	Coronary, lower extremity, carotid
miR-27b	Lipid metabolism, development of lipid-induced atherosclerotic lesions, vascular inflammation, endothelial function, angiogenesis	Lower extremity
miR-29a	Fibrosis and extracellular matrix composition	Coronary, carotid
miR-126	Endothelial function in response to shear stress	Coronary, lower extremity, carotid
miR-146a	Endothelial function in response to inflammatory cytokines, monocyte–macrophage activity	Coronary
miR-218	Endothelial cell migration, angiogenesis	Coronary, carotid

For each candidate microRNA, the main targets (specifically those associated with the regulation of atherosclerosis) are highlighted and the territories of atherosclerosis with a reported dysregulation of these circulating microRNAs are presented [11,18,22,23,36-41].

The main targets of the six candidate microRNAs, concerning atherosclerosis regulation, are herein detailed.

miR-21

Regarding endothelial function, miR-21 inhibits both endothelial cell apoptosis, through PTEN [42], and endothelial cell proliferation, through RhoB [43], and promotes inflammatory activation of endothelial cells by targeting PPARα, which induces the expression of adhesion molecules and cytokines [44]. In addition, miR-21 enhances vascular smooth muscle cell migration and proliferation by targeting TSP-1 and c-Sk [45,46], and reduces vascular inflammation by suppressing macrophage activity through MKK3 [47].

miR-27b

Xie et al. [48] showed that miR-27 reduces vascular lipid accumulation, partially mediated by the suppression of expression of scavenger receptors associated with lipid uptake in vascular macrophages. Interestingly, systemic treatment with miR-27 decreased aortic plaque size and lipid content in mice [48]. Consistently, in another study, miR-27b was identified as a crucial regulatory hub in lipid metabolism in human and mouse liver and was shown to downregulate the expression of key genes involved in lipid metabolism, including Angptl3 and Gpam, which mitigates the accumulation of lipids in circulation [49]. Regarding inflammation, miR-27b downregulates lipoprotein lipase gene expression and thereby reduces vascular inflammatory response, which limits atherogenesis [48]. Moreover, miR-27b restrains the activity of NF-κB and the production of several proinflammatory factors, including interleukin (IL)-1β and IL-6 [50], inhibits IL-17-induced monocyte chemoattractant protein-1 [51], and targets Bcl-2-associated athanogene 2 in macrophages [50]. This contributes to a decreased monocyte–macrophage activation [52], which is atheroprotective as it results in decreased vascular inflammation [4,53]. On the other hand, as miR-27b represses repulsive semaphorins, especially semaphorin 6A, it facilitates the formation of tight endothelial monolayers and stable vessels in response to shear stress [54]. In addition, miR-27b was identified as a proangiogenic microRNA [55], regulating angiogenesis through the angiogenic inhibitor semaphorin 6A and Notch ligand Dll4 [52,56].

miR-29a

miR-29a represses transcripts of several components of the extracellular matrix. Of note, miR-29a downregulates the expression of ELN, COL1A1, and COL3A1, resulting in a reduction of the elastin and collagen content in the atherosclerotic plaque [57,58].

In fact, antagonizing the antifibrotic effect of miR-29a leads to a reduced size of atherosclerotic lesions, enhanced fibrous cap thickness, and reduced necrotic zones [57].

miR-126

miR-126 promotes endothelial cell proliferation by suppressing Dlk1 and thus confers a proliferative reserve that compensates for the antiproliferative effects of hyperlipidemia, thereby limiting atherosclerosis [59]. Interestingly, miR-126 is a mechanosensitive microRNA that is downregulated through the transcription factor klf2a in response to disturbed flow and shear stress [60].

miR-146a

miR-146a is induced in endothelial cells in response to proinflammatory cytokines and acts as a negative feedback regulator of inflammatory signaling in endothelial cells by dampening the activation of proinflammatory transcriptional programs, including the NF-κB, AP-1, and MAPK/EGR pathways, and by promoting eNOS expression [11,18,61]. Moreover, miR-146a was reported to decrease endothelial inflammation by inhibiting NAPDH Oxidase 4 expression in a diabetic atherothrombosis model [62]. On the other hand, the enhancement of miR-146a levels in monocytes and macrophages by cellular apoE was shown to suppress the NF-κB pathway and thus reduce the macrophage activity [63]. Moreover, miR-146a targets the Toll-like receptor 4, reducing the formation of foam cells [64]. Therefore, miR-146a contributes to reduce vascular inflammation and atherosclerosis by targeting endothelial cells and the monocyte–macrophage lineage.

miR-218

miR-218 regulates Slit/Robo signaling through the repression of Robo1, Robo2, and glucuronyl C5-epimerase and thereby regulates endothelial cell migration and vascular patterning during angiogenesis [65,66].

Therefore, the six candidate microRNAs have specific roles in the regulation of atherosclerosis and their simultaneous assessment in single- and multi-territorial atherosclerosis takes into account different, complementary biological pathways associated with atherogenesis.

1.4. Inflammation and atherosclerosis

Atherosclerosis is known to be highly regulated by inflammation [4]. In fact, atherosclerosis is characterized by the activation of proinflammatory pathways [53,67,68]. Of the large diversity of inflammatory mediators associated with atherosclerosis development, soluble CD40 ligand (sCD40L) and tumor necrosis factor alpha (TNF-α) are two proinflammatory mediators with distinct mechanisms of action [53,67,68], which have been studied in patients with atherosclerosis by the team that collaborated with this research project [69-72]. Importantly, prior studies have reported that sCD40L and TNF-α activity is regulated by microRNAs, including the candidate microRNAs selected for this research project [12,73,74]. This highlights the interactions among atherosclerosis, post-transcriptional (microRNA) regulators, and inflammation [53,67,68,73,74].

1.4.1. Soluble CD40 ligand

Activated platelets are the main source of sCD40L [75,76]. After cleavage of the transmembrane glycoprotein CD40 ligand, sCD40L is released into circulation and interacts with the CD40 receptor expressed in endothelial cells, macrophages, and T-cells, among others [75,76]. As a result, sCD40L promotes multiple vascular inflammatory responses, including the activation of endothelial cells, release of inflammatory cytokines, activation of matrix degrading enzymes, and production of tissue factor [75,76]. Furthermore, this research team has previously observed that sCD40L expression is associated with vascular function [70,71]. Altogether, the role of sCD40L in vascular inflammation and function supports sCD40L as a mediator of atherosclerosis expression [70,71,75,76]. Of note, sCD40L expression was downregulated by miR-146a in experimental models [73].

Soluble CD40 ligand in patients with atherosclerosis

Despite the aforementioned data supporting sCD40L as mediator of atherosclerosis expression, and the increased sCD40L levels reported in acute coronary syndromes, less is known about sCD40L expression in patients with stable atherosclerosis [77,78]. In fact, the role of sCD40L and its dysregulation in the chronic setting has recently been questioned [77,78]. In that regard, we assessed whether sCD40L is dysregulated in stable atherosclerosis, irrespective of the diseased arterial territory, and whether this dysregulation differed according to the specific territory through a systematic review and meta-analysis [79]. The review protocol was registered in PROSPERO

under the reference number CRD42020181392. Briefly, major public databases were searched for studies reporting circulating sCD40L levels in individuals with and without atherosclerosis, in one or more specific arterial territories, in humans [79]. Fifty-four studies (59 estimates) including 7,705 patients and 7,841 controls were analyzed and included in the quantitative synthesis [79]. The median QUADAS-2 score was 5 (IQR, 4–6) [79]. sCD40L levels were found to be increased in patients with atherosclerosis, irrespective of the territory (standardized mean difference [SMD] 0.43, 95% confidence interval [CI] 0.29–0.57; 59 estimates; χ^2 heterogeneity $p < 0.001$; $I^2 = 92\%$) [79]. The SMD was greatest in carotid atherosclerosis (SMD 0.58, 95% CI 0.30–0.86; 17 estimates), followed by coronary (SMD 0.43, 95% CI 0.24–0.62; 33 estimates), LE (SMD 0.26, 95% CI -0.02–0.54; 7 estimates), and renal atherosclerosis (SMD -0.07, 95% CI -2.77–2.64; 2 estimates) (χ^2 heterogeneity $p < 0.001$; $I^2 \geq 80\%$ for all) [79]. Interestingly, the subgroup analysis revealed that sCD40L levels were increased in clinical, but not subclinical, atherosclerosis [79].

Regarding sCD40L expression in multi-territorial atherosclerosis, data are scarce. Two studies reporting on the sCD40L levels in patients with stable atherosclerosis included a subgroup with multi-territorial atherosclerosis but the sCD40L levels were not specifically reported in patients with single- and multi-territorial disease [80,81]. In a study on patients with LE atherosclerosis, the sCD40L levels were specifically reported in a subgroup with coexistent CAD; however, not all patients with LE atherosclerosis were screened for CAD in this retrospective study, which limits the interpretation of the results [82]. To the best of our knowledge, no studies reported sCD40L levels in both single- and multi-territorial atherosclerosis with the systematic assessment of different territories.

1.4.2. Tumor necrosis factor alpha

TNF-α is mainly produced by activated macrophages, but several other cells synthesize it as well, such as T lymphocytes and natural killer cells [83,84]. TNF-α is involved in practically every step of inflammation [83-85]. Specifically, in the vessel, TNF-α alters endothelial cell and vascular smooth muscle cell function as well as endothelial cell–blood cell interaction, contributing to vascular dysfunction and promoting atherogenesis [83-85]. Considering the independent role of TNF-α in the development and progression of atherosclerosis, prior studies have investigated this proinflammatory mediator as a therapeutic target in patients with atherosclerosis [86-88].

The mechanisms that regulate TNF-α expression in patients with stable atherosclerosis are not yet fully understood [89]. TNF-α levels increase in response to metabolic dysregulation, including hyperglycemia, dyslipidemia (particularly hypertriglyceridemia), and adiposity [90-95]. Such metabolic abnormalities interact among each other and often coexist in patients with atherosclerosis, and the independent role of each on TNF-α expression is not entirely known [89,96]. On the other hand, post-transcriptional modulators, including microRNAs, may influence TNF-α expression *in vitro* [12]. Specifically, miR-146a downregulated TNF-α expression in experimental studies [12,74]. Whether an interaction between circulating microRNAs and TNF-α expression exists in patients with stable atherosclerosis and whether such potential association is influenced by metabolic abnormalities or coexistent inflammatory dysregulation is unknown.

1.5. Vascular function and atherosclerosis

Vascular endothelial growth factor (VEGF) modulates vascular development [97]. It stimulates the production of nitric oxide and prostacyclin by endothelial cells, increases vascular permeability, stimulates growth, and prevents apoptosis of endothelial cells [97]. In patients with atherosclerosis, which is characterized by endothelial damage, VEGF mediates re-growth in order to repair or replace injured cells [97]. In fact, the team that collaborated with this research project has shown that lower VEGF levels are associated with a higher risk of major adverse cardiac events in patients with CAD [98]. Increased VEGF levels have been observed in patients with atherosclerosis of the coronary and LE arterial territories, probably due to its vascular protective effects in a context of endothelial damage [97,99]. Considering that the degree of endothelial damage may differ in single- and multi-territorial atherosclerosis, the study of VEGF levels in multi-territorial atherosclerosis is potentially relevant [97,99]. Of note, VEGF signaling is affected by post-transcriptional factors; specifically, it is enhanced by miR-126 [100]. The simultaneous assessment of circulating microRNA profiles and VEGF signaling may provide insights into the endothelial regulators associated with single- and multi-territorial atherosclerosis.

1.6. What needs to be explored

Published data are scarce regarding models of clinical investigation of single- and multi-territorial atherosclerosis. In fact, most studies assessing the expression of biomarkers in multi-territorial atherosclerosis were limited by: their retrospective nature; the lack of systematic assessment of atherosclerosis in different territories, including major territories, such as the coronary, LE, and carotid arteries; and the definition of atherosclerosis, which was heterogeneous and in some studies was based on obstructive and non-obstructive atherosclerosis, and on prior revascularization procedures irrespective of the presence of obstructive lesions [24,80-82]. A clinical model of single- and multi-territorial atherosclerosis that may overcome these limitations is valuable.

The expression profile of circulating microRNAs is not known in single- and multi-territorial atherosclerosis and data are scarce regarding the association between the expression levels of circulating microRNAs and severity of atherosclerosis in each territory. It is not entirely clear if the expression of circulating microRNAs is associated with the presence of cardiovascular risk factors and with levels of inflammatory and vascular biomarkers in patients with atherosclerosis. Moreover, data on the expression of inflammatory and vascular biomarkers in single- and multi-territorial atherosclerosis are scarce.

2. Objectives

2.1. Principal objective

- To assess whether the expression of circulating microRNAs is associated with the systemic extent of stable atherosclerosis to a single or multiple arterial territories.

2.2. Secondary objectives

- To assess whether the expression of circulating microRNAs is associated with the severity of stable atherosclerosis in different arterial territories.
- To assess whether the expression of circulating microRNAs is associated with the presence of cardiovascular risk factors, including cigarette smoking.
- To assess whether the expression of inflammatory (sCD40L and TNF-α) and vascular (VEGF) biomarkers is associated with the systemic extent of stable atherosclerosis to a single or multiple arterial territories.
- To assess whether the expression of inflammatory (sCD40L and TNF-α) and vascular (VEGF) biomarkers is associated with the severity of stable atherosclerosis in different arterial territories.
- To assess whether the expression of circulating microRNAs is associated with the expression of inflammatory (sCD40L and TNF-α) and vascular (VEGF) biomarkers.

3. Hypotheses

We hypothesized that:

- The expression levels of atheroprotective and/or proatherogenic circulating microRNAs are associated with the extent of stable atherosclerosis to a single or multiple arterial territories.
- The expression levels of atheroprotective and/or proatherogenic circulating microRNAs are associated with the severity of stable atherosclerosis in different arterial territories.
- Circulating microRNAs mediate the proatherogenic effect of some cardiovascular risk factors.
- The expression of sCD40L, TNF-α, and VEGF is associated with the extent of stable atherosclerosis to a single or multiple arterial territories.
- The expression of sCD40L, TNF-α, and VEGF is associated with the severity of stable atherosclerosis in different arterial territories.
- The expression of circulating microRNAs is associated with levels of inflammatory and vascular biomarkers.

4. Methods

4.1. Ethical and legal issues

The study protocol was approved by the ethics committees of the involved institutions (Faculdade de Ciências Médicas | NOVA Medical School of NOVA University Lisbon, nº24/2015/CEFCM, in 2015, and Centro Hospitalar Universitário de Lisboa Central, nº 245/2015, in 2015). The project was conducted at Centro Hospitalar Universitário de Lisboa Central, according to the institutional research policy (INV. Política de Investigação) and guideline for research of investigator initiative (INV. 101 Realização no CHULC de estudos clínicos da iniciativa do investigador). The investigation conformed to the principles outlined in the Helsinki Declaration. All the participants signed informed consent forms.

After signing the informed consent, participants were recruited and a random numerical code was generated for each participant. Such numerical code was used in a digital anonymized database and in the biological samples. Only the principal investigator had access to the identification of the participants. All data remained anonymized until the end of the study.

All data and material related to participants, including the digital database, the informed consent forms, and the biological samples obtained during the study were stored in a secure site at the Clinical Research Office of the Department of Cardiology, Hospital de Santa Marta, Centro Hospitalar Universitário de Lisboa Central. Upon the end of the study, the digital database and all biological samples were discarded and potential identifiers were removed, according to the study protocol.

4.2. Settings

The study was conducted at the Department of Cardiology (Hospital de Santa Marta, Centro Hospitalar Universitário de Lisboa Central) in collaboration with the Department of Cardiothoracic Surgery & Transplantation and Department of Angiology and Vascular Surgery of the same center, Instituto de Medicina Molecular João Lobo Antunes (Universidade de Lisboa), and Instituto Superior Técnico. The Department of Cardiology is part of the multidisciplinary Cardiovascular & Lung Department of Hospital de Santa Marta, Centro Hospitalar Universitário de Lisboa

Central. This tertiary care center provided the required human and technical resources for conducting the study, including Cardiology, Cardiac Surgery, and Vascular Surgery experts and cardiovascular imaging techniques, including coronary computed tomography angiography, invasive coronary angiography, and Doppler ultrasound (DUS) studies of peripheral arteries.

4.3. Study design

This was a prospective, observational analytic study. A convenience sample was recruited including participants without documented atherosclerosis and with atherosclerosis of a single (coronary) or multiple (coronary plus LE and/or carotid) arterial territories.

4.4. Recruitment of participants

For studying atherosclerosis of a single (coronary) and multiple (coronary and extra-coronary) territories, a clinical model including five groups of age- and sex-matched participants was used: control, with no coronary, LE, or carotid atherosclerosis; group 1, with isolated coronary atherosclerosis; group 2, with coronary and LE atherosclerosis; group 3, with coronary and carotid atherosclerosis; and group 4, with coronary, LE, and carotid atherosclerosis.

The participants were prospectively recruited from the Department of Cardiology, Hospital de Santa Marta, Centro Hospitalar Universitário de Lisboa Central. Controls were recruited from the Outpatient Clinics, of those referred for coronary computed tomography angiography, and other participants (groups 1 to 4) were recruited from those admitted to the Cardiology Ward to perform elective invasive coronary angiography, as scheduled by the attending physician. The recruitment was led by the principal investigator, twice a week (on predefined weekdays), and all potential candidates were invited to participate in the study.

4.4.1. Inclusion and exclusion criteria

Controls were selected if they presented: 1) no effort angina, no evidence of coronary atherosclerosis on coronary computed tomography angiography, including a calcium score of 0 and no soft plaques, and no positive myocardial stress test (the latter was

not mandated to be assessed as per protocol); and 2) no atherosclerotic disease of the LE or carotid arteries. Patients (groups 1 to 4) were selected if they presented obstructive coronary atherosclerosis, with or without obstructive atherosclerosis of the LE and carotid arterial territories, and were assigned to the corresponding study group according to the involved territories. All the participants were screened for obstructive atherosclerotic disease in the three territories. Obstructive CAD was defined as luminal stenosis of at least 50% for the left main artery or at least 70% for other epicardial vessels on invasive coronary angiography [2,101,102]. Obstructive LE atherosclerosis was defined as the combination of chronic claudication and an ankle–brachial index equal to or less than 0.9 [3,103] or a significant (≥ 50%) stenosis on DUS at rest [3,103]. DUS was performed for the characterization of LE arterial disease in all patients with LE atherosclerosis, irrespective of the diagnostic method. Obstructive carotid artery disease was defined as a stenosis of at least 50% on DUS [3,103]. The inclusion criteria for each study group and diagnostic methods are summarized in Table 2.

Table 2. Inclusion criteria and diagnostic methods.

Study group	Territories of atherosclerosis	Inclusion criteria
Control	No atherosclerosis 	- Absence of angina, no coronary atherosclerosis on CCTA (including a calcium score=0 and no soft plaques), and no positive myocardial stress test [a] - No claudication symptoms and ABI >0.9 [b]; or no LE atherosclerosis on DUS - No carotid atherosclerosis on DUS
Group 1	Isolated coronary atherosclerosis 	- Obstructive coronary atherosclerosis (≥50% for the left main artery or ≥70% for other epicardial vessels) on ICA - No claudication symptoms and ABI >0.9 [b]; or no obstructive LE atherosclerosis (≥50% stenosis) on DUS - No obstructive carotid atherosclerosis (≥50% stenosis) on DUS
Group 2	Coronary and LE atherosclerosis 	- Obstructive coronary atherosclerosis (≥50% for the left main artery or ≥70% for other epicardial vessels) on ICA - Claudication symptoms and ABI ≤0.9 [c]; or obstructive LE atherosclerosis (≥50% stenosis) on DUS - No obstructive carotid atherosclerosis (≥50% stenosis) on DUS
Group 3	Coronary and carotid atherosclerosis 	- Obstructive coronary atherosclerosis (≥50% for the left main artery or ≥70% for other epicardial vessels) on ICA - No claudication symptoms and ABI >0.9 [b]; or no obstructive LE atherosclerosis (≥50% stenosis) on DUS - Obstructive carotid atherosclerosis (≥50% stenosis) on DUS
Group 4	Coronary, LE, and carotid atherosclerosis 	- Obstructive coronary atherosclerosis (≥50% for the left main artery or ≥70% for other epicardial vessels) on ICA - Claudication symptoms and ABI ≤0.9 [c]; or obstructive LE atherosclerosis (≥50% stenosis) on DUS - Obstructive carotid atherosclerosis (≥50% stenosis) on DUS

ABI, ankle–brachial index; CCTA, coronary computed tomography angiography; DUS, Doppler ultrasound; ICA, invasive coronary angiography; LE, lower extremity. Red dots represent the involved territory of atherosclerosis. [a] Not mandated to be assessed as per protocol; [b] first-line method for excluding LE atherosclerosis (in cases of doubt, a DUS study was performed to ascertain the diagnosis); [c] in such cases, DUS was also performed for the characterization of LE atherosclerosis.

The exclusion criteria were as follows: patients with acute ischemic events within 12 months, either coronary, LE, or cerebrovascular events; those with coronary artery bypass grafting (CABG) or LE bypass surgery performed within 12 months; those with prior carotid endarterectomy or prior percutaneous intervention of the coronary, LE, or carotid arteries; those with critical limb ischemia (with ischemic rest pain), heart failure, hemodynamically significant valvular heart disease, hematological disorders, active infection, history of malignancy, chronic kidney disease (stage 4 or 5), or severe hepatic dysfunction; those under 18 years of age; or those unable or unwilling to consent to study participation. If performed at least 12 months before inclusion, prior CABG and/or LE bypass surgery were not exclusion criteria since the presence and extent of atherosclerotic lesions, which were the focus of this study, are not modified by the surgical placement of bypass conduits [3,104].

4.4.2. Recruitment steps

The recruitment of candidates included two steps. Firstly, potential candidates signed the informed consent form; their clinical and demographic data were recorded; the methods used in the screening protocol for eligibility and group assignment (*vide* section 4.4.3.) were scheduled, to be completed within one month; and collection and storage of peripheral venous blood was performed within 24 hours (*vide* section 4.6.1.), for later quantification of microRNAs and inflammatory and vascular biomarkers. Secondly, a clinic visit was performed to evaluate and record the results of the screening protocol for eligibility and group assignment, and for clinical evaluation in case of detection of undiagnosed atherosclerosis (*vide* section 4.4.4.).

4.4.3. Screening protocol for eligibility and group assignment

The screening protocol for eligibility and group assignment included the coronary computed tomography angiography in control participants and the invasive coronary angiography in other participants (groups 1 to 4). In addition, the protocol included, in all participants: 1) the assessment of chronic claudication symptoms plus the ankle–brachial index, as a first-line screening of LE arterial disease [3,81,105-107], or a DUS study of the LE in cases of doubt (such as the discordance between claudication symptoms and the ankle–brachial index), for diagnostic ascertainment [3]; 2) a DUS study of the carotid arteries; 3) routine laboratory tests; 4) a 12-lead electrocardiogram; and 5) a transthoracic echocardiogram. As aforementioned, a DUS study was performed in every participant with detected LE atherosclerosis, including cases

diagnosed by claudication symptoms and the ankle–brachial index, in order to characterize LE atherosclerosis [3].

4.4.4. Study monitoring

No adverse events were expected as a result of participation in this observational study. However, undiagnosed atherosclerosis was expected to be detected in some participants as a result of study participation. In cases where obstructive atherosclerosis of the coronary, LE, or carotid arteries were diagnosed a consequence of study participation, antiplatelet and statin therapy was started if appropriate [2,3,108]. Those participants were referred for cardiac consultation in cases of newly diagnosed CAD, or vascular surgery consultation in cases of newly diagnosed LE or carotid atherosclerosis [2,3,108]. In case of detection of nonobstructive atherosclerosis, the attending physician was informed about the results of the atherosclerotic screening and the need for immediate modification of medical therapy was assessed by the principal investigator and performed if necessary [2,3,108].

4.5. Methods used in the screening protocol for eligibility and group assignment

4.5.1. Coronary computed tomography angiography

Coronary computed tomography angiography was used to exclude coronary artery atherosclerosis in control participants since it has a higher negative predictive power than other methods, including invasive coronary angiography [109]. Invasive coronary angiography visualizes only luminal narrowing and may be insensitive to coronary artery lesions with positive remodelling and no compromise of luminal dimensions, which are conversely detectable by coronary computed tomography angiography [109].

The coronary computed tomography angiography scans were performed at the Department of Radiology, Hospital de Santa Marta, Centro Hospitalar Universitário de Lisboa Central, using the 64-slice computed tomography scanner LightSpeed VCT XT (GE Healthcare, Milwaukee, WI, USA). Prior to image acquisition, participants were given a vasodilator and a betablocker, as appropriate, and a prospective ECG-triggered acquisition was performed by default. Two expert cardiologists performed imaging interpretation. The calcium score was calculated using the Agaston score

[110]. The presence of coronary artery atherosclerosis was assessed, including soft plaques, and the percent luminal stenosis was estimated in cases of luminal narrowing.

4.5.2. Invasive coronary angiography

Invasive coronary angiography is the gold standard method for the diagnosis of coronary artery atherosclerosis with luminal narrowing, providing a higher positive predictive value compared with other techniques, including coronary computed tomography angiography [2].

Invasive coronary angiography was performed at the Department of Cardiology, Hospital de Santa Marta, Centro Hospitalar Universitário de Lisboa Central. Two catheterization laboratories were used, which were equipped with the GE Innova 2100-IQ (GE Healthcare, Waukesha, WI, USA) and Artis Zee (Siemens Healthineers, Erlangen, Germany) angiography systems, respectively. The exams were performed and interpreted by a team of seven interventional cardiologists, including the principal investigator. The procedures were performed by radial or femoral approach, using 5 Fr or 6 Fr diagnostic catheters and an intracoronary vasodilator (isosorbide dinitrate) was routinely administered. In patients with prior CABG, the patency of bypasses was assessed. Other procedural aspects remained at the discretion of the operator. Usually, 10 to 15 fps were selected for cineangiography, low- or iso-osmolar contrast media were used and the volume was at least 6 mL for the left coronary artery system and bypass grafts and at least 4 mL for the right coronary artery. Stenosis quantification was assessed visually by the operator by comparison with an adjacent non-diseased segment and, in cases of doubt, quantitative coronary angiography was used. The number of vessels with obstructive disease and number of obstructive lesions were assessed. For the number of vessels with obstructive disease, the left main artery, left anterior descending artery, circumflex artery, and right coronary artery were considered separately, with a total score ranging from 0 to 4. Atherosclerosis in bypass grafts was not considered. In addition, the Gensini score and the SYNergy between percutaneous coronary intervention with TAXus and cardiac surgery (SYNTAX) score were calculated, using acknowledged methods [111-113]. Briefly, for the Gensini score, each lesion is scored according to the percent reduction in luminal diameter, a multiplying factor is applied to each lesion based upon its location in the coronary tree, depending on the functional significance of the area supplied by that segment, and a collateral adjustment factor is applied in cases of

occlusive or sub-occlusive lesions; the final Gensini score is the sum of all the lesion scores [111]. For the SYNTAX score, each lesion is weighted according to its location in the coronary tree and is scored based on parameters reflecting lesion complexity, including the number of segments involved per lesion, involvement of bifurcations or trifurcations, aorto-ostial location, tortuosity, length, calcification, thrombus, diffuse disease / small vessels, and presence and characteristics of chronic total occlusions; the final SYNTAX score is the sum of all the lesion scores [112,113].

4.5.3. Ankle–brachial index

The ankle–brachial index has good accuracy in the detection of LE arterial disease and is indicated as a first-line noninvasive test for screening and diagnosis of LE arterial disease [3]. Therefore, a large number of clinical studies relied on the ankle–brachial index as the diagnostic criterion for the presence of LE atherosclerosis [81,105-107]. In this research project, the accuracy for confirming or excluding LE atherosclerosis was further increased by assessing simultaneously the presence of chronic claudication symptoms and the ankle–brachial index, and by considering only concordant assessments between both [3]. In cases of discordance, the diagnosis was ascertained by DUS, as aforementioned [3].

The ankle–brachial index was assessed at the Department of Angiology and Vascular Surgery, Hospital de Santa Marta, Centro Hospitalar Universitário de Lisboa Central, by the same observer. Systolic blood pressure was measured in the posterior and anterior tibial arteries of each leg and in the brachial arteries of each arm using a manual blood pressure cuff and an 8 MHz transducer [3]. The ankle–brachial index of each leg was calculated by dividing the highest value of systolic blood pressure of each leg by the highest brachial systolic blood pressure [3].

4.5.4. Doppler ultrasound studies

DUS studies were used to assess the presence of obstructive atherosclerosis in the LE and carotid arteries. Overall, DUS studies provide similar diagnostic accuracy for detecting arterial stenoses of at least 50% compared with other methods, including invasive angiography and computed tomography angiography [3,103]. In addition, it is a noninvasive method that does not require the use contrast media or ionizing radiation [3,103]. Therefore, DUS is indicated as an imaging method to confirm and characterize LE and carotid artery disease [3].

The DUS studies of LE and carotid arteries were performed at the Department of Angiology and Vascular Surgery, Hospital de Santa Marta, Centro Hospitalar Universitário de Lisboa Central, by two observers. DUS studies were performed according to a standardized protocol, using the Logiq S7 Expert Ultrasound System (GE Healthcare, Wauwatosa, WI, USA).

For the LE, the external iliac, common femoral, superficial femoral, popliteal, anterior tibial, posterior tibial, and fibular arteries were assessed, on both sides [3,114,115]. Each arterial segment was assessed using B-mode, color flow, and pulsed-wave Doppler [116-118]. A stenosis of at least 50% was defined by a systolic velocity ratio over 2.0 (this ratio was calculated by dividing the peak systolic velocities of the narrowed segment and of a proximal, normal segment); a marked spectral broadening and a monophasic waveform were supporting criteria [116-118]. A total occlusion was defined as absent flow within the artery [116-118]. Each segment, on each side, was assessed for the presence of significant lesions and the number of native obstructive lesions was assessed; lesions in bypass grafts were not considered [119]. LE lesions were classified as proximal if they were located in the popliteal artery or above or distal if they were located below [3].

For the carotid arteries, the common carotid, external carotid, and internal carotid arteries were evaluated [3,115]. Each arterial segment was assessed using B-mode, color flow, and pulsed-wave Doppler [3,115]. A stenosis of at least 50% was defined by a peak systolic velocity over 125 cm/sec; a ratio between internal carotid artery and common carotid artery peak systolic velocities over 2.0 was a supporting criterion [118,120]. A total occlusion was defined as absent flow within the artery [118,120]. Each segment, on each side, was assessed for the presence of significant lesions [118,120]. The mean and maximal intima–media thickness (IMT) were measured in individuals without overt arterial injury (in cases of no significant carotid artery stenosis) [121]. Measurements were performed in the posterior wall of the common carotid artery, on each side, at 1 cm from the carotid bifurcation [121,122]. A 10-mm-in-length straight arterial segment was selected at end-diastole and a semi-automatic border detection was used [121,122].

4.5.5. Additional complementary assessment

Routine laboratory tests, a 12-lead electrocardiogram, and a transthoracic echocardiogram were performed in each participant.

Routine laboratory tests were performed at the Clinical Laboratory of Hospital de Santa Marta, Centro Hospitalar Universitário de Lisboa Central and included: complete blood count, coagulation tests (prothrombin time and activated partial thromboplastin time), and serum levels of creatinine, urea, electrolytes, total bilirubin, alanine transaminase, aspartate transaminase, alkaline phosphatase, gamma glutamyl transpeptidase, fasting glycemia, percentage of glycosylated hemoglobin, total cholesterol, LDL-cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, and C-reactive protein.

The 12-lead electrocardiogram and transthoracic echocardiogram were performed by the principal investigator using the PageWriter TC50 (Philips, Andover, MA, USA) and Vivid E9 (GE Healthcare, Milwaukee, WI, USA) equipment, respectively, and followed published guidelines [2,123].

4.6. MicroRNAs, inflammatory and vascular biomarkers

4.6.1. Collection and storage of peripheral venous blood

Peripheral venous blood was collected prior to any vascular intervention as per protocol, within one day of recruitment of the participant, early in the morning under fasting conditions. Serum was separated by centrifugation (500× g for 10 min) within 15 min of sampling. Aliquots were stored at −80 °C at the Clinical Research Office of the Department of Cardiology, Hospital de Santa Marta, Centro Hospitalar Universitário de Lisboa Central. Samples were thawed only once, at the end of the recruitment of participants, and were used for measurements of the levels of circulating microRNAs and inflammatory and vascular biomarkers, as described below.

4.6.2. MicroRNAs

Six candidate microRNAs (miR-21, miR-27b, miR-29a, miR-126, miR-146a, and miR-218) were selected (*vide* section 1.3.4.) based on the following criteria: microRNAs are associated with the regulation of atherosclerosis development and expression in experimental models [11,18,36]; each of the microRNAs regulates distinct pathways of atherosclerotic disease and/or has distinct mechanisms of action [11,18,36]; and microRNAs were reported to be dysregulated in patients with stable atherosclerosis [22,23,37-41].

The assessment of expression levels of candidate microRNAs was carried out at Instituto de Medicina Molecular João Lobo Antunes, Universidade de Lisboa, by two observers. Total RNA was extracted from serum samples using the miRCURY™ RNA Isolation Kit (Qiagen, Hilden, Germany). Complementary deoxyribonucleic acid (cDNA) was synthesized from total RNA using the Universal cDNA synthesis kit from miRCURY™ LNA microRNA system (Qiagen, Hilden, Germany). MicroRNA amplification was performed using quantitative reverse-transcription polymerase chain reaction (using the miRCURY™ LNA SYBR Green PCR Kit and LNA™ PCR primers, Qiagen, Hilden, Germany), and the melting curve was determined according to the following conditions: 95 °C for 10 min followed by 45 cycles of 95 °C for 10 s and 60 °C for 60 s. All the reactions were performed in triplicates. The amplification data were assessed using DataAssist™ Software v3.01 (Thermo Fisher Scientific, Waltham, MA, USA). Cycle threshold (C_t) values greater than 40 were considered undetermined [37,124-126]. The relative expression levels of the six candidate microRNAs were calculated using the delta cycle threshold (ΔC_t) method, normalizing for the UniSp6 RNA spike-in control [24,37,127,128]. Higher ΔC_t values represent lower circulating levels of the candidate microRNAs [24,37,127,128]. For graphical expression, the relative expression levels of candidate microRNAs were expressed using the Livak method ($2^{-\Delta\Delta Ct}$) [129], for which lower values correspond to lower expression levels.

4.6.3. Inflammatory and vascular biomarkers

Three biomarkers, including two inflammatory mediators (sCD40L and TNF-α) and one marker of vascular function (VEGF) were preselected based on prior investigation [69-72] conducted by the team that collaborated with this research project and the following criteria: the mediators are associated with the regulation of atherosclerosis development and expression [53,89,97,99]; they participate in distinct biological pathways [53,89,97,99]; and they are regulated by at least one of the six candidate microRNAs selected for this research project [12,73,74,100].

Serum levels of sCD40L were measured at the Department of Nuclear Science and Engineering, Instituto Superior Técnico, Universidade de Lisboa, by two observers. An enzyme-linked immunosorbent assay (ELISA) commercial kit (Quantikine® ELISA, Human CD40 Ligand/TNFSF5 Immunoassay, R&D Systems, Minneapolis, MN, USA) was used, following manufacturer guidelines.

