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# **DOCTORAL THESIS**

**NEW HORIZONS IN THE DIAGNOSIS AND TREATMENT OF  
PERIPHERAL ARTERIAL DISEASE OF THE LOWER LIMBS**

## **ABSTRACT**

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## INTRODUCTION

Peripheral arterial disease (PAD) affects more than 200 million adults worldwide, and the incidence of PAD increases by up to 20% in people over the age of 70. Although PAD has traditionally been perceived as a disease that affects men, the prevalence of PAD appears to be equal among older men and women.

Under-diagnosis of PAD in primary care can be a significant problem, as most patients with PAD do not present with the stereotypical symptoms of claudication described in textbooks. Smoking increases the risk of developing PAD four-fold and has the greatest impact on disease severity. Compared to non-smokers, smokers with PAD have a shorter lifespan and progress more frequently to critical limb ischemia and amputation. Additional risk factors for PAD include diabetes, hyperlipidemia, hypertension, race and ethnicity.

There has been a 72% increase in the overall prevalence of PAD from an estimated 65,764,499 people in 1990 to 113,443,016 in 2019. The age-standardized prevalence per 100,000 increased by 13% and the age-standardized prevalence per 100,000 decreased by 22%. Similar patterns were observed for years of life lost, mortality, years lived with disability and disability-adjusted life years. Prevalence and disability were higher among women, while mortality and years of life lost were higher among men. The burden of disease increased with increasing socio-demographic index.

These increases in peripheral artery disease were in contrast to global trends in the overall burden of ischemic heart disease and ischemic stroke, which have seen a decrease in prevalence and disease-related mortality over the same time frame. Overall, only about 55% of the burden of PAD disease could be attributed to the identified risk factors, with tobacco use, diabetes and hypertension being the three major contributors in all investigated groups.

Peripheral arterial disease is an atherosclerotic disease closely associated with high morbidity and mortality from cardiac events. Inflammation is essential in atherosclerosis, both in onset and progression. Numerous inflammatory biomarkers (cytokines, matrix metalloproteinases (MMPs), selectin, intracellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), C-reactive protein (CRP),

fibrinogen) have been measured in atherosclerotic disease, including BAP.

Despite recent advances, PAD continues to be an under-diagnosed and under-treated condition. Recent studies have explored the possibility of using miRNAs as biomarkers for PAD. In a recent study of patients with atherosclerosis, miR-654-5p and miR-409-3p were identified as potential biomarkers, and logistic regression models based on these 2 miRNAs differentiated patients with atherosclerosis from controls.

Furthermore, in a study on peripheral arterial disease, miR-21, miR-130a, miR-27b, let-7f and miR-210 were all significantly elevated, demonstrating that miRNAs can be used as prognostic and diagnostic markers, but also treatment markers in peripheral arterial disease.

Consequently, PAD is often undiagnosed and untreated, especially in the early stages of the pathology or in diabetic patients, presenting silence rates of about 50%, emphasizing the need for early diagnostic and prognostic markers and new therapeutic targets for PAD of the lower limb.

## PHD THESIS ABSTRACT

The thesis aims to investigate in detail the distinct attributes of peripheral arterial disease

1. Epidemiologic and risk factor study in critical ischemia
2. A biomarker profile to predict amputation-free survival in patients with critical ischemia
3. miRNA in the diagnosis and treatment of critical ischemia

The proposed scientific objectives were:

- Identification of risk factors and their association with the progression of peripheral vascular disease in the general population of Romania
- Identification of a panel of biomarkers useful in the diagnosis of peripheral arterial disease in general and critical ischemia in particular
- Identification of miRNAs useful in the diagnosis and treatment of critical ischemia and peripheral vascular disease
- Setting new scientific research directions in the diagnosis and treatment of peripheral arterial disease

The **general part** has 3 chapters.

The first chapter deals with clinical, paraclinical and epidemiologic data related to peripheral arterial disease. It contains sub-chapters related to definition and epidemiology, classification, clinical signs, clinical and paraclinical investigations, management of risk factors, principles of drug, surgical and endovascular treatment.

The second chapter extensively discusses the details of inflammatory markers in peripheral arterial disease. Peripheral arterial disease is an atherosclerotic disease closely associated with high morbidity and mortality from cardiac events. Inflammation is essential in atherosclerosis, both in onset and progression. Numerous inflammatory biomarkers (cytokines, matrix metalloproteinases (MMPs), selectin, intracellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), C-reactive protein (CRP), fibrinogen) have been measured in atherosclerotic disease, including BAP. