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PHD THESIS SUMMARY

IDENTIFICATION OF NEW PREDICTIVE BIOMARKERS FOR THE APPEARANCE OF CRITICAL EVENTS IN PATIENTS WITH MULTIPLE VASCULAR PATHOLOGIES

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Introduction and general objectives of the thesis

The elaboration of this thesis started from the great problem of modern medicine, namely atherosclerosis, and more precisely from the totality and severity of atherosclerotic determinations at the peripheral vascular level (carotid and lower limbs levels).

The severity of atherosclerotic disease at the peripheral level, and not only, is influenced by the presence of risk factors (diabetes, dyslipidemia, smoking, age, combination of other atherosclerotic determinations, obesity) and their complexity. The evolution of atherosclerotic disease is rampant in the absence of correction of these risk factors or neglect of medication or unhealthy lifestyle. This doctoral thesis aims to find correlations between plasma lipid and inflammatory parameters and atherosclerotic disease, as well as the identification of markers of the evolution of atherosclerotic disease. The persistence of risk factors in the pro-atherogenic arterial areas determines the appearance and progression of a cascade of events that translates into the appearance and progression of the atheroma plaque.

The stages of atheroma plaque formation begin with the subendothelial accumulation of oxidatively modified lipoproteins, which trigger the activation of endothelial cells (EC), followed by endothelial dysfunction, with the appearance of cell adhesion molecules responsible for triggering a strong inflammatory response. Inflammatory cells entering the endothelium, through the factors they secrete, cause the accumulation of smooth muscle cells in the arterial intima, with the formation of fibrous head. Smooth muscle cells also undergo a process of transformation into a secretory phenotype, giving rise to a hyperplastic, multilayered

basal lamina, with an extracellular matrix rich in collagen fibers and fibrils (Simionescu and Sima, 2012). Finally, the atheroma plaque is formed, which can be stable or unstable, depending on its cellular composition. Erosion or thinning of the fibrous head exposes the subendothelial extracellular matrix rich in proinflammatory and procoagulant cells to the cells in the bloodstream, thus initiating the process of thrombosis. This cascade of events is amplified and accelerated in the case of diabetes, starting with the early onset of endothelial dysfunction (Toma et al 2021), atherosclerotic plaque and its thrombosis.

The structure of the doctoral thesis

In **the first part** of the doctoral thesis we addressed the issue of the "vascular" patient, i.e. the patient who associates one or more atherosclerotic determinations, usually with multiple associated and neglected risk factors, in a high proportion. The patient goes to the vascular surgeon because he can no longer bear the pain of rest or at night, or when the trophic lesion in the foot acquires a foul odor that the patient or family can no longer bear, or when the vascular lesions in the carotid arteries result in a stroke and then a specialist examination is needed. We also briefly described how atheroma plaque forms and the mechanisms and molecules involved in this atherosclerotic process.

The second part of the paper, the original contributions, includes:

- the criteria for enrolling patients and the description of the types of surgery applied to patients enrolled in the study;
- biochemical and immunochemical methods for the characterization of plasma proteins and lipoproteins and molecular biology methods for the analysis of epigenetic factors (microRNAs) in plasma and atheroma samples;
- identification of biochemical and epigenetic parameters as potential markers of the severity of carotid stenotic disease and peripheral arterial disease in two specific studies.

We established the groups of patients with carotid stenosis and peripheral arterial disease, the control group for each of the two categories of patients, the anthropometric data, the secondary diagnoses, the drug treatment of these categories of patients. We studied two vascular territories (carotid arteries and peripheral arteries of the lower limbs) whose atherosclerotic damage is common and produces morbidity and mortality to the highest degree.

In the first study, we aimed to evaluate the role of miR-223 as a potential biomarker for worsening SAC in patients over 65 years of age and correlated its levels with parameters

associated with high-density lipoprotein (HDL). The conclusion of the study is that patients with SAC over 65 years of age had a significant increase in miR-223-3p levels in both plasma and carotid atheroma compared to patients with SAC under 65 years of age.

In the second study, we aimed to identify changes in the composition of HDL2 and HDL3 subfractions that are associated with the severity of peripheral arterial disease (BAP) and cause impaired HDL function measured as anti-inflammatory effects on human EC activated by tumor necrosis factor alpha (TNFα). We aimed to determine which of the identified changes in HDL constituents are detectable in plasma proteins and could predict the severity of the disease in patients with BAP for transposition in clinical laboratories. Thus, we found an increase in clusterin levels (CLU) and specific activity of myeloperoxidase (MPO) and decreased specific activity of paraoxonase (PON1) in plasma, which reflected the alteration of HDL proteins and their functionality. The results could be translated into clinical laboratories and could be used to predict the severity of BAP. Also, in the same study we analyzed the expression in atherosclerotic plaque and plasma level of hsa-miR-142-3p, hsa-miR-223-3p, hsa-miR-155-5p, hsa-miR-92a-3p and we correlated with the postoperative evolution during one year of patients with BAP. We demonstrated for the first time the link between the levels of miRNAs in atherosclerotic plaque and the expression of the corresponding miRNAs in atherosclerotic plaque.

Current state of knowledge

In this chapter we have included known data about the structure of the arterial wall in physiological conditions (types of arteries and the structure of the normal vascular wall), what happens in pathological conditions, how atherosclerosis occurs, the stages of appearance and development of atheroma plaque, and well-known risk factors that determine the atherosclerotic process. We also presented data on the role of epigenetic factors (microRNAs) in atherosclerosis.

Cardiovascular disease (CVD), the leading cause of death in the world, is a complex process, with major clinical manifestations including myocardial infarction, stroke and BAP, which may be unique in location or with simultaneous determinations at two or three arterial levels. The atherosclerotic process is the one that generates CVD. The atherosclerotic plaque is the result of risk factors, considered classic (dyslipidemia, oxidative stress, proinflammatory cytokines, vasoconstrictor factors incriminated in hypertension, glycoxidation products

associated with hyperglycemia and smoking) or in the absence of a systemic hypercholesterolemia, the initiation of atheroma plaque formation is an inflammatory reaction.

Individual cardiovascular risk assessment involves measuring of risk factors related to cardiovascular risk (lipids, blood sugar, age, sex, smoking and blood pressure). These classic factors are used to establish correlations between markers plasma and atherosclerotic disease, as well as the predictability of atherosclerotic disease progression. It is known that vascular disease affects the arteries at different levels and is characterized by harmful effects on the vascular endothelium, causing endothelial dysfunction. Endothelial dysfunction is the response to the action of cardiovascular risk factors. It is a key early stage in the development of atherosclerosis, but is then involved in all stages of atherosclerotic plaque progression and its complications. Regardless of the factors that generate it, the formation of atheroma plaque is a process in which the gradual involvement of different cell types and their secretory products leads to a series of sequences from lipid layer formation to fibrolipid plaque, rupture and atherothrombosis.

Atherosclerosis is a multifactorial process induced and favored by risk factors such as dyslipidemia, oxidative and inflammatory stress, diabetes mellitus (DM), hypertension, smoking, aging and genetic mutations (Authors/Task Force et al., 2019) and can be located at several levels at the same time (coronary, carotid and lower limb levels).

Dyslipidemia is defined by high blood concentrations of total cholesterol (TC) and LDL-cholesterol (LDL-C) and/or triglycerides (TG) and by decreased cholesterol in HDL (Musunuru, 2010).

HDL are macromolecular complexes composed of lipids and proteins, produced in the liver and small intestine, which carry free and esterified cholesterol, phospholipids (PL), TG and apoproteins (apolipoproteins - apoAI, apoAII, apoCIII, apoE, enzymes - paraoxonase 1 (PON1), myeloperoxidase (MPO)) in circulation (Camont et al., 2011, Eren et al., 2012, Larach et al., 2012). In human plasma, HDL are represented by two major subfractions: HDL2 and HDL3, with different size, density and composition (Eren et al., 2012). HDL have anti-atherosclerotic properties due to their ability to takeup excess cholesterol from peripheral tissues by transporting it to the liver, the so-called reverse cholesterol transport (RCT), but also due to their anti-oxidant, anti-inflammatory and anti-thrombotic potential (Arora et al., 2016). An atero-protective effect of HDL is the preservation of the EC function (Li et al., 2000, Kuvin et al., 2003). In recent years, HDL quality and function have become a consistent indicator of CVD risk (Carnuta et al., 2017, Parhofer, 2015). When oxidative stress increases, HDL becomes dysfunctional and has an increased number of MPO molecules that replace PON1

molecules (Variji et al., 2019). Thus, dysfunctional HDL can no longer protect LDL from oxidation, these changes being the key risk factors for the onset and progression of atherosclerotic plaques. The activity of PON1 in serum and HDL in patients with BAP is low compared to healthy subjects, the conclusion being that HDL oxidizes and becomes dysfunctional (Tan et al., 2014, Shen et al., 2015, Luscher et al., 2014). The attempts to develop new therapies for the treatment of patients with BAC based on stimulation of the liver and small intestine to produce functional HDL are highly relevant.

MicroRNAs (miRNAs) are short, non-coding RNA sequences of 20-25 nucleotides, involved in the control of gene expression. These miRNAs orchestrate gene expression at the post-transcriptional level, regulating the synthesis of proteins at the messenger RNA (mRNA) level by degrading it. Thus, each miRNA can become a transcription factor and a regulator of gene expression with implications for cell proliferation and differentiation, apoptosis and inflammatory processes (Vogiatzi et al., 2018). These miRNAs are described as regulators in various cellular processes encountered in the pathophysiology of CVD (Vogiatzi et al., 2018), cancer (Rupaimoole and Slack, 2017), autoimmune diseases (Yan et al., 2019) and other conditions. Moreover, miRNAs are found in various tissues and blood cells, their plasma level being proposed as a potential biomarker in the diagnosis of cardiovascular events, such as myocardial infarction (Devaux et al., 2012). There are some data on the level of miRNA in atherosclerotic plaque (Parahuleva et al., 2018), but no studies have been reported on the correlation of the level of miRNA expression in plaque and plasma, as well as the corresponding expression of pri-miRNA in plaque.

The major vascular event in carotid artery stenosis (SAC) is represented by ischemic stroke, and the development of lesions in the arteries of the lower limbs generates BAP, with important repercussions on the patient's quality of life and reintegration into society.

Atherosclerosis of the extracranial carotid arteries is a major health problem, with ischemic stroke mortality ranging from 10-30%. The signs and symptoms of extracranial cerebrovascular disease include fleeting amaurosis, transient ischemic attack, developing ischemic attack, and stroke. Imaging scans that establish the diagnosis and therapeutic conduct are Doppler ultrasound, angiography or MRI angiography, carotid angiography.

The treatment of extracranial cerebrovascular disease involves several approaches, namely: lifestyle change, drug treatment and, where appropriate, surgical treatment. Carotid endarterectomy (EAC) is addressed to patients with symptomatic SAC, but also to a group of asymptomatic patients at high risk of cerebral vascular event (according to international guidelines).

Atherosclerosis of the peripheral arteries of the lower limbs is a small percentage of the overall mortality of cardiovascular causes, about 1-2%, but is a common cause of morbidity caused by reduced mobility, intermittent claudication, resting pain or acute ischemia. BAP of the lower limbs may be symptomatic or asymptomatic, located at various levels of the peripheral arterial axis (common, deep, superficial femoral arteries, popliteal and leg trunks) and may cause various clinical manifestations, from intermittent claudication pain, with progressive reduction of the perimeter of walking to rest pain (which does not allow the patient to rest and which usually requires the use of a significant amount of opiates) and the appearance of minimal or extensive trophic lesions such as gangrene. The association of diabetes also has important implications for the evolution of patients with BAP (Abou-Zamzam et al., 2007). The diagnosis of BAP is made by performing a thorough anamnesis, clinical examination of the patient with the assessment of peripheral pulses, measurement of the ankle-arm index, performing a Doppler ultrasound and angiography of the lower limbs or a CT/MRI scan.

BAP treatment involves the control of risk factors (conservative treatment) and surgical treatment, the latter can be represented by revascularization (bypass, angioplasty) or amputation.

Original contributions

This chapter includes:

1. Identification of biochemical and epigenetic parameters as potential markers of carotid artery stenosis (SAC) severity.

2. Identification of biochemical and epigenetic parameters as potential markers of peripheral arterial disease (BAP) severity.

The identification of biochemical and epigenetic parameters as potential markers of carotid artery stenosis (SAC) severity aimed to assess the association of hsa-miR-223-3p (miR-223) with the age in patients with SAC after EAC surgery, i.e. the estimation of the role of miR-223 as a potential biomarker for worsening SAC in patients over 65 years of age and its association with HDL-associated parameters.

The study included 32 patients with SAC > 70% hospitalized for the EAC procedure and 28 healthy control subjects (N). The 32 patients with SAC, hospitalized for the EAC procedure, were divided into 2 groups, according to age: SAC patients under 65 years of age (n = 13) and SAC patients over 65 years of age (n = 19). We selected the patients according to

the inclusion and exclusion criteria we established before the start of the studies, and the surgeries followed the recommendations of the profile companies.

Plasma parameters were determined and plasma proteins were characterized in patients with SAC (Figure 1) by specific methods and Western blot analysis of samples from SAC patients was also performed.

We processed carotid atherosclerotic plaque specimens for light microscopy and applied molecular biology methods for epigenetic factors (microRNA) analysis in plasma and atherosclerotic plaque samples from patients with SAC, then statistically analyzed miRNA results from carotid artery stenosis patients (SAC). .

Out of the total number of patients with SAC who underwent surgery to remove the carotid plaque, a percentage of 66.7% showed a good evolution and 33.3% had an unfavorable evolution. Patients with SAC with an unfavorable course of the disease showed an increase in the plasma concentration of TG, CRP, the oxidized form of ApoAI (MDA-ApoAI) and sialic acid, compared to those with a favorable evolution (Figure 1).

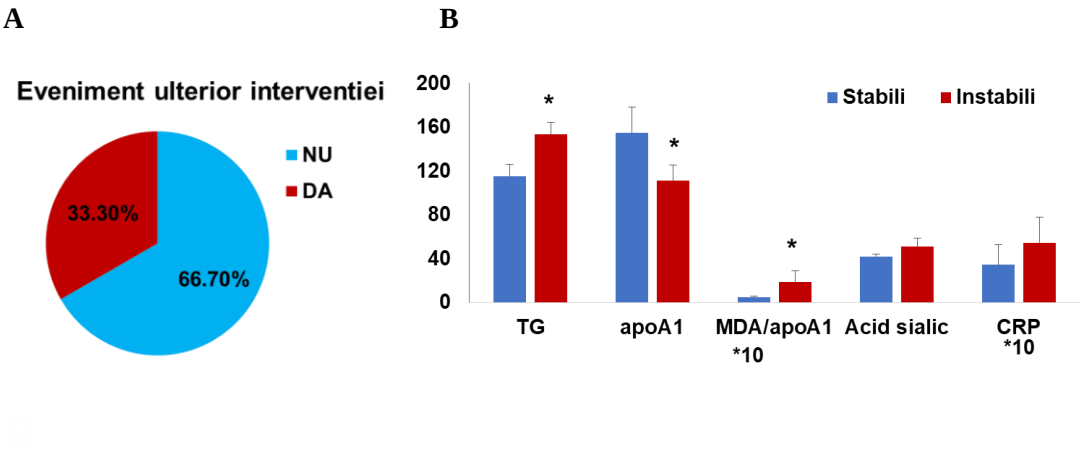

Figure 1. Correlations between the evolution of patients with carotid artery stenosis 1 year after surgery (A) and the initially determined plasma parameters (B). Data are expressed as mean ± standard error of the mean (SEM). * $p < 0,05$ SAC stable vs. unstable.

The novelty of our study is that the patients with SAC over 65 years of age compared to patients with SAC under 65 years of age showed a significant increase in miR-223-3p levels in both plasma and carotid atheroma plaque (Figure 2).

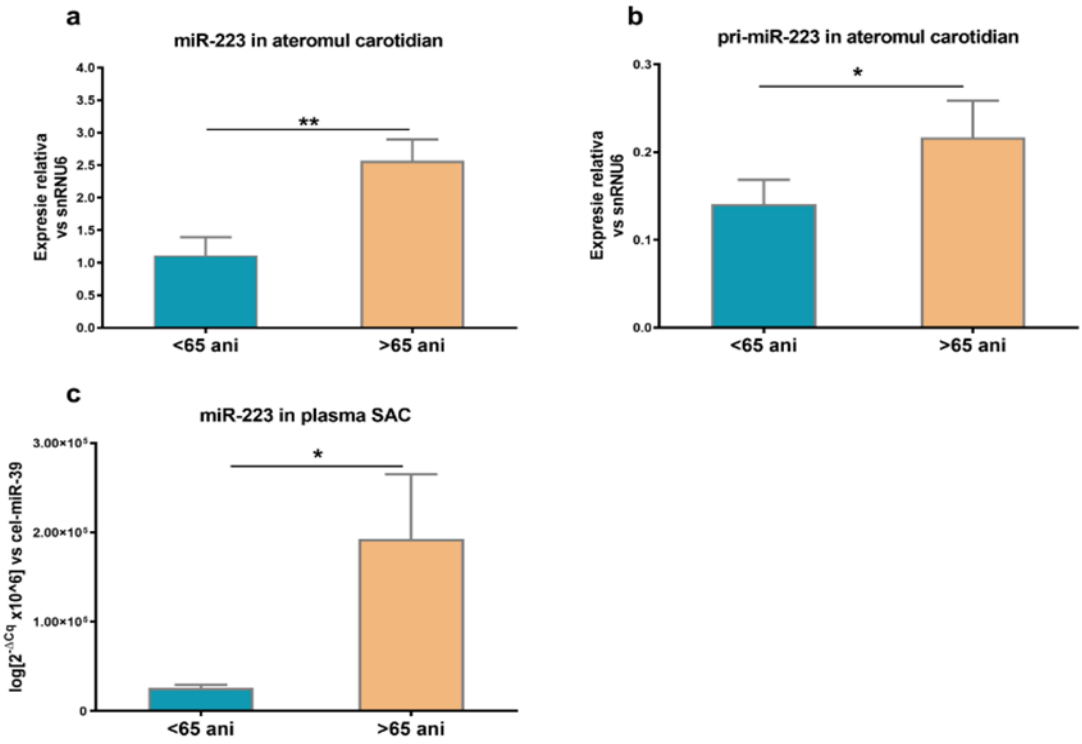

Figure 2. MiR-223 levels in SAC patients. Levels of miR-223 (a) and pri-miR-223 (b) in the carotid atheroma, and miR-223 in the plasma (c) of SAC patients aged over 65 years (> 65 years) compared to those under 65 years (< 65 years). Data are illustrated as boxplots with Tukey whiskers and median line. * $p < 0,05$, ** $p < 0,01$ vs. < 65 years group.

As far as we know, this is the first study to report a correlation between miR-223-3p levels in plasma and carotid atheroma plaque with the age of patients with SAC.

We have demonstrated in this chapter an increased production of miR-223-3p, local and specific in the cells of the carotid atheroma in elderly patients with SAC, associated with the locally increased expression of pri-miR-223, these data correlating with the observations of Li and colab. (Li et al., 2017) who support the hypothesis that in elderly patients with SAC, an increased production of miR-223 occurs in atherosclerotic plaques in response to various oxidative and inflammatory factors that could affect the activity of the miR-223 promoter.

Together, our data and that of the other groups suggest that:

1. Elevated plasma miR-223-3p levels observed in elderly patients with SAC correlate with elevated local miRNA production in the carotid atheroma,

2. Elevated levels of miR-223-3p are correlated with oxidative and inflammatory processes that occur during aging,

3. Modulation of miR-223-3p expression could be used as a possible therapeutic approach to prevent restenosis after endarterectomy in patients with SAC.

A limitation of this study is the relatively small number of patients with SAC analyzed. Despite this, the statistical analyzes used, together with the distribution of miR-223 in plasma and carotid atheroma, gave statistically significant results. Another limitation of our and others study on miRNA analysis is the lack of standardized methods for measuring absolute plasma miRNA levels, which makes comparison with other data in the literature quite difficult.

The second study involved the identification of biochemical and epigenetic parameters as potential markers of peripheral arterial disease (BAP) severity.

In this study, we aimed to identify changes in the composition of HDL_2 and HDL_3 subfractions that are associated with BAP severity and cause impaired HDL function measured as an anti-inflammatory effect on TNFα-activated human EC. Another aim of the study was to determine which of the identified changes in HDL constituents are detectable in plasma and could predict the severity of the disease in patients with BAP for transposition in clinical laboratories.

We analyzed the levels of a set of 4 miRNAs (hsa-miR-142-3p, hsa-miR-223-3p, hsa-miR-155-5p, hsa-miR-92a-3p) in plasma and femoral atherosclerotic plaques in patients with BAP who developed cardiovascular events within one year of primary surgery. We chose this set of miRNAs based on previous studies from the Lipidomics department of IBPC-NS and other data published in the literature. Thus, we analyzed the expression levels in the atherosclerotic plaque and plasma of hsa-miR-142-3p, hsa-miR-223-3p, hsa-miR-155-5p, hsa-miR-92a-3p and we made correlations with the evolution patients with BAP. We reported that hsa-miR-142-3p can be considered a predictive biomarker for cardiovascular events.

The study included 47 patients diagnosed with BAP (4 women and 43 men), aged 32-83 years, with lower limb bypass and 28 healthy control subjects (N). We selected the patients according to the inclusion and exclusion criteria we established before the start of the studies, and the surgeries followed the recommendations of the profile companies.

We determined plasma parameters, isolated by ultracentrifugation the HDL subfractions from plasma of patients with BAP, measured biochemical parameters in HDL subfractions, determined MDA and AGE protein adducts in HDL subfractions, performed Western blot analysis and analyzed miRNA in plasma and atherosclerotic plaque in patients with BAP.

Patients with BAP were divided into two groups according to the presence of DM as a pathology associated with BAP: (i) BAP without DM (BAP, n = 32) and (ii) BAP known to have DM (BAP-DZ, n = 21). HDL2 and HDL3 isolated by ultracentrifugation of plasma from healthy subjects (N) and BAP patients with and without DM (BAP-DZ/BAP) were characterized by:

1. lipid and apolipoprotein content: cholesterol, phospholipids (PL), apolipoprotein AI (ApoAI), ApoAII, ApoCIII, clusterin (CLU), and ApoE,
2. PON1 and MPO content and activity: PON1 protein and activity, MPO protein and activity,
3. presence of AGE-protein adducts: AGE-ApoAI, AGE-CLU, AGE-ApoE and AGE-PON1,
4. the presence of markers of oxidative stress: malondialdehyde-ApoAI (MDA-ApoAI), MDA-CLU, MDA-ApoE, MDA-PON1, ceruloplasmin (CP) and conjugated dienes.

The new results of our study are that in patients with BAP, DM induces a differentiated alteration of the protein components of HDL2 and HDL3, as follows:

1. HDL2 shows a decrease in PON1 protein and activity, along with decreased apoAI and increased apoAII and apoCIII;
2. a modest increase in MPO protein in HDL2 is accompanied by a significant increase in MDA and -AGE protein adducts in ApoAI, CLU, ApoE and PON1;
3. in HDL3 CLU and apoCIII increased and apoAII levels decreased;
4. HDL3 has low PON1 activity, in parallel with elevated levels of MPO and CP, and elevated levels of MDA- and AGE-PON1, -CLU and AGE-apoAI (Figures 3-5).

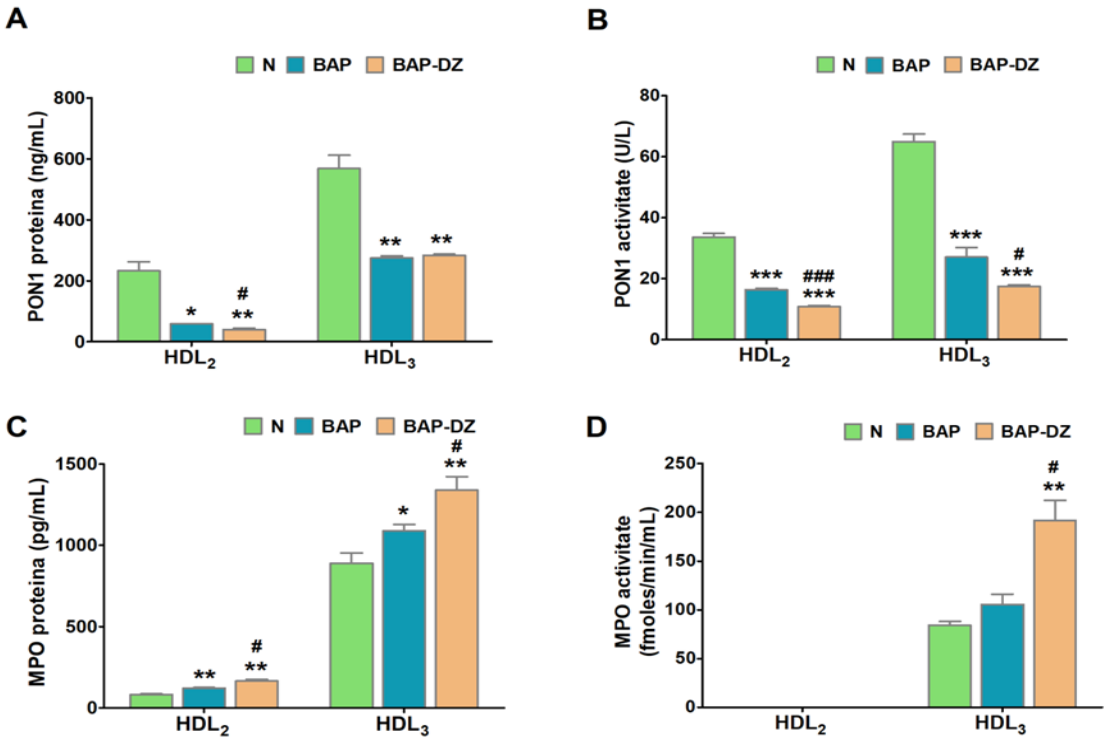

Figure 3. Protein and activity levels of paraoxonase 1 (PON1) and myeloperoxidase (MPO) in HDL subfractions. HDL2 and HDL3 isolated by ultracentrifugation from plasma of healthy subjects (N) and BAP patients with and without DM (BAP-DZ/BAP) were characterized in terms of PON1 and MPO content and activity: PON1 protein (A) and activity (B), MPO protein (C) and activity (D). Data are expressed as mean ± SEM; * $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$ vs. N; $^{\#}p < 0,05$, $^{\#\#\#}p < 0,001$ vs. BAP.

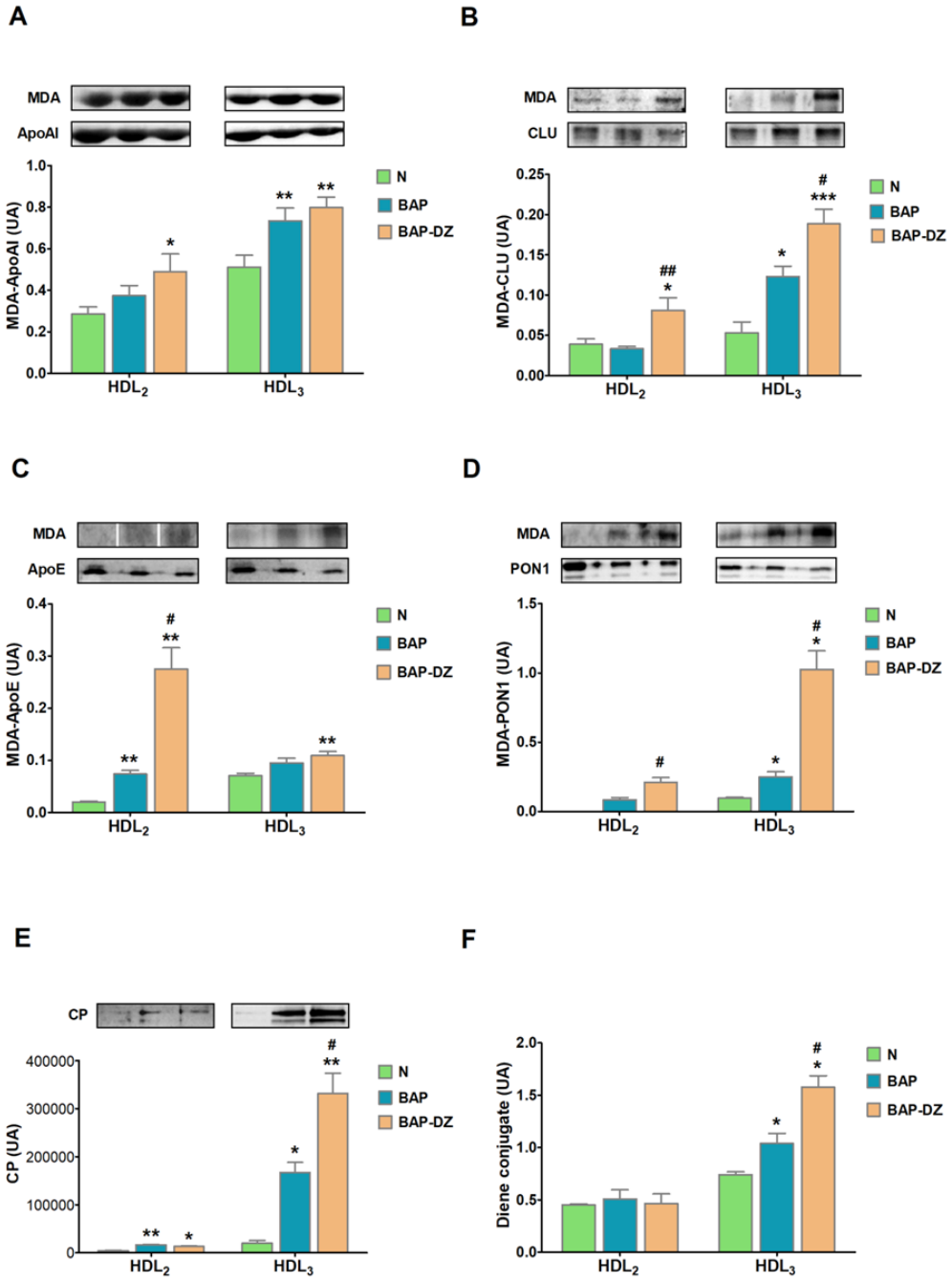

Figure 4. Oxidative stress parameters in HDL subfractions. HDL2 and HDL3 isolated by ultracentrifugation from plasma of healthy subjects (N) and BAP patients with and without DM (BAP-DZ/BAP) were characterized by the presence of oxidative stress markers: adducts malondialdehyde-apolipoprotein AI (MDA-ApoAI) (A), MDA-clusterin (MDA-CLU) (B), MDA-ApoE (C), MDA-paraoxonase 1 (MDA-PON1) (D), ceruloplasmin (CP) (E) and conjugated dienes (F). Data are expressed as mean ± SEM; * $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$ vs. N; $^{\#}p < 0,05$, $^{\#\#}p < 0,01$ vs. BAP.

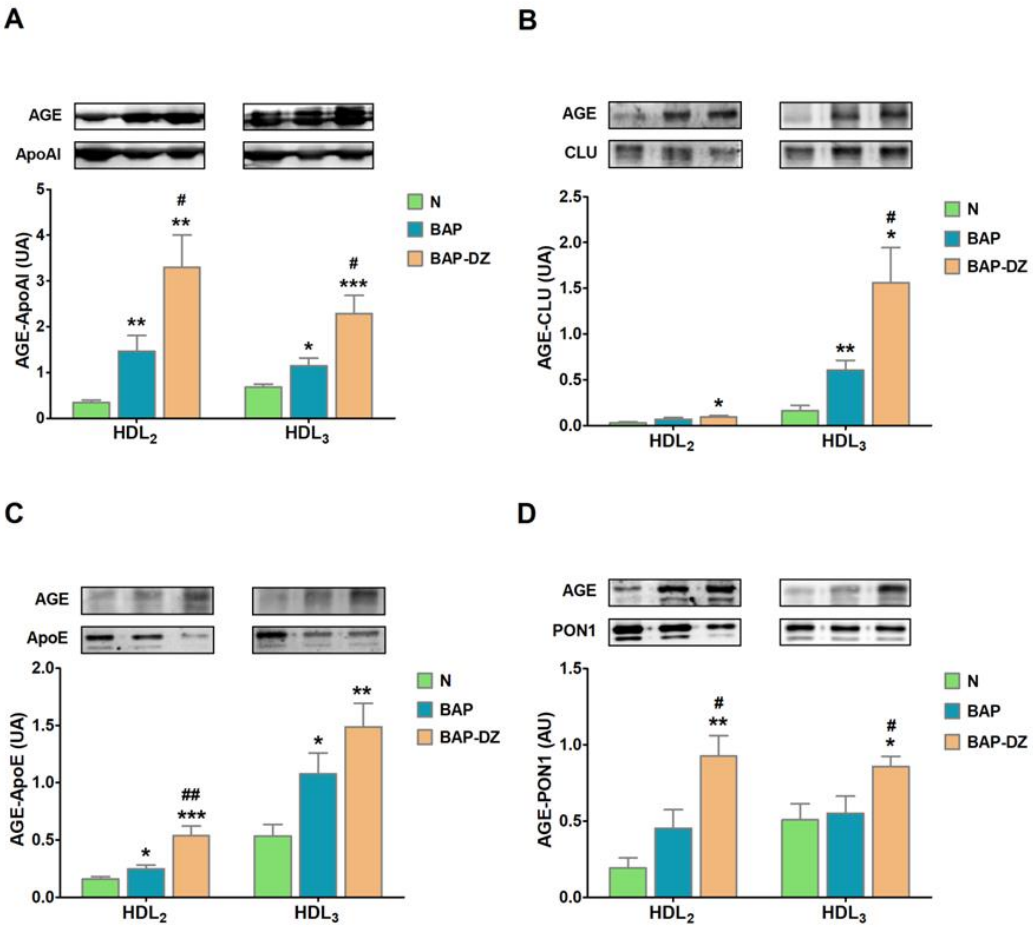

Figure 5. Advanced glycation end products (AGEs) form adducts with proteins from HDL subfractions. HDL2 and HDL3 isolated by ultracentrifugation from plasma of healthy subjects (N) and BAP patients with and without DM (BAP-DZ/BAP) were characterized by the presence of AGE-protein adducts: AGE-apolipoprotein AI (AGE-ApoAI) (A), AGE-clusterin (AGE-CLU) (B), AGE-ApoE (C) and AGE-paraoxonase 1 (AGE-PON1) (C). Data are expressed as mean ± SEM; * $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$ vs. N; $^{\#}p < 0,05$, $^{\#\#}p < 0,01$ vs. BAP.