Serum levels of TNF-α were measured at Instituto de Medicina Molecular João Lobo Antunes, Universidade de Lisboa, by the same observer. An ELISA commercial kit

(Quantikine® ELISA, Human TNF-α Immunoassay, R&D Systems, Minneapolis, MN, USA) was used, following manufacturer guidelines.

Serum levels of VEGF were measured at Instituto de Medicina Molecular João Lobo Antunes, Universidade de Lisboa, by the same observer. An ELISA commercial kit (Quantikine® ELISA, Human VEGF Immunoassay, R&D Systems, Minneapolis, MN, USA) was used, following manufacturer guidelines.

All measurements were performed in duplicates. The intra-assay variation among the duplicates for all samples was less than 10%.

Preliminary exploratory analysis

The sCD40L, TNF-α, and VEGF levels were quantified in a preliminary exploratory analysis with the aim of detecting trends in their expression levels, in order to decide whether these biomarkers would be assessed in the whole recruited sample, considering the associated cost-effectiveness (Supplementary Materials, sections 1.1. and 1.2.). This analysis included a subgroup of 24 participants with atherosclerosis of different territories. Based on the results of this preliminary exploratory analysis, only the sCD40L measurements were extended to the entire recruited sample.

4.7. Sample size calculation

Sample size calculation was based on the principal objective of the study. However, no previous studies have reported data on circulating microRNAs in both single- and multi-territorial atherosclerosis with the systematic screening of atherosclerotic lesions in different territories, which would be valuable for supporting the sample size estimation in our study. The sample size of studies assessing expression levels of circulating microRNAs in single-territorial atherosclerosis was considered for sample size calculation [23,130-132]. Therefore, we planned to recruit at least 20 control participants, 20 patients with isolated coronary atherosclerosis (group 1), and 40 patients with coronary and extra-coronary atherosclerosis (groups 2 to 4), including at least 10 patients per group in groups 2 to 4. The recruitment of participants for each group continued even after meeting the minimum number of participants until the minimum sample size was achieved for all groups.

4.8. Statistical analysis

Discrete variables are presented as frequency (percentage) and continuous variables are presented as the mean (standard deviation) in normally distributed data or median (IQR) in variables without a normal distribution (Shapiro–Wilk test). Categorical variables were analyzed using the chi-square or Fisher's exact tests. Continuous variables were analyzed using Student's t-test or the Mann–Whitney test when the normality was not verified. Comparisons between multiple groups were performed using the analysis of variance (ANOVA) in normally distributed data and Kruskal–Wallis test in variables without a normal distribution; the Bonferroni *post hoc* correction was used for multiple pairwise comparisons. Pearson's correlation was used to test correlations between continuous variables. The multivariate logistic regression analysis was used to assess independent predictors of categorical variables and the multivariate linear regression analysis was used to assess independent predictors of continuous variables. Of the tested variables, those with a p-value < 0.10 in the univariate analyses were tested in the multivariate models after a correction for collinearity, when applicable. Outliers were excluded, as appropriate [32]. For analysing the diagnostic accuracy of potential biomarkers, the area under the receiver operating characteristics (ROC) curve was assessed. The level of significance considered was $\alpha = 0.05$. Analyses were conducted using the SPSS software, version 26.0 (IBM Corp, Armonk, NY, USA).

5. Results

5.1. Participants included

A total of 106 individuals were invited to participate in the study and signed the informed consent form. Of these, 12 were excluded due to percutaneous arterial treatment before collection and storage of peripheral venous blood (4 participants), heart failure (1 participant), valvular heart disease (2 participants), active infection (1 participant), renal dysfunction (2 participants), and patient withdraw of consent to study participation (2 participants). Therefore, 94 participants were included, meeting the criteria of estimated sample size (section 4.7.): 26 control participants, 20 with isolated coronary atherosclerosis (group 1), 18 with coronary and LE atherosclerosis (group 2), 12 with coronary and carotid atherosclerosis (group 3), and 18 with atherosclerosis of the coronary, LE, and carotid territories (group 4).

5.1.1. Clinical characteristics, laboratory results, and atherosclerosis data of participants

The clinical characteristics, laboratory results, and atherosclerosis data of the participants are presented in Table 3. The differences in clinical characteristics and laboratory data among groups were driven by control participants, where classical cardiovascular risk factors and the use of antiplatelet and statin therapy were less prevalent; the neutrophil count, neutrophil/lymphocyte ratio, and creatinine levels were lower; and the HDL-cholesterol levels were higher than those in patients with atherosclerosis. The distribution of these parameters did not differ among groups 1 to 4.

Regarding atherosclerosis data, the distribution of CAD parameters did not differ significantly among groups 1 to 4, including the number of vessels with obstructive disease, number of obstructive lesions, Gensini score, SYNTAX score, or rates of prior CABG. Among all patients with coronary atherosclerosis, the median number of vessels with obstructive disease was 3 (IQR, 2–4), the median number of obstructive lesions was 4 (IQR, 3–5), the median Gensini score was 90 (IQR, 42–123), and the mean SYNTAX score was 26.5 (9.3).

Among patients with LE atherosclerosis (groups 2 and 4), group 4 showed a higher prevalence of bilateral LE atherosclerosis and a higher number of LE arterial segments

with obstructive disease. The prevalence of proximal LE lesions and rates of prior LE bypass surgery did not differ between groups 2 and 4, although the revascularization rates were nonsignificantly higher in the latter (11.1% vs. 33.3%, respectively, $p = 0.109$). Among all patients with LE atherosclerosis, the median number of LE lesions was 3 (IQR, 2–5).

Regarding patients with carotid atherosclerosis (groups 3 and 4), 36.7% had bilateral carotid atherosclerosis and this rate did not differ between the two groups. The mean and maximal IMT did not differ among controls and groups 1 and 2.

Table 3. Characteristics of the participants according to the involved territories of atherosclerosis.

	Controls	**Group 1**	**Group 2**	**Group 3**	**Group 4**	***p*-value**
Territories of atherosclerosis	None	Coronary	Coronary + LE	Coronary + Carotid	Coronary + LE + Carotid	
n	26	20	18	12	18	
Clinical characteristics						
Age, years	59 (53–69)	65 (56–70)	67 (57–72)	59 (51–73)	69 (60–75)	0.079
Male, n (%)	23 (88.5)	18 (90.0)	16 (88.9)	10 (83.3)	17 (94.4)	0.912
Hypertension, n (%)	14 (53.8)	17 (85.0) [a]	18 (100.0) [a]	11 (91.7) [a]	18 (100.0) [a]	<0.001
Dyslipidemia, n (%)	18 (69.2)	19 (95.0) [a]	18 (100.0) [a]	11 (91.7)	17 (94.4) [a]	0.010
Diabetes mellitus, n (%)	3 (11.5)	6 (30.0)	8 (44.4) [a]	6 (50.0) [a]	9 (50.0) [a]	0.036
Smoking history, n (%)	6 (23.1)	9 (45.0)	12 (66.7) [a]	4 (33.3)	12 (66.7) [a]	0.014
LVEF > 50%, n (%)	26 (100.0)	20 (100.0)	18 (100.0)	12 (100.0)	18 (100.0)	–
Antiplatelet therapy, n (%)	6 (23.1)	20 (100.0) [a]	17 (94.4) [a]	11 (91.7) [a]	18 (100) [a]	<0.001
Statin therapy, n (%)	13 (50.0)	18 (90.0) [a]	16 (94.1) [a]	11 (91.7) [a]	16 (88.9) [a]	0.001
Laboratory parameters						
Hemoglobin, g/dL	13.9 (12.9–15.0)	14.53 (10.0–15.1)	14.1 (13.2–14.6)	12.0 (11.4–13.4) [b]	12.9 (12.1–14.2)	0.017
Leukocyte count, 10^9/L	6.4 (1.7)	7.4 (1.9)	7.3 (1.7)	7.5 (2.2)	8.1 (1.7)	0.080
Neutrophil count, 10^9/L	3.2 (2.5–4.8)	4.1 (3.4–5.2)	3.9 (3.4–4.8)	4.0 (3.4–6.7)	4.7 (3.6–6.0) [a]	0.043
Lymphocyte count, 10^9/L	1.9 (1.7–2.2)	2.1 (1.6–2.4)	2.1 (1.6–2.8)	1.7 (1.2–2.3)	2.2 (1.6–2.6)	0.401
Neutrophil/lymphocyte ratio	1.9 (0.7)	2.3 (1.1)	2.1 (1.0)	2.9 (1.1) [a]	2.4 (1.0)	0.026

Platelet count, 10^9/L	242 (191–274)	209 (176–269)	219 (195–264)	229 (137–251)	227 (203–263)	0.854
Fasting glycaemia, mg/dL	89 (80–98)	94 (86–129)	94 (83–125)	99 (84–157)	85 (75–123)	0.385
Percentage of glycosylated hemoglobin	5.6 (5.2–5.9)	5.9 (5.6–6.7)	5.9 (5.5–6.1)	5.8 (5.4–7.4)	5.9 (5.3–7.7)	0.185
Creatinine, mg/dL	0.8 (0.7–0.9)	0.9 (0.8–1.1)	0.8 (0.8–1.2)	0.9 (0.8–1.4)	1.1 (0.9–1.5) [a]	0.002
Total cholesterol, mg/dL	186 (51)	164 (38)	172 (50)	153 (50)	173 (49)	0.329
LDL-cholesterol, mg/dL	99 (77–141)	95.0 (71–120)	106 (83–120)	65 (56–132)	117 (82–142)	0.297
HDL-cholesterol, mg/dL	51 (44–58)	35.0 (31–41) [a]	35 (31–45) [a]	40 (27–44) [a]	40 (32–42) [a]	<0.001
Triglycerides, mg/dL	106 (67–144)	142 (98–206)	115 (83–204)	100 (62–177)	117 (95–171)	0.423
C-reactive protein, mg/L	4.1 (2.0)	3.7 (1.4)	3.8 (1.1)	4.1 (2.3)	3.3 (2.0)	0.151
Coronary atherosclerosis						
Nr. of vessels with obstructive disease	0 (0–0)	3 (2–3) [a]	3 (2–4) [a]	3 (3–3) [a]	3 (2–4) [a]	<0.001
Nr. of obstructive lesions	0 (0–0)	4 (2–5) [a]	4 (3–5) [a]	4 (3–5) [a]	4 (3–5) [a]	<0.001
Gensini score	0 (0–0)	81 (41–97) [a]	83 (42–118) [a]	48 (40–116) [a]	43 (36–87) [a]	<0.001
SYNTAX score	0.0 (0.0)	23.3 (8.4) [a]	29.6 (9.9) [a]	25.3 (7.2) [a]	28.1 (10.6) [a]	<0.001
Prior CABG, n (%)	0 (0.0)	7 (35.0) [a]	7 (38.9) [a]	6 (50.0) [a]	3 (16.7) [a]	0.002
LE atherosclerosis						
Bilateral disease, n (%)	0 (0.0)	0 (0.0)	10 (55.5) [a,b,d,e]	0 (0.0)	15 (83.3) [a–d]	<0.001
Any proximal lesion, n (%)	0 (0.0)	0 (0.0)	10 (55.5) [a,b,d]	0 (0.0)	12 (66.7) [a,b,d]	<0.001
Nr. of segments with obstructive disease	0 (0–0)	0 (0–0)	2 (1–4) [a,b,d,e]	0 (0–0)	4 (3–5) [a–d]	<0.001
Prior bypass surgery, n (%)	0 (0.0)	0 (0.0)	2 (11.1) [a,b,d]	0 (0.0)	6 (33.3) [a,b,d]	<0.001
Carotid atherosclerosis						
Bilateral disease, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	3 (25.0) [a–c]	8 (44.4) [a–c]	<0.001
Mean IMT, mm	0.68 (0.11)	0.67 (0.14)	0.76 (0.06)	–	–	0.296
Maximal IMT, mm	0.80 (0.13)	0.86 (0.18)	0.94 (0.09)	–	–	0.105

Categorical variables are expressed as frequency (percentage) and continuous variables as the mean (standard deviation) or median (interquartile range). CABG, coronary artery bypass grafting; HDL, high-density lipoprotein; IMT, intima–media thickness; LDL, low-density lipoprotein; LE, lower extremity; LVEF, left ventricular ejection fraction; Nr., number; SYNTAX, SYNergy between percutaneous coronary intervention with TAXus and cardiac surgery. [a] *p*-

value < 0.05 vs. controls; [b] *p*-value < 0.05 vs. group 1; [c] *p*-value < 0.05 vs. group 2; [d] *p*-value < 0.05 vs. group 3; [e] *p*-value < 0.05 vs. group 4.

5.2. MicroRNA profiles according to single- and multi-territorial atherosclerosis and severity of atherosclerosis in different territories

The results regarding microRNA profiles according to single- and multi-territorial atherosclerosis and severity of atherosclerosis in different territories were published [133] and are specified in sections 5.2.1 to 5.2.4.

5.2.1 Circulating microRNAs according to the systemic extent of atherosclerosis

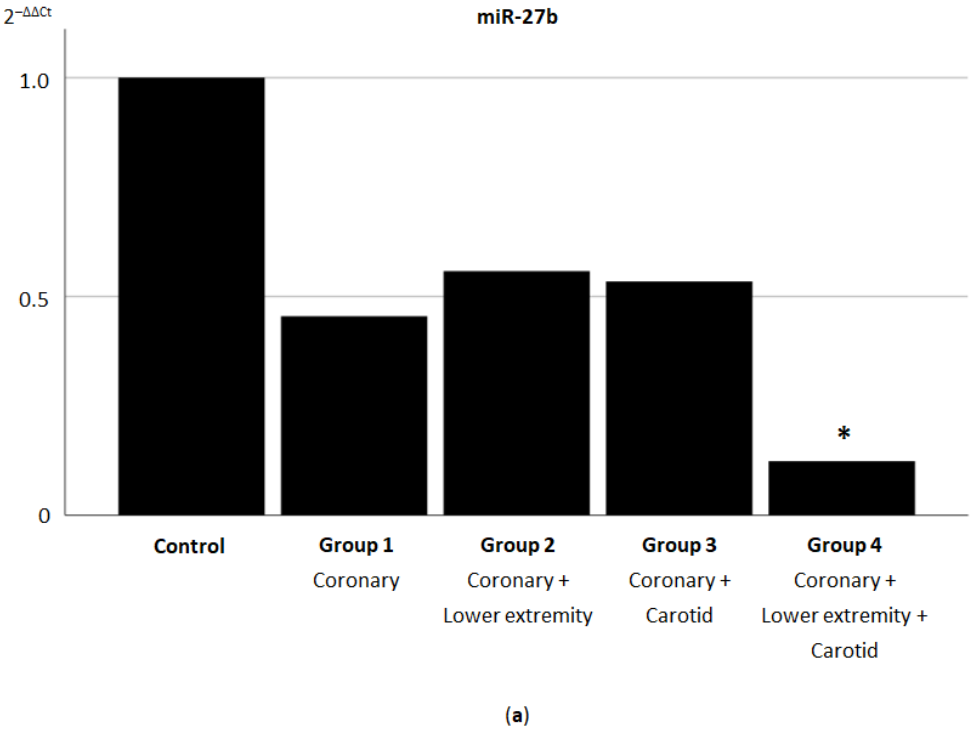
The expression levels (ΔC_t) of miR-27b and miR-146a differed across groups (ANOVA $p < 0.05$ for both microRNAs), whereas the expression levels of other microRNAs did not (Table 4).

Table 4. Expression of circulating microRNAs according to the involved territories of atherosclerosis.

Study Group	**Control**	**Group 1**	**Group 2**	**Group 3**	**Group 4**	***p*-value**
Territories of atherosclerosis	None	Coronary	Coronary + LE	Coronary + Carotid	Coronary + LE + Carotid	
MicroRNAs						
miR-21	14.73 (4.63)	15.60 (4.22)	14.14 (4.41)	13.83 (5.06)	18.22 (4.01)	0.064
miR-27b	17.82 (3.43)	19.39 (4.06)	19.03 (4.37)	18.71 (5.45)	22.34 (3.93) [a]	0.041
miR-29a	20.90 (3.52)	21.09 (2.02)	20.67 (2.90)	20.99 (5.06)	23.50 (2.79)	0.121
miR-126	17.38 (15.12–21.89)	17.79 (14.99–22.88)	16.79 (15.57–22.74)	14.85 (13.36–21.34)	23.32 (17.88–24.86)	0.102
miR-146a	18.06 (3.00)	19.63 (3.34)	19.43 (4.39)	18.76 (4.21)	22.20 (3.47) [a]	0.048
miR-218	22.63 (10.01–23.51)	23.15 (16.18–26.12)	22.63 (10.87–25.71)	22.67 (9.19–23.36)	24.70 (23.88–25.68)	0.744

The ΔC_t values are presented for each microRNA (higher ΔC_t values correspond to lower microRNA expression levels) and are expressed as the mean (standard deviation) or median (interquartile range). LE, lower extremity; ΔC_t, delta cycle threshold. [a] *p*-value < 0.05 vs. controls.

The ΔC_t values of both miR-27b and miR-146a showed a stepwise increase (corresponding to a decrease in their expression levels) from control participants to patients with atherosclerosis of one or two territories (groups 1–3), and further to patients with atherosclerosis of the three territories (group 4), with significant differences between the controls and patients with atherosclerosis of the three territories. The relative expression of miR-27b and miR-146a is presented in Figure 2.



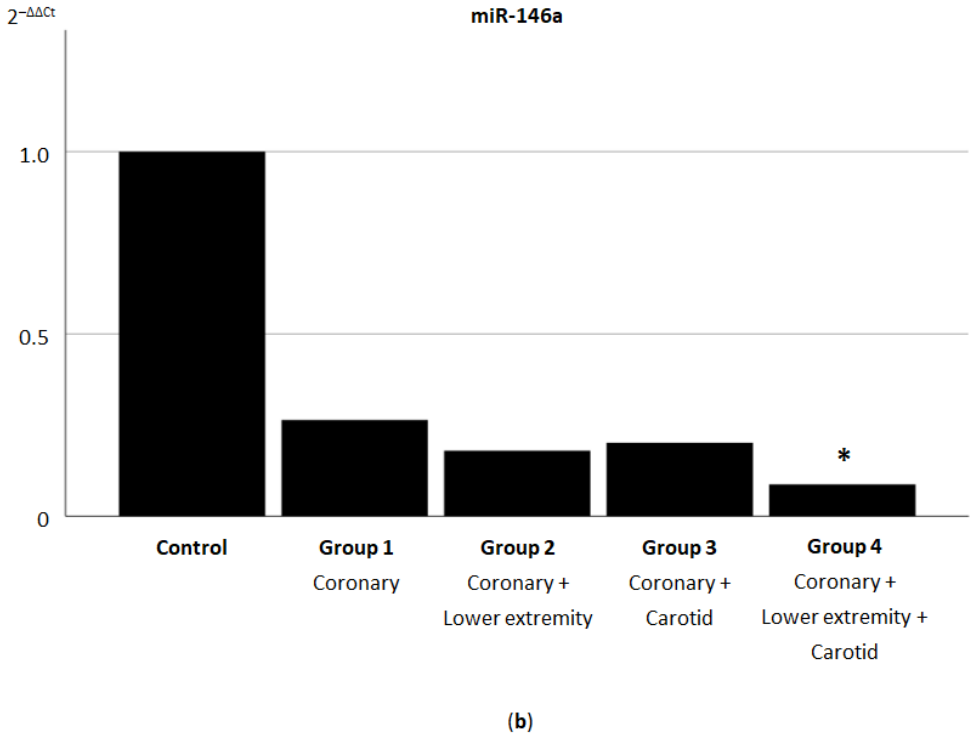


Figure 2. Relative expression of miR-27b and miR-146a according to the systemic extent of atherosclerosis to different territories. The mean relative expression levels of (**a**) miR-27b and (**b**) miR-146a are presented using the Livak method ($2^{-\Delta\Delta Ct}$) [129], with lower values corresponding to lower expression levels. * *p*-value <0.05 vs controls.

5.2.2. Circulating microRNAs according to the atherosclerosis severity in different territories

Coronary atherosclerosis

For both miR-27b and miR-146a, there was a weak positive correlation between the ΔC_t and the number of coronary arteries with obstructive disease (r = 0.241, p = 0.043 for miR-27b; and r = 0.242, p = 0.036 for miR-146a), number of coronary artery lesions (r = 0.241, p = 0.043 for miR-27b; and r = 0.289, p = 0.012 for miR-146a), and the SYNTAX score (r = 0.286, p = 0.019 for miR-27b; and r = 0.257, p = 0.037 for miR-146a), indicating decreased expression levels of both microRNAs with an increase in the severity of coronary atherosclerosis. No correlation was found between expression levels of candidate microRNAs and the Gensini score.

Lower extremity atherosclerosis

For miR-27b, miR-126, and miR-146a, there was a weak positive correlation between the ΔC_t and the number of obstructive lesions in the LE (r = 0.320, p = 0.008 for miR-27b; r = 0.254, p = 0.043 for miR-126; and r = 0.352, p = 0.003 for miR-146a), indicating decreased expression levels of the three microRNAs with an increase in the severity of LE atherosclerosis.

Regarding the number of sides involved in the LE, ΔC_t miR-27b was significantly higher in bilateral LE atherosclerosis (24.01 [17.18–25.72]) compared with unilateral disease (20.32 [15.11–23.22], p = 0.041 vs. bilateral LE atherosclerosis) and with absent LE atherosclerosis (18.32 [14.84–21.59], p = 0.004 vs. bilateral LE atherosclerosis). Such results corresponded to a 4.0- and 52.0-fold reduction in miR-27b expression levels in bilateral LE atherosclerosis compared with unilateral and absent LE atherosclerosis, respectively. ΔC_t miR-146a was nonsignificantly higher in bilateral LE atherosclerosis (21.98 [19.46–25.11]) compared with unilateral disease (18.84 [16.37–22.34], p = 0.068 vs. bilateral LE atherosclerosis), and was significantly higher compared with absent LE atherosclerosis (18.75 [15.62–21.42], p = 0.008 vs. bilateral LE atherosclerosis). Such results corresponded to a 12.7-fold reduction in miR-146a in bilateral compared with absent LE atherosclerosis.

Carotid atherosclerosis

ΔC_t miR-27b showed a stepwise increase from individuals with no carotid atherosclerosis (18.64 [3.89]) to patients with unilateral disease (19.56 [5.88]), and further to patients with bilateral disease (21.66 [4.89]), with a significant difference between individuals with no carotid atherosclerosis and those with bilateral disease

(p = 0.030), corresponding to lower expression levels of miR-27b in bilateral carotid atherosclerosis (8.1-fold reduction in bilateral compared with absent carotid atherosclerosis).

5.2.3. Atherosclerosis severity and microRNAs expression levels in multivariate analysis

Multivariate linear regression analysis was performed considering all clinical, laboratory, and atherosclerosis data (including the systemic extent of atherosclerosis, severity of atherosclerosis in different territories, and prior revascularization) as independent variables, and ΔC_t miR-27b and ΔC_t miR-146a as dependent variables. The univariate analysis assessing the parameters associated with ΔC_t miR-27b and ΔC_t miR-146a is presented in Tables S3 and S4, respectively (Supplementary Materials, section 2.). In the multivariate linear regression analysis, the presence of multi-territorial atherosclerosis involving the coronary, LE, and carotid territories, and indexes of CAD severity were independently associated with ΔC_t miR-27b and ΔC_t miR-146a (Table 5).

Table 5. Parameters associated with miR-27b and miR-146a expression levels in multivariate linear regression analysis.

Predictors of microRNA expression levels	β	95% CI	*p*-value
ΔC_t miR-27b			
Coexistence of coronary, LE, and carotid atherosclerosis	3.41	0.55–6.27	0.020
SYNTAX score	0.072	0.001–0.143	0.049
ΔC_t miR-146a			
Coexistence of coronary, LE, and carotid atherosclerosis	3.10	0.73–5.46	0.011
Number of coronary artery lesions	0.37	0.02–0.73	0.041

95% CI, 95% confidence interval; LE, Lower extremity; SYNTAX, SYNergy between percutaneous coronary intervention with TAXus and cardiac surgery; ΔC_t, delta cycle threshold.

5.2.4. Accuracy of miR-27b and miR-146a for predicting severe systemic atherosclerosis

ΔC_t miR-27b and ΔC_t miR-146a provided areas under the ROC curve of 0.76 (95% CI 0.60–0.91, p = 0.004) and 0.75 (95% CI 0.59–0.91, p = 0.009), respectively, for predicting severe systemic atherosclerosis with concomitant involvement of the coronary, LE, and carotid arteries (Figure 3). The cut-off (21.5) of ΔC_t miR-27b indicated 77% sensitivity and 72% specificity, and the cut-off (21.6) of ΔC_t miR-146a indicated 73% sensitivity and 77% specificity for predicting severe systemic atherosclerosis with concomitant involvement of the three territories.

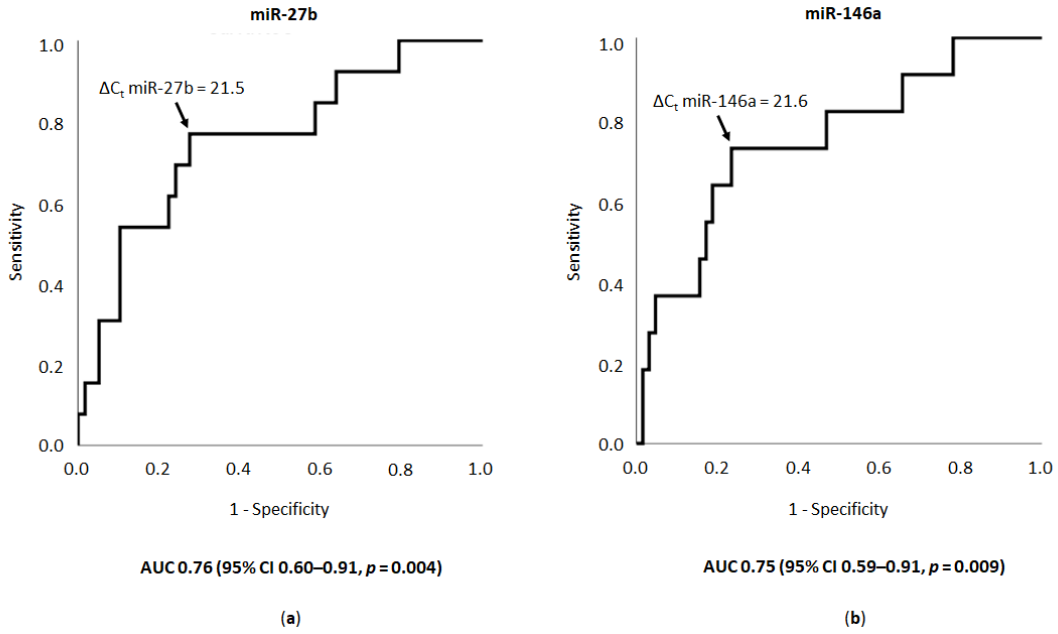


Figure 3. Accuracy of miR-27b and miR-146a for predicting severe systemic atherosclerosis. The receiver operating characteristics curve is presented for (**a**) ΔC_t miR-27b and (**b**) ΔC_t miR-146a, for predicting severe systemic atherosclerosis involving the coronary, lower extremity, and carotid territories. The optimal cut-off for (**a**) ΔC_t miR-27b was 21.5 and for (**b**) ΔC_t miR-146a was 21.6, presenting a Youden's index [134] of 0.49 and 0.50, respectively. 95% CI, 95% confidence interval; AUC, area under the curve; ΔC_t, delta cycle threshold.

In summary, lower expression levels of miR-27b and miR-146a were associated with an increase in the systemic extent of atherosclerosis to multiple territories, particularly if involving the coronary, LE, and carotid territories, and with an increase in the atherosclerosis severity in the three territories. The coexistence of atherosclerosis in the coronary, LE, and carotid territories was independently associated with the miR-

27b and miR-146a expression levels and both microRNAs presented areas under the ROC curve ≥ 0.75 to predict atherosclerosis of the three territories.

5.3. Cigarette smoking toxicity, microRNAs, and lower extremity atherosclerosis

The results regarding cigarette-smoking toxicity, microRNA profiles, and LE atherosclerosis were published [135] and are specified in sections 5.3.1 to 5.3.5.

5.3.1. Characteristics of participants according to cigarette-smoking status

Of the 94 recruited participants, 51 were never-smokers, 28 were prior smokers (cases of smoking cessation at least six months before recruitment), and 15 were active smokers (cases of daily cigarette smoking, irrespective of the number of cigarettes [136]) (Table 6). The mean pack-year was 53 (22) in prior smokers and 45 (21) in active smokers, with a median time from cigarette-smoking cessation of 10 years (IQR, 7–11 years) in prior smokers. The prevalence of LE atherosclerosis and the proportion of bilateral LE atherosclerosis increased from never-smokers to prior smokers and active smokers (Table 6). Moreover, the leukocyte, neutrophil, and lymphocyte counts were higher in active smokers compared with other groups. miR-27b was the only dysregulated microRNA according to cigarette-smoking status. ΔC_t miR-27b values were significantly higher in active smokers compared with prior smokers (p = 0.004; corresponding to a downregulation of miR-27b in active smokers), and there was a (nonsignificant) trend for higher ΔC_t miR-27b values in active smokers compared with never-smokers (p = 0.053; corresponding to a trend for downregulation of miR-27b in active smokers).

Table 6. Characteristics of participants according to cigarette-smoking status.

	Never-smokers	**Prior smokers**	**Active smokers**	***p*-value**
n	51	28	15	
Clinical characteristics				
Age, years	65 (56–73)	67 (58–71)	59 (53–68)	0.415
Male, n (%)	43 (84.3)	28 (100.0)	13 (86.7)	0.090
Hypertension, n (%)	36 (70.6)	28 (100.0)	14 (93.3)	0.002

Dyslipidemia, n (%)	43 (84.3)	26 (92.9)	14 (93.3)	0.424
Diabetes mellitus, n (%)	17 (33.3)	10 (35.7)	5 (33.3)	0.975
LVEF > 50%, n (%)	51 (100.0)	28 (100.0)	15 (100.0)	–
Antiplatelet therapy, n (%)	36 (70.6)	24 (85.7)	12 (80.0)	0.298
Statin therapy, n (%)	37 (72.5)	24 (85.7)	13 (93.3)	0.156
Coronary artery disease, n (%)	31 (60.8)	24 (85.7)	13 (93.3)	0.024
Carotid artery disease, n (%)	14 (27.5)	10 (35.7)	6 (40.0)	0.576
LE atherosclerosis				
Number of patients	12 (23.5)	13 (46.4)	11 (73.3)	0.001
Bilateral disease, n (%)	8 (15.7)	11 (39.3)	6 (40.0)	0.002
Prior bypass surgery, n (%)	2 (3.9)	5 (17.9)	1 (6.7)	0.101
Laboratory parameters				
Hemoglobin, g/dL	13.4 (1.5)	14.1 (1.4)	14.1 (1.4)	0.063
Leukocyte count, 10^9/L	6.6 (1.5)	7.3 (1.8)	9.2 (1.8)	<0.001 [a,b]
Neutrophil count, 10^9/L	3.8 (3.0–4.4)	3.9 (3.2–5.3)	4.6 (3.7–5.8)	<0.001 [a,b]
Lymphocyte count, 10^9/L	1.8 (1.5–2.2)	2.1 (1.6–2.4)	2.3 (1.8–3.5)	0.027 [a]
Neutrophil/lymphocyte ratio	2.0 (1.5–3.0)	2.2 (1.7–2.7)	2.3 (1.9–2.8)	0.568
Platelet count, 10^9/L	219 (51)	218 (54)	255 (51)	0.058
Fasting glycemia, mg/dL	92 (83–116)	91 (85–112)	89 (72–117)	0.565
Percentage of glycosylated hemoglobin	5.7 (5.3–7.1)	5.8 (5.4–6.2)	5.9 (5.6–6.1)	0.787
Creatinine, mg/dL	0.86 (0.78–1.08)	0.86 (0.76–1.00)	1.01 (0.75–1.71)	0.447
Total cholesterol, mg/dL	178 (51)	154 (43)	172 (48)	0.060
LDL-cholesterol, mg/dL	108 (41)	89 (38)	116 (32)	0.059
HDL-cholesterol, mg/dL	42 (34–51)	41 (29–46)	35 (31–42)	0.236
Triglycerides, mg/dL	116 (79–156)	104 (73–178)	158 (112–243)	0.139
C-reactive protein, mg/L	3.5 (1.6)	3.7 (1.8)	4.0 (1.9)	0.339
MicroRNAs [c]				
miR-21	15.44 (4.40)	14.55 (4.62)	16.42 (5.09)	0.454
miR-27b	19.29 (4.42)	17.44 (3.98)	22.00 (4.35)	0.014 [b]
miR-29a	21.01 (3.04)	19.74 (3.86)	23.19 (3.12)	0.059
miR-126	17.42 (15.20–23.98)	16.10 (14.89–19.48)	22.40 (17.65–24.15)	0.075
miR-146a	20.03 (15.74–22.21)	17.79 (16.26–20.12)	21.69 (18.23–23.58)	0.150
miR-218	22.31 (15.67–23.02)	22.31 (15.67–23.02)	8.45 (-8.46–21.50)	0.120

Categorical variables are expressed as frequency (percentage) and continuous variables as the mean (standard deviation) or median (interquartile range). HDL, high-density lipoprotein; LDL,

low-density lipoprotein; LE, lower extremity; LVEF, left ventricular ejection fraction. [a] *p*-value < 0.05, active smokers vs. never-smokers; [b] *p*-value < 0.05, active smokers vs. prior smokers; [c] delta cycle threshold (ΔC_t) values are presented for each microRNA (higher ΔC_t values correspond to lower expression levels of candidate microRNAs).

Active smokers presented significantly higher ΔC_t miR-27b values (22.00 [4.35]) compared with nonactive smokers, including never-smokers and prior smokers (18.66 [4.33], p = 0.014 vs. active smokers), corresponding to a 10.0-fold downregulation of miR-27b expression levels in active smokers (Figure 4).

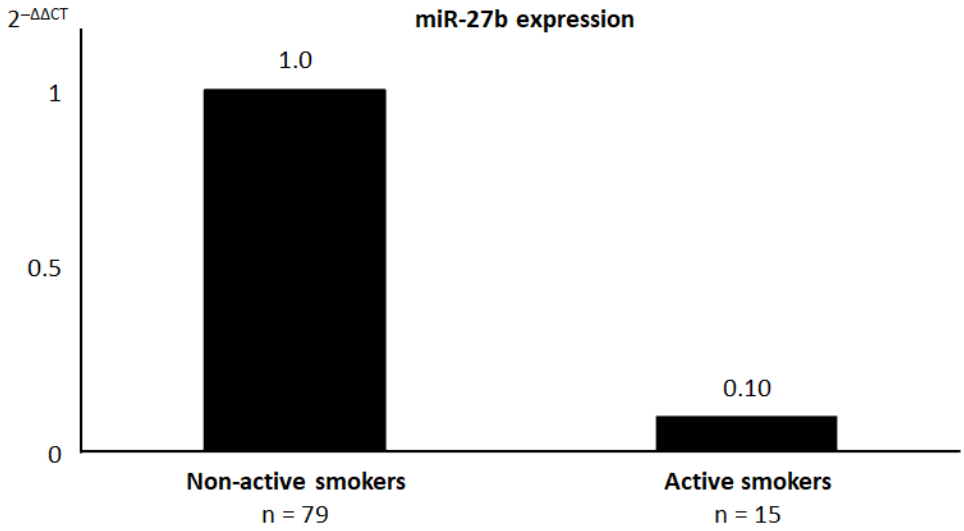


Figure 4. Relative expression of miR-27b according to the cigarette-smoking status. The relative expression of miR-27b ($2^{-\Delta\Delta Ct}$ [129]) is presented for nonactive smokers, including never-smokers and prior smokers, and for active smokers.