In the present study, we show for the first time that HDL subfractions in patients with BAP and DM have a distinct composition compared to those in patients with BAP without DM. Thus, HDL2 has lower levels of TC and PL in BAP-DZ than BAP.

Another novelty of our study is the redistribution of CLU and apoAII between HDL subfractions in BAP-DZ compared to BAP patients. Thus, the CLU level is reduced in HDL2 and increased in HDL3, while the apoAII level is increased in HDL2 and decreased in HDL3 (Maskanakis et al., 2018, Rodriguez-Rivera et al., 2021). Our results show that in severe pathological conditions such as diabetes, CLU production is higher in patients with BAP-DZ compared to those with BAP, its affinity for HDL3 being increased compared to HDL2, according to previously published data (Davidson et al., 2009).

We report here for the first time that in patients with BAP-DZ versus those with BAP, the PON1 protein decreases in HDL2, while its activity decreases in both HDL subfractions, probably due to the significant increase in MPO levels that displace and oxidize PON1 (especially in HDL3), thus contributing to HDL dysfunction. Previously, the ratio between MPO and PON1 proteins or between their activities has been proposed as markers for HDL functionality (Arora et al., 2016).

Our data show for the first time that in diabetic conditions, the attachment of CLU to HDL3 increases, despite the decrease in PON1. In addition, pro-oxidative CP attached to HDL3 is greatly increased in diabetic conditions, thus contributing to HDL dysfunction, in line with our previous results (Carnuta et al., 2017).

Thus, another novelty of our results is that CLU and CP are more attached to HDL3 in patients with BAP-DZ than in those with BAP, suggesting that in diabetic conditions their coexistence in HDL3 is favored. This imbalance between the antioxidant and pro-oxidant enzymes associated with HDL, in favor of the latter, generates a significant increase in the oxidative changes of apolipoproteins. All of these changes are reflected in elevated levels of conjugated dienes, MDA- and AGE-apolipoproteins and -PON1, revealing excessive alteration in HDL quality in patients with BAP-DZ compared to those with BAP. Of the changes that occur in HDL components under pathological conditions, the MDA-HDL change has the strongest negative impact on RCT (Bornfeldt et al., 2021).

We highlight the new and very interesting results obtained in our study, namely that of all the HDL2 and HDL3 components analyzed, only CLU levels, PON1 and MPO specific activities show significant differences in plasma of patients with BAP-DZ compared to those with BAP and show a strong predictive association with diabetes. The small number of patients with BAP-DZ and BAP are a limitation of our study; however, we reached statistical significance for the parameters proposed as markers for BAP severity.

In conclusion, increasing CLU levels and MPO-specific activity and decreasing specific PON1 activity in plasma- reflect impaired HDL functionality. They could be translated into clinical laboratories and could predict the severity of BAP in hyperglycemic conditions. In addition to the established markers measured in clinical laboratories, these three parameters could be considered candidate biomarkers for BAP severity and used in the future for better and earlier prediction of disease progression.

In a parallel study, we sought to identify potential predictive miRNAs for postoperative cardiovascular events in patients with BAP, measuring miRNA levels in plasma and atherosclerotic plaque, and correlated with cardiovascular events within one year of surgery. The new data (Figure 6) show that unlike BAP patients without EV, patients with BAP with EV show:

1. a significant increase in plasma levels of miR-142-3p and miR-223-3p;
2. significant increases in miR-142-3p, miR-155-5p and miR-223-3p expression in atherosclerotic plaque, which correlates positively with their associated pri-miRNA levels;
3. a significant decrease in miR-92a expression in plaque, while the pri-miR-92a level is increased;
4. a significant association between oxidative and inflammatory parameters, plasma lipids and plasma miRNA levels;
6. plasma levels of miR-142 have been shown to be an independent predictive factor for cardiovascular events in patients with BAP.