5.3.2. Characteristics of participants according to lower extremity atherosclerosis

Of the 94 participants, 36 presented LE atherosclerosis and 58 did not (Table 7). Patients with LE atherosclerosis presented a higher prevalence of classical cardiovascular risk factors (including cigarette smoking), concomitant coronary and carotid atherosclerosis, and use of antiplatelet and statin therapy, as well as higher creatinine levels, compared with participants without LE atherosclerosis. The ΔC_t miR-27b and ΔC_t miR-146a values were significantly higher in patients with LE atherosclerosis (Table 7), corresponding to a 17.0- and 3.4-fold downregulation of miR-27b and miR-146a, respectively, in patients with LE atherosclerosis.

Table 7. Characteristics of participants without and with lower extremity atherosclerosis.

	Without LE atherosclerosis	**With LE atherosclerosis**	***p*-value**
n	58	36	
Clinical characteristics			
Age, years	61 (53–70)	68 (60–73)	0.009
Male, n (%)	51 (87.9)	33 (91.7)	0.419
Hypertension, n (%)	42 (72.4)	36 (100.0)	<0.001
Dyslipidemia, n (%)	48 (82.8)	35 (97.2)	0.031
Diabetes mellitus, n (%)	15 (25.9)	17 (47.2)	0.029
Cigarette-smoking status, n (%)			0.001
Never-smoker	39 (67.2)	12 (33.3)	
Prior smoker	15 (25.9)	13 (36.1)	
Active smoker	4 (6.9)	11 (30.6)	
LVEF > 50%, n (%)	58 (100.0)	36 (100.0)	–
Antiplatelet therapy, n (%)	37 (63.8)	35 (97.2)	<0.001
Statin therapy, n (%)	42 (72.4)	32 (88.9)	0.023
Coronary atherosclerosis, n (%)	32 (55.2)	36 (100.0)	<0.001
Carotid atherosclerosis, n (%)	12 (20.7)	18 (50.0)	0.006
LE atherosclerosis			
Bilateral disease, n (%)	0 (0.0)	25 (69.4)	<0.001
Prior bypass surgery, n (%)	0 (0.0)	8 (22.2)	<0.001
Laboratory parameters			
Hemoglobin, g/dL	13.8 (1.5)	13.6 (1.6)	0.586
Leukocyte count, 10^9/L	7.0 (1.9)	7.7 (1.7)	0.081
Neutrophil count, 10^9/L	4.0 (1.7)	4.5 (1.4)	0.192
Lymphocyte count, 10^9/L	1.9 (1.6–2.3)	2.2 (1.6–2.7)	0.100
Neutrophil/lymphocyte ratio	2.2 (1.0)	2.2 (1.0)	0.943
Platelet count, 10^9/L	223 (58)	227 (44)	0.749
Fasting glycemia, mg/dL	92 (83–104)	90 (82–121)	0.978
Percentage of glycosylated hemoglobin	5.7 (5.4–6.2)	5.9 (5.5–7.4)	0.295
Creatinine, mg/dL	0.85 (0.77–0.97)	0.94 (0.80–1.34)	0.036
Total cholesterol, mg/dL	164 (130–206)	166 (147–205)	0.781
LDL-cholesterol, mg/dL	92 (72–130)	109 (83–135)	0.250
HDL-cholesterol, mg/dL	43 (34–51)	36 (31–43)	0.062
Triglycerides, mg/dL	115 (71–163)	117 (88–178)	0.429
C-reactive protein, mg/L	4.0 (1.9)	3.6 (1.5)	0.529
MicroRNAs [a]			
miR-21	14.89 (4.51)	15.99 (4.65)	0.282

miR-27b	18.23 (14.58–21.59)	22.32 (16.50–24.13)	0.032
miR-29a	20.42 (3.57)	21.80 (3.12)	0.152
miR-126	16.89 (14.89–22.46)	19.69 (16.28–24.06)	0.060
miR-146a	18.70 (3.40)	20.48 (4.23)	0.048
miR-218	22.69 (22.48–23.33)	14.49 (-8.6–23.50)	0.186

Categorical variables are expressed as frequency (percentage) and continuous variables as the mean (standard deviation) or median (interquartile range). HDL, high-density lipoprotein; LDL, low-density lipoprotein; LVEF, left ventricular ejection fraction. [a] Delta cycle threshold (ΔC_t) values are presented for each microRNA (higher ΔC_t values correspond to lower microRNA expression levels).

5.3.3. Association between cigarette smoking and lower extremity atherosclerosis

Considering all the risk factors for the development of cardiovascular disease that differed according to the presence of LE atherosclerosis in the univariate analysis, the age, cigarette smoking, and creatinine levels were independently associated with the presence of LE atherosclerosis in the multivariate logistic regression analysis (Table 8).

Table 8. Parameters associated with lower extremity atherosclerosis in multivariate logistic regression analysis.

Parameters associated with LE atherosclerosis	**β**	**95% CI**	***p*-value**
Age, years	1.11	1.04–1.18	0.002
Cigarette smoking	4.11	1.89–8.95	0.031
Creatinine, mg/dL	6.29	1.12–33.49	<0.001

95% CI, 95% confidence interval.

5.3.4. Association between cigarette smoking and microRNAs expression

Considering the metabolic and inflammatory parameters and the microRNAs dysregulated in active smokers in the univariate analysis, the leukocyte count and ΔC_t miR-27b were independently associated with active smoking in the multivariate logistic regression analysis (Table 9).

Table 9. Parameters dysregulated in active smokers in multivariate logistic regression analysis.

Parameters dysregulated in active smokers	β	95% CI	*p*-value
Leukocyte count	2.33	1.37–3.94	0.002
ΔC_t miR-27b	1.33	1.02–1.72	0.034

95% CI, 95% confidence interval; ΔC_t, delta cycle threshold.

5.3.5. Association between microRNAs expression and lower extremity atherosclerosis

Considering all the metabolic and inflammatory parameters and the microRNA (miR-27b) dysregulated in active smokers in the univariate analysis, only ΔC_t miR-27b was independently associated with the presence of LE atherosclerosis (β = 1.13, 95% CI 1.01–1.28, p = 0.037). Moreover, as detailed in section 5.2.2., miR-27b was downregulated in more severe presentations of LE atherosclerosis, as assessed by the number of LE lesions and number of sides involved in the LE.

In summary, in multivariate models, cigarette smoking was associated with the presence of LE atherosclerosis, cigarette smoking was associated with miR-27b downregulation, and miR-27b downregulation was associated with the presence of LE atherosclerosis. Active smokers, but not prior smokers presented a downregulation of miR-27b.

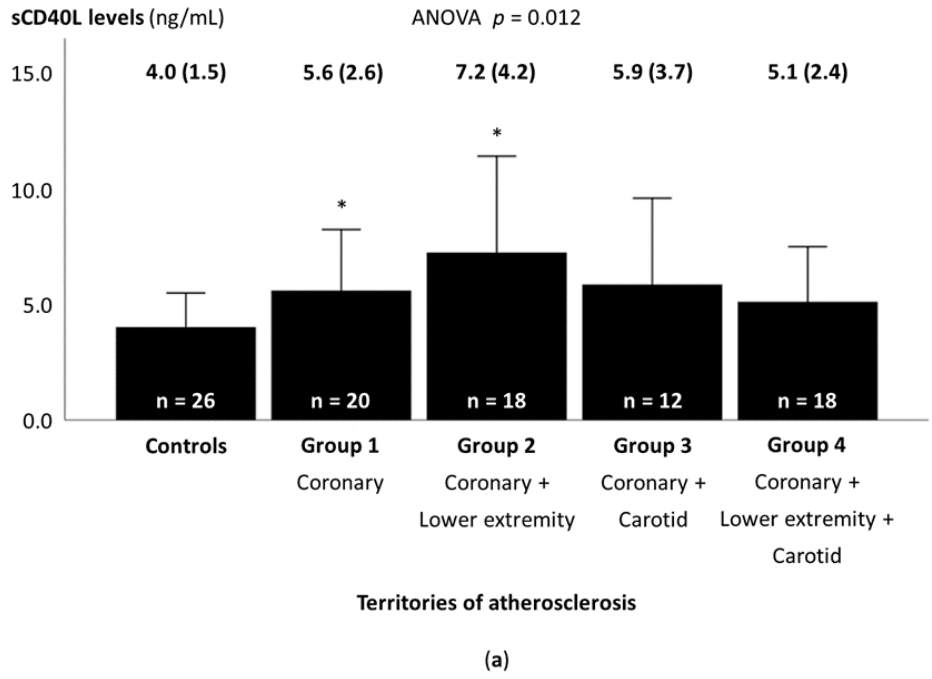
5.4. Soluble CD40 ligand according to single- and multi-territorial atherosclerosis and severity of atherosclerosis in different territories

The results regarding sCD40L levels according to single- and multi-territorial atherosclerosis and severity of atherosclerosis in different territories were published [137,138] and are specified in sections 5.4.1 to 5.4.4.

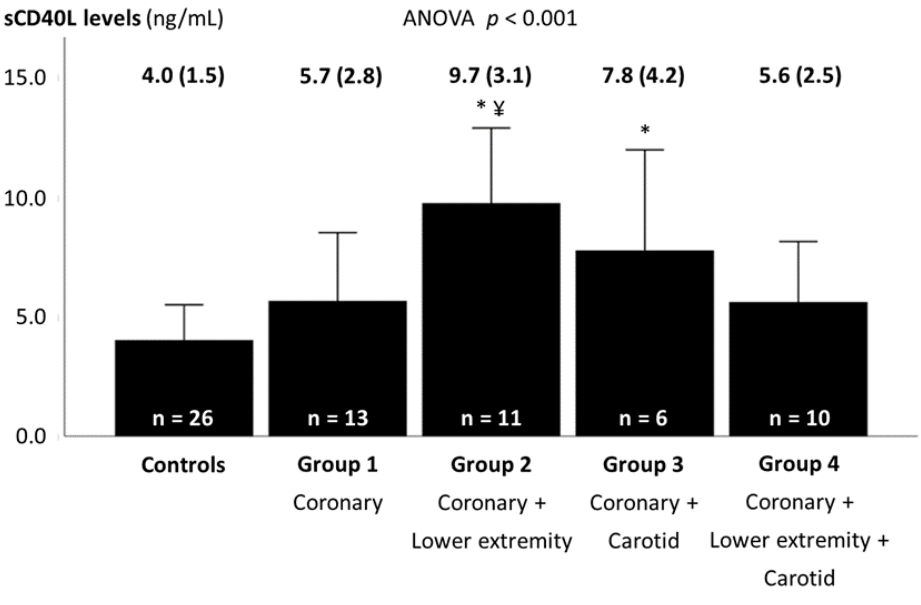
5.4.1 Soluble CD40 ligand according to the systemic extent of atherosclerosis

The sCD40L levels differed across groups (ANOVA p = 0.012) (Figure 5a). Patients from groups 1 (isolated CAD) and 2 (coronary and LE atherosclerosis) showed significantly higher sCD40L levels than control participants, and those from groups 3 (coronary

and carotid atherosclerosis) and 4 (atherosclerosis of the three territories) showed nonsignificantly higher sCD40L levels than controls. In a *post hoc* analysis, excluding patients with prior CABG and/or LE bypass surgery (which are associated with reduced sCD40L levels, as presented in section 5.4.3.), the sCD40L levels were significantly higher in patients from group 2 (coronary and LE atherosclerosis) compared with those in patients from group 1 (isolated CAD) (Figure 5b).



(a)



(b)

Figure 5. Soluble CD40 ligand levels according to the systemic extent of atherosclerosis to different territories. Soluble CD40 ligand levels are presented (**a**) including the whole sample, and (**b**) excluding patients with prior revascularization of the coronary and/or lower extremity arterial territories. Soluble CD40 ligand values are expressed as the mean (standard deviation). sCD40L, soluble CD40 ligand. * *p*-value < 0.05 vs. controls; ¥ *p*-value < 0.05 vs. isolated coronary artery disease.

5.4.2. Soluble CD40 ligand according to the atherosclerosis severity in different territories

Coronary atherosclerosis

Clinically, the sCD40L levels showed a stepwise increase with increasing severity of angina among patients with CAD (ANOVA *p* = 0.001) (Figure 6).

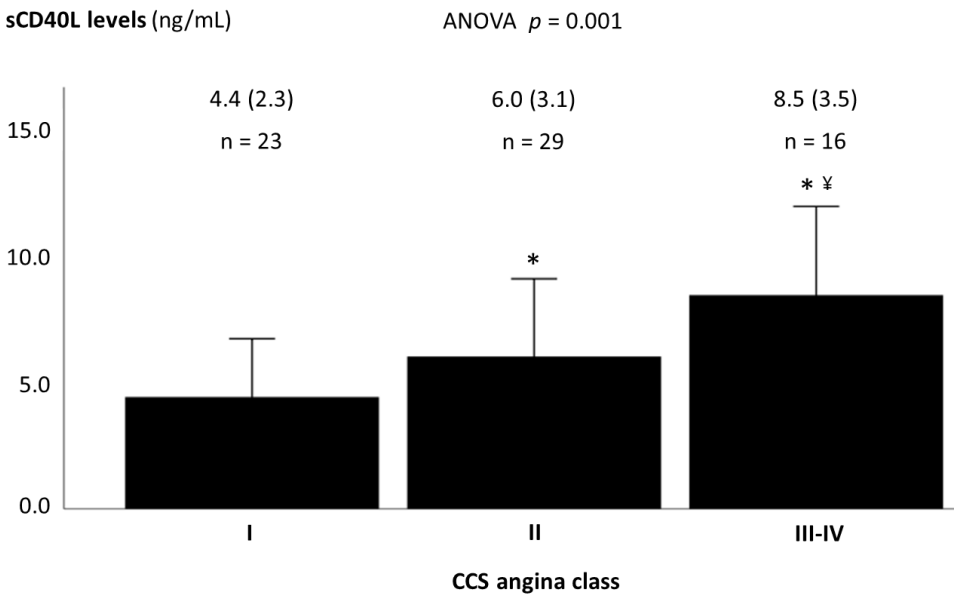


Figure 6. Soluble CD40 ligand levels according to angina class, among patients with coronary artery disease. Soluble CD40 ligand values are expressed as the mean (standard deviation). CCS, Canadian Cardiovascular Society (angina class); sCD40L, soluble CD40 ligand. * *p*-value < 0.05 vs. angina class I; ¥ *p*-value < 0.05 vs. angina class II.

Anatomically, there was a weak positive correlation between sCD40L levels and the number of coronary arteries with obstructive disease (r = 0.285, *p* = 0.006), number of obstructive coronary artery lesions (r = 0.238, *p* = 0.022), Gensini score (r = 0.279, *p* = 0.007), and SYNTAX score (r = 0.265, *p* = 0.015). Considering that prior studies have reported a more consistent association between sCD40L levels and the Gensini score [139] compared with the SYNTAX score [140,141], we further explored the association between sCD40L levels and the Gensini score. The sCD40L levels were increased in higher categories of the Gensini score (ANOVA *p* = 0.026) (Figure 7).

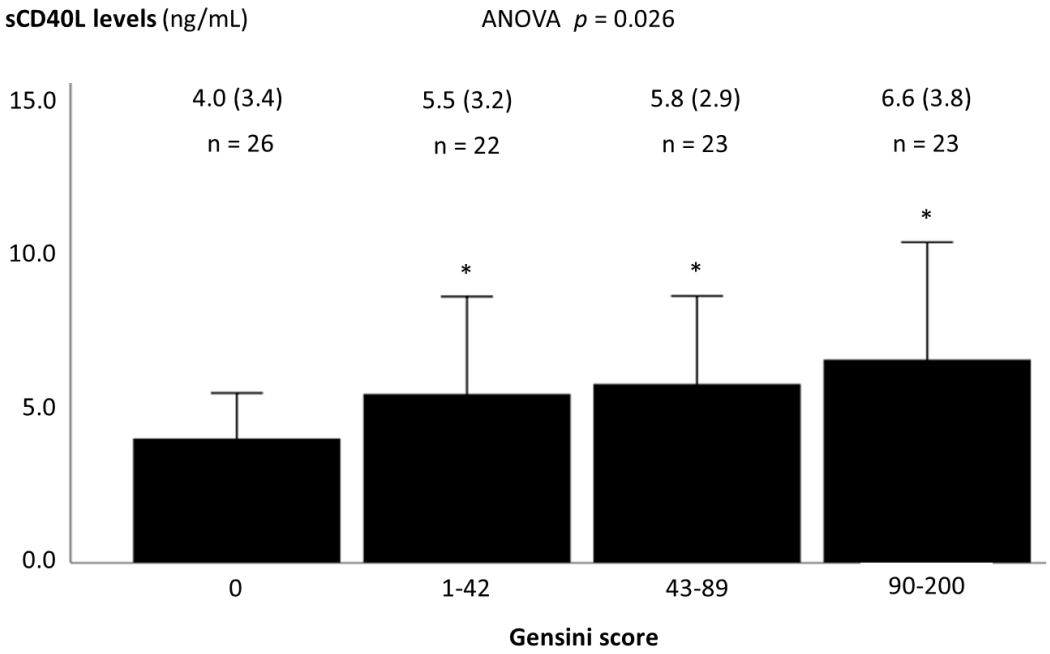


Figure 7. Soluble CD40 ligand levels according to the Gensini score. The first group corresponds to controls (all of which had a Gensini score of zero), and the remaining participants were equally divided into three groups according to the Gensini score. Soluble CD40 ligand values are expressed as the mean (standard deviation). sCD40L, soluble CD40 ligand. * *p*-value < 0.05 vs. Gensini score of zero.

The univariate analysis assessing parameters associated with the Gensini score (as a continuous variable) is presented in Table S5 (Supplementary Materials, section 3.). In the multivariate linear regression analysis, sCD40L was an independent predictor of the Gensini score, in addition to neutrophil/lymphocyte ratio, creatinine levels, and HDL-cholesterol levels (Table 10).

Table 10. Predictors of the Gensini score by multivariate linear regression analysis.

Predictors	**β**	**95% CI**	***p*-value**
Neutrophil/lymphocyte ratio	12.82	4.02–21.63	0.005
Creatinine, mg/dL	33.28	4.39–62.18	0.024
HDL-cholesterol, mg/dL	-1.21	-1.99–[-0.42]	0.003
sCD40L, ng/mL	3.18	0.28–6.08	0.032

95% CI, 95% confidence interval; HDL, high-density lipoprotein; sCD40L, soluble CD40 ligand.

Lower extremity atherosclerosis

For the LE disease, no significant correlation was found between the sCD40L levels and number of arterial segments with obstructive disease (r = 0.157, *p* = 0.147). However, a weak positive correlation between the sCD40L levels and number of arterial segments with obstructive disease was observed excluding patients with prior LE bypass surgery (r = 0.238, *p* = 0.034) and those with prior CABG and/or LE bypass surgery (r = 0.281, *p* = 0.027). The median number of LE segments with obstructive disease was 3 (IQR, 2–5), as aforementioned (section 5.1.1); classifying patients with LE disease according to the median number of diseased segments, the sCD40L levels were significantly higher in patients with three or more LE diseased segments, but not in patients with less than three, compared with participants with no LE disease (Figure 8). sCD40L levels did not differ according to the presence of bilateral or proximal LE disease (Supplementary Materials, section 4., Table S6).

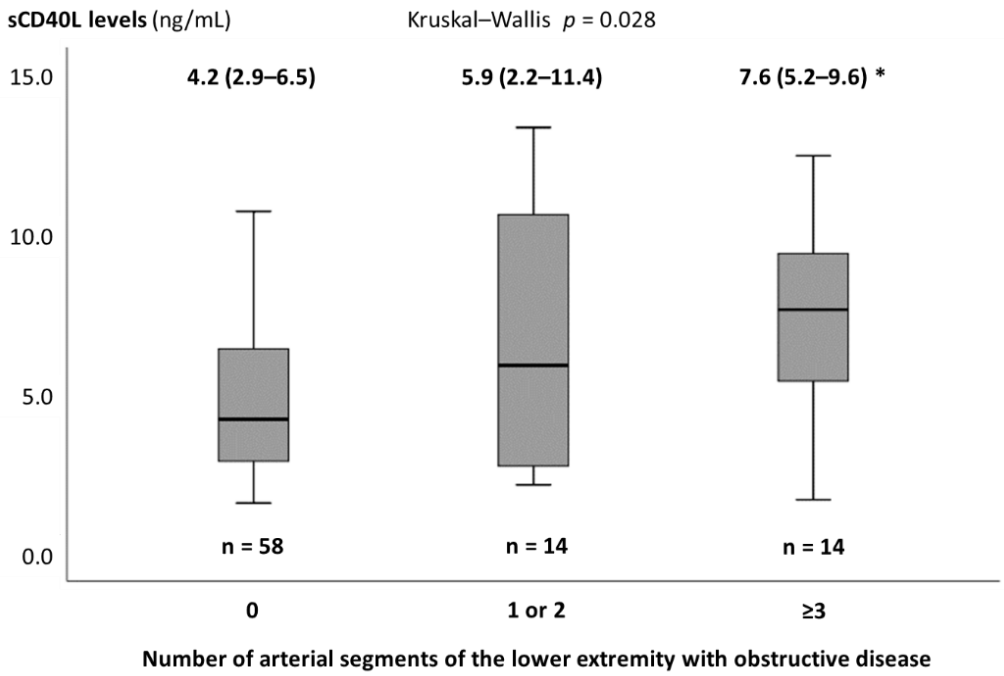


Figure 8. Soluble CD40 ligand levels according to the number of segments of the lower extremity with obstructive disease in participants without prior lower extremity bypass surgery. Soluble CD40 ligand values are expressed as the median (interquartile range). sCD40L, soluble CD40 ligand. * *p*-value < 0.05 vs. no segments with obstructive disease.

Carotid atherosclerosis

For carotid artery disease, the sCD40L levels showed no association with the presence of bilateral disease and no correlation with the mean or maximal IMT (Supplementary Materials, section 4., Table S6). Furthermore, no associations were found after excluding patients with prior CABG and/or LE bypass surgery.

5.4.3. Soluble CD40 ligand and prior revascularization

Among the 68 patients with CAD (groups 1 to 4), 23 had previously undergone CABG and the median time elapsed from CABG was 36 months (IQR, 14 months to 10 years). Patients with prior CABG showed lower levels of sCD40L (Figure 9a). Leukocyte, neutrophil, and platelet counts were also significantly lower in patients with prior CABG (Supplementary Materials, section 5.1., Table S7). There were no other differences between groups of participants with and without prior CABG, including the extent of native CAD (Supplementary Materials, section 5.1., Table S7). In the multivariate logistic regression analysis, sCD40L was the only parameter independently associated with prior CABG (β 0.74, 95% CI 0.60–0.91, p = 0.004). There was no correlation between time elapsed from CABG and sCD40L levels (r = -0.263, p = 0.225).

Among the 36 patients with LE atherosclerosis, 8 had previously undergone LE bypass surgery and the median time elapsed from LE bypass surgery was 4 years (IQR, 2–9 years). Prior LE bypass surgery was associated with lower sCD40L levels (Figure 9b). There were no differences between patients with and without prior LE bypass surgery regarding clinical characteristics, other laboratory data, CAD severity, rates of prior CABG, or proportion of bilateral or of proximal LE disease; there was a trend for a higher number of LE diseased segments in patients with prior LE bypass surgery (Supplementary Materials, section 5.2., Table S8). There was no correlation between time elapsed from LE bypass surgery and sCD40L levels (r = -0.473, p = 0.236).

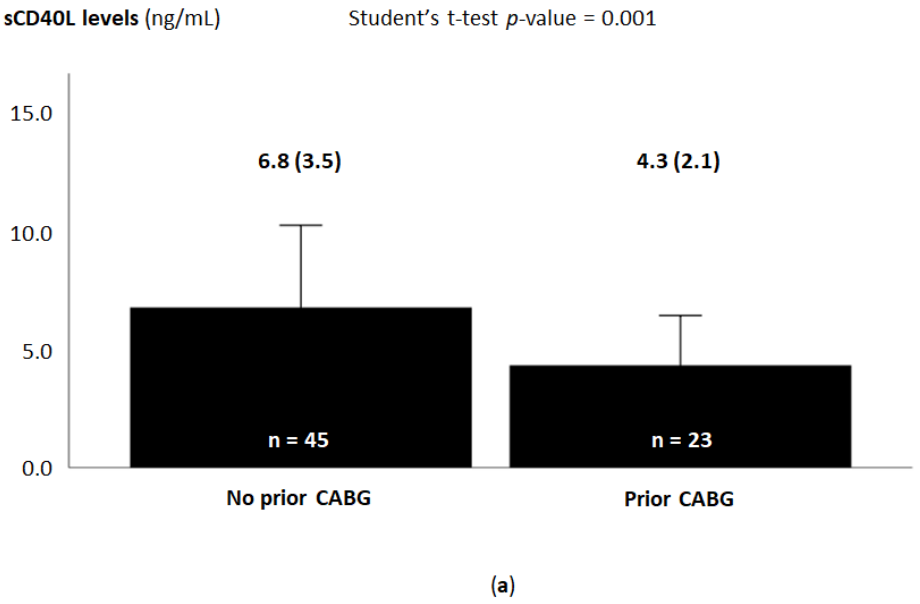


Figure 9. Soluble CD40 ligand levels according to prior revascularization. Soluble CD40 ligand levels are presented for (**a**) patients with coronary atherosclerosis, with and without prior coronary artery bypass grafting; and (**b**) patients with lower extremity atherosclerosis, with and without prior lower extremity bypass surgery, irrespective of coexistent atherosclerosis in other territories. Soluble CD40 ligand values are expressed as the mean (standard deviation). CABG, coronary artery bypass grafting; sCD40L, soluble CD40 ligand.

5.4.4. Predictors of soluble CD40 ligand levels

A detailed univariate analysis on parameters associated with the sCD40L levels is presented in Table S6 (Supplementary Materials, section 4.). In the multivariate linear regression analysis, the independent predictors of the sCD40L levels were coexistent coronary and LE obstructive atherosclerosis, prior CABG, and leukocyte count (Table 11).

Table 11. Predictors of soluble CD40 ligand levels by multivariate linear regression analysis.

Predictors	β	95% CI	*p*-value
Coexistent coronary and lower extremity atherosclerosis	2.51	1.05–3.97	0.001
Prior coronary artery bypass grafting	-1.76	-3.160–[-0.36]	0.015
Leukocyte count	0.42	0.10–0.73	0.010

95% CI, 95% confidence interval.

In summary, sCD40L levels were associated with the systemic extent of atherosclerosis to multiple territories, particularly the coronary and LE territories, and severity of atherosclerosis in those territories; prior revascularization was associated with lower sCD40L levels.

5.5. Tumor necrosis factor alpha and microRNAs expression

The results regarding the association between TNF-α levels and microRNAs expression were published [142] and are specified in sections 5.5.1 to 5.5.3.

5.5.1. Clinical characteristics and laboratory data of patients

The subgroup of 24 participants included in the preliminary exploratory analysis (section 4.6.3. and Supplementary Materials, section 1.1.) was used for assessing which parameters were associated with TNF-α expression. These participants presented a mean age of 65 (9) years, 21 (87.5%) were male and 13 (54.2%) were diabetic (Table 12). The median of TNF-α levels was 1.0 pg/mL (IQR, 0.7–1.1 pg/mL).

Table 12. Characteristics of participants included in the analysis of tumor necrosis factor alpha expression.

Clinical characteristics	
Age, years	65 (9)
Male, n (%)	21 (87.5)
Hypertension, n (%)	22 (91.7)
Dyslipidemia, n (%)	22 (91.7)
Diabetes mellitus, n (%)	13 (54.2)
Smoking history, n (%)	12 (50.0)
Left ventricular ejection fraction > 50%, n (%)	24 (100.0)
Antiplatelet therapy, n (%)	24 (100.0)
Statin therapy, n (%)	22 (91.7)
Coronary atherosclerosis, n (%)	24 (100.0)
Lower extremity atherosclerosis, n (%)	15 (62.5)
Carotid atherosclerosis, n (%)	9 (37.5)
Laboratory data	
Hemoglobin, g/dL	13.6 (1.6)
Leukocyte count, 10^9/L	8.0 (1.5)
Neutrophil count, 10^9/L	4.7 (1.5)
Lymphocyte count, 10^9/L	2.2 (1.7–2.7)
Neutrophil/lymphocyte ratio	1.0 (0.7–1.1)
Platelet count, 10^9/L	236 (41)
Fasting glycaemia, mg/dL	99 (85–166)
Percentage of glycosylated hemoglobin	6.1 (5.6–7.9)
Creatinine, mg/dL	0.9 (0.8–1.3)
Total cholesterol, mg/dL	158 (40)
LDL-cholesterol, mg/dL	92 (29)
HDL-cholesterol, mg/dL	35 (29–43)
Triglycerides, mg/dL	117 (87–162)
Soluble CD40 ligand, ng/mL	8.4 (2.5)
Vascular endothelial growth factor, pg/mL	305 (193–531)
C-reactive protein, mg/L	4.0 (3.6–4.7)
MicroRNAs [a]	
miR-21	17.6 (3.2)
miR-27b	23.4 (20.9–24.4)
miR-29a	22.9 (2.8)

miR-126	23.1 (16.9–24.9)
miR-146a	22.5 (2.5)
miR-218	20.9 (19.5–24.3)

Categorical variables are expressed as frequency (percentage) and continuous variables as the mean (standard deviation) or median (interquartile range). HDL, high-density lipoprotein; LDL, low-density lipoprotein. [a] Delta cycle threshold (ΔC_t) values are presented for each microRNA (higher ΔC_t values correspond to lower microRNA expression levels).

5.5.2. Parameters associated with tumor necrosis factor alpha levels in univariate analysis

The parameters associated with TNF-α levels in the univariate analysis are presented in Table 13. The percentage of glycosylated hemoglobin, serum triglyceride levels, and C-reactive protein levels were positively correlated with TNF-α levels, and there was a trend for a positive correlation between fasting glycemia and TNF-α levels. sCD40L and VEGF levels were not associated with TNF-α levels.

Regarding the candidate microRNAs, ΔC_t miR-146a showed a positive correlation with TNF-α levels, indicating an inverse correlation between miR-146a expression levels and TNF-α levels (Table 13). The expression levels of other microRNAs were not associated with TNF-α levels. Of note, miR-146a expression levels were not associated with other metabolic or inflammatory parameters, including the percentage of glycosylated hemoglobin, serum triglyceride levels, or C-reactive protein levels (Supplementary Materials, section 1.3., Table S2).

Table 13. Parameters associated with tumor necrosis factor alpha levels in univariate analysis.

		TNF-α, pg/mL	***p*-value**
Clinical characteristics			
Age, years [a]		r = -0.145	0.500
Sex [b]	Male	0.9 (0.7–1.1)	1.000
	Female	1.0 (0.5–1.0)	
Hypertension [b]	No	1.5 (1.1–1.5)	0.145
	Yes	0.9 (0.7–1.1)	
Dyslipidemia [b]	No	1.5 (1.1–1.5)	0.181
	Yes	0.9 (0.7–1.1)	
Diabetes mellitus [b]	No	0.9 (0.8–1.2)	0.820

	Yes	1.0 (0.6–1.3)	
Smoking history [b]	No	0.9 (0.6–1.4)	0.713
	Yes	1.0 (0.8–1.1)	
LVEF [b]	≤ 50%	–	–
	> 50%	1.0 (0.7–1.1)	
Antiplatelet therapy [b]	No	–	–
	Yes	1.0 (0.7–1.1)	
Statin therapy [b]	No	0.8 (0.7–0.8)	0.587
	Yes	1.0 (0.7–1.2)	
Coronary atherosclerosis [b]	No	–	–
	Yes	1.0 (0.7–1.1)	
LE atherosclerosis [b]	No	1.0 (0.9–1.6)	0.174
	Yes	0.8 (0.6–1.1)	
Carotid atherosclerosis [b]	No	0.9 (0.8–1.0)	0.861
	Yes	1.1 (0.5–1.7)	
Laboratory data			
Hemoglobin, g/dL [a]		r = -0.343	0.101
Leukocyte count, 10^9/L [a]		r = 0.167	0.436
Neutrophil count, 10^9/L [a]		r = 0.186	0.385
Lymphocyte count, 10^9/L [a]		r = -0.352	0.870
Neutrophil/lymphocyte ratio [a]		r = 0.115	0.592
Platelet count, 10^9/L [a]		r = 0.195	0.360
Fasting glycaemia, mg/dL [a]		r = 0.395	0.056
Percentage of glycosylated hemoglobin [a]		r = 0.418	0.042
Creatinine, mg/dL [a]		r = 0.362	0.082
Total cholesterol, mg/dL [a]		r = 0.129	0.549
LDL-cholesterol, mg/dL [a]		r = -0.094	0.662
HDL-cholesterol, mg/dL [a]		r = -0.271	0.201
Triglycerides, mg/dL [a]		r = 0.429	0.037
Soluble CD40 ligand, ng/mL [a]		r = 0.170	0.427
Vascular endothelial growth factor, pg/mL [a]		r = 0.123	0.568
C-reactive protein, mg/L [a]		r = 0.407	0.048
MicroRNAs [a]			
miR-21		r = 0.278	0.199
miR-27b		r = 0.328	0.198
miR-29a		r = 0.189	0.627
miR-126		r = 0.374	0.139
miR-146a		r = 0.500	0.035
miR-218		r = 0.408	0.423

[a] Correlations between TNF-α levels and continuous variables were tested and the correlation coefficient (r) is presented for each; [b] TNF-α levels were compared between groups for categorical variables and are expressed as the mean (standard deviation) or median (interquartile range). HDL, high-density lipoprotein; LDL, low-density lipoprotein; LE, lower extremity; LVEF, left ventricular ejection fraction; TNF-α, tumor necrosis factor alpha.

5.5.3. Parameters associated with tumor necrosis factor alpha levels in multivariate analysis

In the multivariate analysis, serum triglyceride levels and miR-146a expression levels were independently associated with TNF-α levels (Table 14). Lower miR-146a expression levels and higher serum triglyceride levels were associated with increased TNF-α levels.

Table 14. Predictors of tumor necrosis factor alpha levels by multivariate linear regression analysis.

Predictors of TNF-α levels	β	95% CI	*p*-value
Serum triglyceride levels	0.003	0.001–0.004	0.008
ΔC_t miR-146a	0.111	0.026–0.196	0.014

95% CI, 95% confidence interval; TNF-α, tumor necrosis factor alpha; ΔC_t, delta cycle threshold.

In summary, in patients with stable atherosclerosis, metabolic and post-transcriptional (microRNA) factors were associated with TNF-α expression. Lower miR-146a expression levels were associated with higher TNF-α levels, irrespective of other metabolic and inflammatory parameters.