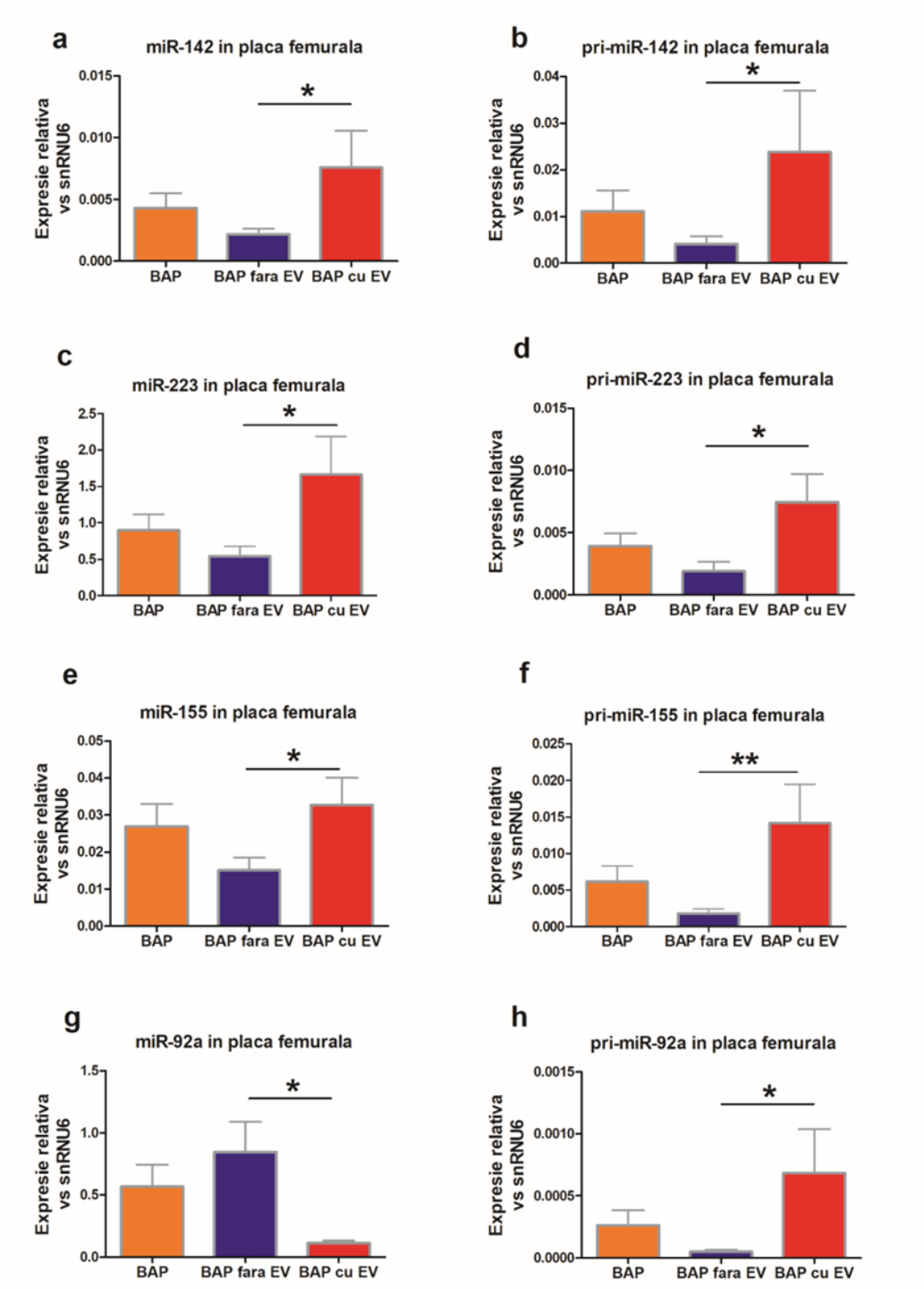

Figure 6. MiRNA and primary transcript (pri-miRNA) levels in atherosclerotic plaques in the femoral arteries in patients with peripheral artery disease (BAP). Levels of miR-142 (a), miR-223 (c), miR-155 (e), miR-92a (g), pri-miR-142 (b), pri-miR-223 (d), pri-miR -155 (f), and pri-miR-92a (h) in femoral atherosclerotic plaques from postoperative BAP patients with cardiovascular events (BAP with EV) compared with those without EV (BAP without EV). Data are illustrated as error bar histograms (SEM). Legend: * p values < 0.05, ** p values < 0.01 compared to BAP without EV.

To our knowledge, the present study is the first to address the prediction of disease progression using comparative analysis of plasma and atheroma plaque levels of miRNA in patients with BAP. We first demonstrated the link between the level of miRNA in atherosclerotic plaque and the expression of pri-miRNA in plaque.

Early and rapid prediction of the evolution of BAP can optimize the clinical decision to adjust individual interventions in the treatment of these patients. By practicing a personalized medicine, miR-142 can help to stratify the risk of patients with BAP by predicting post-operative cardiovascular events. These new data increase the possibility of using miRNA (in vivo miR-142 inhibition) therapy to supplement statin treatment in atherosclerotic disease by modulating the miRNA-related mechanism.

The most important and new result of the study is the highlighting of the potential of miR-142 as an independent predictive factor for the postoperative occurrence of cardiovascular events in patients with BAP.

In conclusion, we have shown that miR-142, miR-155, miR-223 and miR-92a are produced in atherosclerotic plaque as evidenced by the specific expression of pri-miRNA and their positive correlations with key components of the miRNA processing complex (Dicer and Drosha enzymes). We also showed that the expression of miR-142, miR-155, miR-223 and miR-92a in atherosclerotic plaque is associated with postoperative cardiovascular events in patients with BAP and correlated with pro-atherogenic risk factors: metabolic, oxidative and proinflammatory. Finally, based on data obtained from statistical regression modeling, we propose miR-142 as an independent biomarker for predicting cardiovascular events in BAP patients.

General conclusions

1. SAC patients show an altered profile of parameters associated with oxidative (reduced PON1, increased MPO and CP) and inflammatory (increased IL-1β and CRP) stress compared to healthy controls.

2. Patients with SAC over 65 years of age have a significant increase in miR-223-3p levels in both plasma and carotid atheroma plaque compared to patients with SAC under 65 years of age.

3. Elevated levels of miR-223-3p in carotid atheroma plaque in patients with SAC over 65 years of age is associated with increased expression of pri-miR-223 in the atherosclerotic plaque.

4. Type II diabetes mellitus induces a differentiated alteration of the protein components of HDL2 and HDL3 subfractions in the plasma of patients with BAP, as follows:

• HDL2 shows a reduction in both protein and enzymatic activity of PON1, along with decreased apoAI and increased apoAII and apoCIII; MPO protein levels show a modest increase.

• HDL3 has reduced enzymatic activity of PON1, in parallel with elevated levels of MPO and CP, associated with elevated levels of MDA- and AGE-PON1, MDA- and AGE-CLU and AGE-apoAI.

5. Changes in plasma concentrations of HDL-altered proteins, such as CLU, PON1 and MPO, could be considered indicators of disease severity in patients with BAP.

6. MiR-142 has the potential to be considered an independent predictive factor for the postoperative occurrence of cardiovascular events in patients with BAP.

7. From the point of view of the vascular surgeon, access to the measurement of miRNA levels in patients with SAC would allow the indication of early measures to assess the condition of patients by short-term ultrasound monitoring to detect restenosis in patients with SAC surgery and apply appropriate treatment.

8. The composition and function of HDL are altered in patients with BAP and to a greater degree in patients with BAP with type II diabetes. Modified levels of CLU and specific PON1 and MPO activities are detectable in the plasma of patients with BAP. Because HDL isolation and characterization is a laborious procedure, we can consider plasma levels of CLU, PON1, and MPO as potential indicators of HDL dysfunction that can be used for better risk stratification in patients with BAP.

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