6. Discussion

In this research project, we assessed the expression of circulating microRNAs and inflammatory mediators according to the presence of single- or multi-territorial atherosclerosis. Four main result sets stood out: the expression levels of circulating microRNAs (miR-27b and miR-146a) were associated with the presence of multi-territorial atherosclerosis and severity of atherosclerosis in different territories; of these, miR-27b appeared to be a mediator of cigarette smoking-induced toxicity, specifically LE atherosclerosis; sCD40L levels were associated with the systemic extent of atherosclerosis to multiple territories, atherosclerosis severity in different territories, and prior arterial revascularization; and TNF-α levels were inversely correlated with miR-146a expression levels.

Multi-territorial atherosclerosis is frequently encountered in clinical practice and is associated with a higher morbidity and risk of mortality compared with single-territorial atherosclerosis [5,6]. Little is known about the mechanisms that regulate the atherosclerosis extent to single or multiple arterial beds [1,2]. Although there are common drivers for atherosclerosis development irrespective of its systemic extent, multi-territorial atherosclerosis may have a different pathophysiology than that of single-territorial atherosclerosis [1,2]. The heterogeneity in the systemic extent of atherosclerosis is only partially attributable to acquired cardiovascular risk factors and genetic Mendelian inheritance [8,9], and post-transcriptional and inflammatory mediators contribute independently to such heterogeneity [4,11]. Understanding the molecular phenotypes of single- and multi-territorial atherosclerosis may provide insights into its pathophysiology and contribute to identify biomarkers for use in clinical practice [4,11,16,18]. Of note, the relevance of identifying key molecular regulators of atherosclerosis has regained interest following the publication of favourable clinical results of a pure anti-inflammatory agent in patients with atherosclerosis, highlighting the potential role of such molecular regulators as therapeutic targets in stable atherosclerosis [13].

The observed signature of post-transcriptional (microRNAs) and inflammatory mediators according to the extent of atherosclerosis, particularly the extent to multiple territories, is herein discussed.

6.1. MicroRNA profiles according to single- and multi-territorial atherosclerosis and severity of atherosclerosis in different territories

In the analysis of microRNA profiles according to single- and multi-territorial atherosclerosis and severity of atherosclerosis in different territories, the four main findings were: lower expression levels of miR-27b and miR-146a were associated with an increase in the systemic extent of atherosclerosis to multiple territories, particularly if involving the coronary, LE, and carotid territories; lower expression levels of miR-27b and miR-146a were associated with an increase in the atherosclerosis severity in the three territories; the coexistence of atherosclerosis in the coronary, LE, and carotid territories was independently associated with miR-27b and miR-146a expression levels; and both miR-27b and miR-146a were reasonably accurate in predicting severe systemic atherosclerosis with concomitant involvement of the three territories.

To the best of our knowledge, we provide the first description of the circulating microRNA expression profile in single- and multi-territorial atherosclerosis with a systematic screening of atherosclerotic lesions in three major territories of atherosclerosis. We observed that multi-territorial atherosclerosis was associated with a specific microRNA expression profile, particularly in multi-territorial atherosclerosis with simultaneous involvement of the coronary, LE, and carotid territories. In such a scenario, both the atheroprotective miR-27b and miR-146a were significantly downregulated. The systemic extent of atherosclerosis with involvement of the three territories was a major determinant of miR-27b and miR-146a expression levels in multivariate analysis, independently of the atherosclerosis severity in each territory. Moreover, miR-27b and miR-146a showed reasonable accuracy for predicting severe systemic atherosclerosis with involvement of the three territories. The results highlight miR-27b and miR-146a as potential biomarkers that can contribute to detect multi-territorial atherosclerosis noninvasively. Screening of multi-territorial atherosclerosis in daily clinical practice by assessing simultaneously different arterial territories using currently available methods is potentially laborious [2,3]. Therefore, miR-27b and miR-146a may simplify the stratification of systemic atherosclerotic burden [2,3]. Considering the high morbidity and risk of mortality associated with multi-territorial atherosclerosis [5,6], the identification of severe systemic atherosclerosis using a simple, noninvasive tool may be clinically useful as it could facilitate an early intensification of the atheroprotective regimens, such as antithrombotic and lipid-lowering therapies [29-33]. Further studies are needed to confirm these hypotheses.

Consistent with the results of the systemic extent of atherosclerosis, the expression levels of miR-27b and miR-146a decreased with an increase in the severity of atherosclerosis in different territories, as assessed by different, complementary indexes, including the number of coronary arteries with obstructive disease, number of coronary artery lesions, SYNTAX score, number of obstructive lesions in the LE, number of sides involved in the LE, and number of sides involved in the carotid territory. Interestingly, there was no association with any of the candidate microRNAs and the Gensini score, conversely to the SYNTAX score. The Gensini score is mostly associated with the myocardial ischemic burden since it grades, for each lesion, the percent stenosis and weights the lesion according to the amount of myocardium it supplies [111]. The SYNTAX score is also associated with the myocardial ischemic burden but mainly reflects the complexity of coronary artery lesions and the atherosclerotic plaque burden [112,113]. The SYNTAX score includes, among other parameters, the number of segments involved per lesion, lesion length, presence of diffuse disease, and severe calcification, which do not necessarily reflect the myocardial ischemic burden but are associated with the complexity of CAD and atherosclerotic plaque burden [112,113]. The association between expression levels of miR-27b and miR-146a and the SYNTAX score, but not the Gensini score, suggest that the expression of these microRNAs is more related with the complexity of CAD and the atherosclerotic plaque burden rather than the myocardial ischemic burden.

Published data regarding the roles of miR-27b and miR-146a in experimental studies and expression levels of these microRNAs in patients with atherosclerosis are discussed below. Atheroprotective mechanisms of miR-27b and miR-146a have been described in experimental models, which are consistent with the observed dysregulation of both microRNAs in our sample [11,18,48-52,54,56,61-63]. In summary, miR-27b has complementary atheroprotective effects: it contributes to a downregulation of the expression of key genes involved in lipid metabolism, mitigation of the accumulation of lipids in circulation, and blockage of lipid-induced atherogenesis [48,49]; reduces vascular inflammation by suppressing the release of proinflammatory factors [48,50,51], leading to a decrease in monocyte–macrophage activation [52]; contributes to endothelial integrity as it facilitates the formation of tight endothelial monolayers and stable vessels in response to shear stress [54]; and is proangiogenic [52,56]. miR-146a is induced in endothelial cells in response to proinflammatory cytokines and acts as a negative feedback regulator of inflammatory signalling in endothelial cells, promotes eNOS expression, and inhibits NAPDH Oxidase 4 expression [11,18,61,62]. In addition, miR-146a reduces atherogenesis by

inhibiting monocyte–macrophage activation, including the formation of foam cells [63]. Considering the miR-27b and miR-146a dysregulation observed in our study and their specific biological roles described in the aforementioned experimental studies, the extent of atherosclerosis to multiple territories and atherosclerosis severity in each territory appear to be influenced by a post-transcriptional regulation of the lipid metabolism and lipid accumulation in the vessel, inflammatory response involving endothelial and monocyte–macrophage cells, and endothelial function. The expression levels of miR-21, miR-29a, miR-126, and miR-218 did not differ according to the systemic extent of atherosclerosis to multiple territories. Possibly, vascular smooth muscle cell function (miR-21), fibrosis and extracellular matrix composition (miR-29a), endothelial function in response to shear stress (miR-126), and endothelial cell migration and angiogenesis (miR-218) are not the main regulators of the extent of atherosclerosis to multiple territories, despite their described role in atherogenesis in experimental studies [11,18,36]. Of note, miR-126 was downregulated in association with an increasing number of LE lesions. Since miR-126 is a mechanosensitive microRNA that is downregulated in response to disturbed flow and shear stress, the results point to the relevance of shear stress in the expression of LE atherosclerosis [60].

Published data about miR-27b and miR-146a expression in patients with atherosclerosis are scarce. Regarding miR-27b, Signorelli et al. [143] reported an upregulation of miR-27b in patients with LE atherosclerosis compared with controls. On the other hand, Stather et al. [131] reported a downregulation of miR-27b in patients with LE atherosclerosis, and our findings are consistent with their results. Contrary to the study by Signorelli et al. [143], Stather et al. [131] confirmed the presence of LE atherosclerosis using imaging methods, used one derivation and two validation sample sets, reported the diagnostic accuracy of miR-27b for detecting LE atherosclerosis, and presented a pathway enrichment analysis. There is a known biological variation in microRNAs expression levels, occasionally with conflicting results between different studies focused on the same disease [144]. The miR-27b downregulation observed in our study reinforces the results of the study by Stather et al. [131], where a very robust methodology was used. Additional data on miR-27b expression in patients with stable atherosclerosis are lacking. Regarding miR-146a, although it was reported to be upregulated in patients with coronary atherosclerosis [145], which conflicts with the experimental data, it was also reported to be downregulated in more severe forms of coronary atherosclerosis, including acute coronary syndromes, compared with stable CAD [146], and coronary atherosclerosis

associated with poor collateral circulation [147]. No data have been published on the expression levels of these two microRNAs in multi-territorial disease. As previously discussed, not only data from experimental studies but also studies in humans are consistent with the downregulation of miR-27b and miR-146a in more severe forms of atherosclerosis, including multi-territorial atherosclerosis.

6.2. Cigarette smoking toxicity, microRNAs, and lower extremity atherosclerosis

In the analysis of the expression of circulating microRNAs according to cigarette-smoking status, three main findings stood out: cigarette smoking was associated with the presence of LE atherosclerosis, cigarette smoking was associated with miR-27b downregulation, and miR-27b downregulation was associated with the presence and severity of LE atherosclerosis. These results suggest that miR-27b mediates the proatherogenic effects of cigarette smoking.

The expression levels of circulating microRNAs may be influenced by exogenous factors, such as cigarette smoking [148]. We observed that the atheroprotective miR-27b was downregulated in active smokers compared with non-active smokers (including never-smokers and prior smokers), independently of other metabolic and inflammatory parameters. These results suggest a detrimental effect of active cigarette smoking on miR-27b expression, similar to the reported effect of cigarette smoking on other atheroprotective microRNAs [149]. miR-27b was significantly downregulated in active smokers compared with prior smokers but not with never-smokers, although there was a trend towards miR-27b downregulation in active smokers compared with never-smokers. The latter may be explained by a limited sample size. Based on an extensive *post hoc* analysis, the absence of differences in miR-27b expression levels between active smokers and never-smokers is difficult to explain and we did not find additional factors that could contribute to such results. Nevertheless, the numerical differences between active smokers and never-smokers were close to the margin of statistical significance and are consistent with a culprit effect of active smoking in downregulating miR-27b compared with prior smokers and, likely, never-smokers as well. There were no significant differences in miR-27b expression levels between prior smokers and never-smokers. Such results may be explained by a sensitivity of miR-27b expression levels to active smoking without a legacy effect [149,150]. Specifically, smoking cessation was reported to completely revert the dysregulation of some microRNAs observed in active smokers [149,150], and

this may also be the case of miR-27b. These data reinforce the atheroprotective effects of smoking cessation. Published data addressing the effect of smoking on miR-27b expression are limited [149-151]. A nonsignificant downregulation of miR-27b was reported in human oral keratinocytes in association with cigarette smoke exposure [151]. In our study, the downregulation of circulating miR-27b in active smokers was significant, adjusting for other metabolic and inflammatory parameters.

Cigarette smoking is a recognized causal risk factor for the development of atherosclerosis, particularly in the LE territory [10,152]. Some of the mechanisms associated with cigarette-smoking-induced atherogenesis include the activation of inflammation, dysregulation of the lipid metabolism, increase in oxidative stress, and endothelial dysfunction [10,152]. Nevertheless, the pathophysiology associated with the initiation and progression of atherosclerosis secondary to cigarette smoking, particularly the post-transcriptional regulation, is not entirely known [10,152]. The dysregulation of circulating microRNAs is known to be associated with the development of atherosclerosis, as previously discussed [11,18]. In our study, a downregulation of miR-27b expression was associated with the presence and severity of LE atherosclerosis. This suggests that miR-27b contributed to atherogenesis in the LE, and this hypothesis is supported by published data regarding the atheroprotective roles of miR-27b in experimental studies and miR-27b expression levels in patients with atherosclerosis, as discussed in section 6.1.

In summary, the downregulation of miR-27b in active smokers and the independent association between miR-27b and the presence of LE atherosclerosis in this study suggest that miR-27b was downregulated due to active cigarette smoking and that such dysregulation contributed to LE atherosclerosis (Figure 10). The results are, therefore, consistent with miR-27b acting as a mediator of cigarette-smoking toxicity, specifically in the development of LE atherosclerosis.

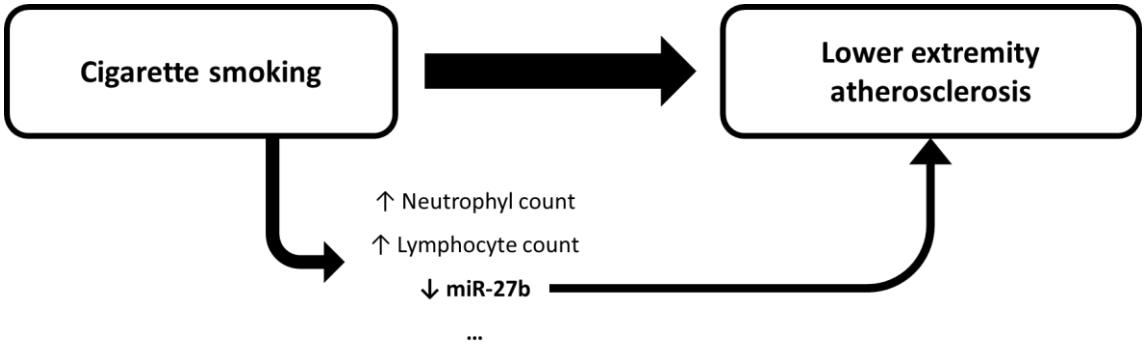


Figure 10. Putative role of miR-27b in the toxicity induced by cigarette smoking. Cigarette smoking was associated with the presence of lower extremity atherosclerosis and with miR-27b downregulation; downregulation of the atheroprotective miR-27b was associated with the presence and severity of lower extremity atherosclerosis; miR-27b may mediate the atherogenesis induced by cigarette smoking.

The results indicate associations among cigarette smoking, miR-27b dysregulation, and LE atherosclerosis, but not a causal effect. Nevertheless, the adjustment for confounders in the multivariate analyses and the consistency of the results with the aforementioned experimental data [48-52,54] suggest that miR-27b is likely a mediator of cigarette-smoking toxicity, specifically in what regards LE atherosclerosis.

6.3. Soluble CD40 ligand according to single- and multi-territorial atherosclerosis and severity of atherosclerosis in different territories

In this analysis, three main findings stood out: sCD40L levels varied according to the systemic extent of atherosclerosis to a single or multiple arterial territories, higher sCD40L levels were associated with higher severity of atherosclerosis in the coronary and LE territories, and prior bypass surgery of the coronary and LE territories was associated with lower sCD40L levels.

To the best of our knowledge, this is the first study assessing sCD40L levels in single- and multi-territorial atherosclerosis with the systematic screening of atherosclerotic lesions in three major territories of atherosclerosis. The higher sCD40L levels in atherosclerosis of multiple territories (coronary and LE) suggest that sCD40L is a common denominator of the atherosclerosis expression and is associated with the systemic extent of atherosclerosis. Multi-territorial atherosclerosis was a major

determinant of sCD40L levels in the multivariate analysis, independently of the atherosclerosis severity in each territory.

The sCD40L levels were higher in combined CAD and LE atherosclerosis, but not in combined CAD and carotid atherosclerosis, compared with CAD alone. In a systematic review and meta-analysis conducted by the team that collaborated with this research project, carotid atherosclerosis was associated with a higher dysregulation of sCD40L levels, followed by the coronary, LE, and renal artery territories, although the differences between groups were not significant [79]. These results were based on single-territorial atherosclerosis and differ from the analysis in our sample on multi-territorial atherosclerosis [79]. We speculate that single-territorial atherosclerosis has a different pathophysiology compared with that of multi-territorial atherosclerosis; likely, in our sample, the regulation of sCD40L levels was mainly associated with the presence of CAD and the coexistence of carotid atherosclerosis did not impact further on sCD40L levels, in the contrary to LE atherosclerosis. The local expression of inflammatory markers differs in carotid and femoral atherosclerotic plaques [153], and the stimulated LE iliac arteries may express more intensively CD40L *in situ* than the stimulated carotid arteries [154]. On the other hand, obstructive atherosclerosis of the LE could result in a higher degree of oxidative stress and inflammation compared with carotid atherosclerosis, considering the highly demanding LE muscles during physical effort and bilateral carotid blood supply to the cerebral territory [23]. For instance, the expression of miR-210, an adaptive microRNA to oxidative stress and inflammation, is altered in the presence of LE atherosclerosis but not in carotid atherosclerosis [23]. This could explain the higher levels of sCD40L in the presence of LE atherosclerosis compared with carotid atherosclerosis, in patients with CAD. In addition, bifurcations increase shear stress, which upregulates the expression of CD40 ligand on platelets (the main source of sCD40L), thereby accelerating atherogenesis and further increasing shear stress [155-157]. The higher number of bifurcations in the LE may also have contributed to higher dysregulation of sCD40L levels in the presence of atherosclerosis, compared with the carotid territory, in a background of CAD.

The sCD40L levels were nonsignificantly higher in group 4 (atherosclerosis of the three arterial territories) compared with controls, corresponding to a trend consistent with the results of increased sCD40L levels in group 2 (atherosclerosis of the coronary and LE territories) compared with controls. On the other hand, group 4 presented similar sCD40L levels compared with group 1 (CAD alone). This finding is difficult to explain based on the extensive *post hoc* analysis performed. Nevertheless, group 4

presented a slightly better metabolic control compared with group 2, as reflected by nonsignificant lower fasting glycemia levels, higher HDL-cholesterol levels (which inhibit platelet activity through scavenger receptor B type I), and lower serum triglyceride levels, which may have contributed to lower sCD40L levels in group 4 [150,158-161]. We acknowledge that possible unmeasured confounders may have contributed to lower sCD40L levels in group 4.

Regarding the severity of atherosclerosis in each territory, higher sCD40L levels were associated with higher CAD severity. Of the few data available on this subject, no association was reported between sCD40L levels and the anatomical severity of CAD, as assessed by an angiographic score [162,163] or by the coronary artery calcium score [164]. Theses scores reflect the atherosclerotic plaque burden and not necessarily the area of myocardium supplied by each lesion or the ischemic burden [162-164]. In our research project, the sCD40L levels were positively correlated with the Gensini and SYNTAX scores, both of which indirectly reflect the myocardial ischemic burden, particularly the former score [111-113]. Accordingly, a prior study reported a positive correlation between sCD40L levels and the Gensini score [139], although the association between sCD40L levels and the SYNTAX score was not consistent in prior studies [140,141]. Probably, sCD40L levels are primarily associated with the myocardial ischemic burden, as reflected by the Gensini score, rather than the atherosclerotic plaque burden. This hypothesis is further supported by our results of decreased sCD40L levels in patients with prior CABG and/or LE bypass surgery (as discussed below) and the results of increased sCD40L levels in clinical but not subclinical atherosclerosis (defined by atherosclerosis without documented functional significance) in a systematic review and meta-analysis conducted by our research team [79]. Of note, to the best of our knowledge, there are no published data on the association between angina severity, as a measure of the clinical severity of CAD, and sCD40L levels. Our results of increased sCD40L levels in association with increased angina severity may add to the knowledge on the coupling between inflammation and clinical status. Regarding LE atherosclerosis, sCD40L levels were associated with disease severity in non-revascularized patients. These results are consistent with the very few studies describing the sCD40L levels according to the severity of LE disease, as assessed by the lesion length [82] or by an angiographic score based on the degree of luminal stenosis in each arterial segment [114]. We acknowledge that the association between sCD40L levels and the severity of LE atherosclerosis observed in our study may be affected by a confounding effect of baseline CAD, in patients with LE atherosclerosis. For the carotid arteries, the association between sCD40L levels and

atherosclerosis severity is less well established and further studies are needed to assess such an association, particularly because the carotid territory was associated with the greatest dysregulation of sCD40L levels in single-territorial atherosclerosis [79,165].

To the best of our knowledge, we provide the first description of lower sCD40L levels in patients with prior coronary and LE surgical revascularization. The scarce data available on the long-term effects of bypass surgery in patients with atherosclerosis point to an attenuation of the acute-phase reaction, reflected by a reduction in C-reactive protein levels [166]. Specifically, for sCD40L, the levels were reported to rise immediately after CABG, probably due to endothelial disruption, platelet function dysregulation, and inflammatory activation related to the surgery itself, followed by a decrease in the first month after surgery [167]. Nevertheless, no prior studies reported a reduction in sCD40L levels in the long-term after surgical bypass placement. We speculate that the association between sCD40L and atherosclerosis is bidirectional, with inflammation mediated by sCD40L contributing to atherogenesis, as demonstrated in animal models [156], and with ischemia driven by atherosclerosis exacerbating sCD40L-mediated inflammation [81]. It is known that severe chronic ischemia promotes the release of reactive oxygen species, cytokines and other inflammatory markers [168,169]. It is possible that the reduction of ischemic stress after coronary and LE bypass grafting may have reduced inflammation, which translated into lower sCD40L release into the peripheral blood [104]. The fact that prior bypass surgery of two distinct territories (coronary and LE) was associated with lower sCD40L levels, and the independent role of prior CABG on sCD40L expression, as highlighted in the multivariate analysis, add consistency to the association between revascularization status and sCD40L expression. Of note, the variation of sCD40L levels according to revascularization treatments was a *post hoc* analysis. Further studies are needed to confirm the hypothesis of bidirectional interaction between atherosclerosis and sCD40L.

6.4. Tumor necrosis factor alpha and microRNAs expression

In this analysis, two main findings were noted: in patients with CAD, metabolic and post-transcriptional (microRNA) factors were associated with TNF-α expression, and higher TNF-α levels were associated with lower miR-146a expression levels irrespective of other metabolic and inflammatory parameters.

TNF-α is a proatherogenic agent and closely associated with prognosis in patients with CAD [53,170,171]. Nevertheless, the regulators of TNF-α expression in patients with stable atherosclerosis are not fully understood [89]. In our study, metabolic dysregulation, characterized by a higher percentage of glycosylated hemoglobin or higher serum triglyceride levels, was associated with higher TNF-α levels, which is consistent with the reports from preclinical and clinical studies [90-95]. Of note, TNF-α may itself promote hyperglycemia and dyslipidemia, thereby increasing further the cardiovascular risk [172,173]. Data on the association between post-transcriptional mediators and TNF-α expression are scarce [12,174,175]. There are reports that miR-146a suppresses the inflammatory response by downregulating TNF-α expression in experimental models [12]. In humans, a negative correlation between miR-146a expression levels and TNF-α levels has been described in patients with noncardiac inflammatory diseases, although such an association has not been reported in patients with stable CAD [174,175]. Consistently, we observed a negative correlation between miR-146a expression levels and TNF-α levels. Of note, the association was independent of other metabolic and inflammatory parameters, which are frequently abnormal in CAD and influence TNF-α expression [90-96]. The results are in line with the reported atheroprotective role of miR-146a and add to the consistency of miR-146a expression levels according the systemic extent of atherosclerosis to multiple territories (section 5.2.1.) and atherosclerosis severity in each territory (section 5.2.2.) observed in our sample.

The results support an independent role of miR-146a in the regulation of TNF-α-induced inflammation in stable CAD and suggest miR-146a as a potential therapeutic target, complementary to other disease-modifying strategies, such as glycemic and lipid control. The use of miR-146a mimics in patients with stable CAD presenting enhanced inflammatory activation based on TNF-α levels may be a potential field for investigation [14].

7. Conclusions

This research project was focused on the evaluation of circulating microRNA profiles according to the expression of stable atherosclerosis, particularly its systemic extent to a single or multiple arterial territories, and was complemented with the assessment of inflammatory markers (sCD40L and TNF-α), considering the independent and interdependent roles of both types of mediators (microRNAs and inflammatory markers) in the regulation of atherosclerosis. The conclusions of this investigation and what the results added to the knowledge about atherosclerosis and to clinical practice are highlighted.

Lower expression levels of the atheroprotective miR-27b and miR-146a were associated with the presence of multi-territorial atherosclerosis, particularly if involving the coronary, LE, and carotid territories, and with higher severity of atherosclerosis in the three territories. The simultaneous involvement of the coronary, LE, and carotid territories was independently associated with miR-27b and miR-146a dysregulation. Moreover, both microRNAs showed reasonable accuracy for predicting severe systemic atherosclerosis involving the three territories. These results provide insights into the pathophysiology of multi-territorial atherosclerosis, pointing to the relevance of described microRNA modulation of specific pathways in the regulation of atherosclerosis extent to multiple territories, including lipid metabolism, lipid accumulation in the vessel, inflammatory response involving endothelial and monocyte–macrophage cells, and endothelial function. In addition, miR-27b and miR-146a seem to be promising noninvasive biomarkers for refining the stratification of systemic atherosclerotic burden in clinical practice and, therefore, may contribute to the tailoring of primary prevention strategies.

Cigarette smoking was associated with the presence of LE atherosclerosis. Active smokers, but not prior smokers, presented a downregulation of miR-27b expression levels, and such dysregulation was associated with the presence and severity of LE atherosclerosis. These data suggest that miR-27b mediates the proatherogenic effects of cigarette smoking and that cigarette-smoking cessation may be associated with an attenuation of miR-27b dysregulation. These data provide insights into the regulation of cigarette-smoking toxicity and associated LE atherosclerosis, and reinforce the potential benefits of cigarette-smoking cessation on the post-transcriptional (microRNA) regulation.

CONCLUSIONS

The sCD40L levels were higher in patients with atherosclerosis, particularly in those with multi-territorial disease involving the coronary and LE territories. The sCD40L levels increased with higher severity of coronary and LE atherosclerosis, while prior CABG and/or LE surgical revascularization were associated with lower sCD40L levels. These results provide insights into the inflammatory signature associated with the expression of atherosclerosis, including multi-territorial disease. Furthermore, sCD40L seems to be a promising noninvasive tool for refining the stratification of systemic atherosclerotic burden and could contribute to individualize the primary prevention strategies.

Finally, metabolic and post-transcriptional (miR-146a) factors were associated with TNF-α expression in patients with CAD. miR-146a expression levels were negatively correlated with TNF-α levels and this association was independent of other metabolic or inflammatory parameters. These data highlight the coupling of post-transcriptional regulation and inflammatory activation in stable atherosclerosis.

8. Strengths and limitations

8.1. Strengths

To the best of our knowledge, we used a new clinical model for investigating the expression of circulating biomarkers in single- (coronary) and multi-territorial (coronary and extra-coronary) atherosclerosis. This model was based on obstructive atherosclerosis, which may be more relevant for clinical practice than subclinical atherosclerosis, due to the higher atherosclerotic plaque burden and the associated symptomatic, therapeutic, and prognostic implications [2,3]. Three major arterial territories of atherosclerosis were prospectively and systematically screened in all participants, including the coronary, LE, and carotid territories. Of note, all patients (groups 1–4) presented native obstructive atherosclerosis, as prior endarterectomy and percutaneous revascularization procedures were exclusion criteria, in order to reduce bias. Moreover, exclusion of CAD in control participants was based on coronary computed tomography angiography, which is a very sensitive method compared with other invasive and noninvasive methods [109].

Importantly, as far as we know, we provide the first description of the circulating microRNAs signature according to the presence of single- and multi-territorial atherosclerosis (specifically, miR-27b and miR-146a downregulation in multi-territorial atherosclerosis), with a systematic screening of different arterial territories. The consistency of results was reinforced for the following reasons: the multivariate analysis carried out confirmed the independent association between multi-territorial atherosclerosis and miR-27b and miR-146a expression levels; analyses of the severity of atherosclerosis in different territories showed a similar direction of miR-27b and miR-146a dysregulation compared with the analyses of the systemic extent of atherosclerosis to multiple territories (specifically, miR-27b and miR-146a downregulation in more severe presentations of atherosclerosis); miR-27b downregulation was likely associated with smoking-induced toxicity, specifically LE atherosclerosis, supporting miR-27b as an atheroprotective mediator; miR-146a downregulation was associated with an increased inflammatory activation based on TNF-α levels, independently of metabolic parameters, supporting miR-146a as an atheroprotective mediator; and the results of miR-27b and miR-146a dysregulation according to the atherosclerosis severity are in line with data from experimental studies and studies in patients with atherosclerosis [11,18,48-52,54,56,61-63,131,146,147].

In addition, we provide the first report on the diagnostic accuracy of circulating microRNAs, specifically miR-27b and miR-146a, for the detection of severe, multi-territorial atherosclerosis. The results have potential implications for a condition that is frequent in clinical practice and associated with considerable morbimortality [5,6], as both microRNAs may consist of simple noninvasive tools for stratifying the systemic atherosclerotic burden.

To the best of our knowledge, the variation of sCD40L levels according to single- and multi-territorial atherosclerosis, with a prospective and systematic assessment of different territories, has not yet been described. The further analyses added to the consistency of results: the multivariate analysis indicated an independent association between multi-territorial (coronary and LE) atherosclerosis and sCD40L levels; the analyses of the severity of atherosclerosis in the coronary and LE territories also showed an increase in sCD40L levels in association with increased severity of atherosclerosis; and sCD40L was as an independent predictor of the anatomical severity of CAD in the multivariate analysis. As far as we know, the association between prior CABG and/or LE surgical revascularization and lower sCD40L levels has not yet been reported.

8.2. Limitations

There are some limitations to be acknowledged. Firstly, the sample may be of limited size. However, as this study pioneered the investigation of the expression of circulating microRNAs in single- and multi-territorial atherosclerosis with a systematic screening of atherosclerotic lesions in different territories, no similar data were available to support the sample size estimation. In this exploratory pilot study, the sample size was sufficient to analyze the microRNA expression profile in such a clinical scenario, allowing to detect differences in the expression levels of circulating microRNAs in multi-territorial atherosclerosis.

Secondly, as the definition of atherosclerosis was based on obstructive atherosclerosis, some participants may have presented non-obstructive atherosclerosis in territories without obstructive atherosclerosis. We acknowledge that the presence of non-obstructive atherosclerosis may have influenced the levels of the studied biomarkers. However, non-obstructive atherosclerosis is associated with a lower atherosclerotic plaque burden compared with obstructive atherosclerosis [2,3,176]. Therefore, the impact of non-obstructive atherosclerosis on biomarkers expression is less consistent

compared with that of obstructive atherosclerosis, as shown in a systematic review and meta-analysis on sCD40L levels, which were not dysregulated in subclinical atherosclerosis, contrary to the increased sCD40L levels in obstructive atherosclerosis [2,3,79]. We focused on obstructive atherosclerosis since it may be more relevant for clinical practice, considering that obstructive atherosclerosis is associated with a higher morbidity, including a higher prevalence of ischemic symptoms, is associated with a worse prognosis, including a higher risk of ischemic events, and has a distinct treatment, including medical and revascularization therapies, compared with non-obstructive atherosclerosis [2,3].

Thirdly, the definition of obstructive atherosclerosis was based on anatomical criteria in the coronary territory and on functional criteria in the LE and carotid territories. Although there are discrepancies between the anatomical and functional classifications of coronary lesions, the anatomical criteria used in this project are long-time established criteria used for clinical decision-making, and such criteria were in fact validated against functional methods, with a reasonable correlation between both [2,101,102]. Importantly, the positive predictive value of the anatomical criteria used in the coronary territory for detecting lesions with a high plaque burden is high [2,101,102,177]. In fact, the plaque burden of lesions associated with anatomically significant stenoses on invasive coronary angiography is substantial, and is usually higher than expected due to vascular remodeling and the Glagov phenomena [177]. Regarding the exclusion of CAD in control participants, it was based on coronary computed tomography angiography, which has a very high negative predictive value, as aforementioned [109].

Fourthly, the atherosclerosis extent to multiple territories and disease severity in each territory are commonly associated and both may influence the levels of studied biomarkers [11,24,178], which could have been a potential source of bias regarding the studied association between atherosclerosis extent to multiple territories and the levels of studied biomarkers. However, CAD, which was the common factor to all patients, presented a well-balanced severity among the different groups of patients. Moreover, the multivariate analysis confirmed an independent association between the atherosclerosis extent to multiple territories and microRNAs (miR-27b and miR-146a) expression levels, irrespective of disease severity in each territory, and the same was verified for sCD40L. This suggests that atherosclerosis severity in each territory may not have been a significant source of bias for the analyses of microRNAs and sCD40L.

Finally, this is a single-center research project that included only Portuguese participants, which may limit the applicability of results to other clinical settings. Populations with different ethnicities may express distinct microRNA profiles, either in healthy individuals or in specific disease subsets [179,180]. Therefore, further multicentric studies recruiting participants from distinct geographical areas are warranted for an external validation of our findings.

9. Future directions

Based on the results of this research project, some fields deserve further investigation for increasing the knowledge about atherosclerotic disease and potentially improving clinical care of patients with stable atherosclerosis.

The observed dysregulation of miR-27b and miR-146a according to the systemic severity of atherosclerosis and the accuracy of both microRNAs to predict multi-territorial atherosclerosis suggest that these microRNAs are potential diagnostic biomarkers for such a clinical scenario. A prospective external validation of miR-27b and miR-146a in the diagnosis of multi-territorial atherosclerosis should be carried out before considering these microRNAs as biomarkers for use in clinical practice.

The expression of miR-27b and miR-146a according to the extent of atherosclerosis to multiple territories and atherosclerosis severity in each territory conveys prognostic information, as the severity of stable atherosclerosis is closely associated with the risk of acute ischemic events [5,6]. However, we suggest that both microRNAs need to be studied as prognostic biomarkers in properly sized cohorts for assessing clinical events, as this may further guide the intensification of atheroprotective strategies.

Importantly, miR-27b and miR-146a deserve to be studied in experimental models as targets for atheroprotective strategies. This is supported by the observed dysregulation of miR-27b and miR-146a according to the extent of atherosclerosis to multiple territories and severity in each territory, and by the atheroprotective roles of both microRNAs reported in experimental studies. These data are further reinforced by the observed associations between expression of both microRNAs and specific cardiovascular risk factors (cigarette smoking for miR-27b) and/or inflammatory mediators (TNF-α levels for miR-146a) in our sample. Systemic delivery of miR-27b and miR-146a mimics may be tested in animal models of atherosclerosis as potential strategies aimed at preventing and/or delaying the development of atherosclerosis [35].

Regarding sCD40L, based on the observed dysregulation according to the systemic extent of atherosclerosis to multiple territories and severity in different territories, a prospective validation of this inflammatory mediator as a diagnostic biomarker of severe systemic atherosclerosis deserves to be assessed. Based on our results, sCD40L is more likely to be used in clinical practice as a biomarker of obstructive rather than

nonobstructive atherosclerosis, particularly for detecting combined coronary and LE atherosclerosis.

Finally, the reduced sCD40L levels in patients with prior revascularization suggest that the impact of revascularization procedures on the inflammatory activation, including sCD40L, deserves to be prospectively assessed in patients with stable atherosclerosis. This could provide insights into potential pleiotropic effects of revascularization procedures, in addition to the recognized reduction of ischemia and/or symptoms [104,168].

10. References

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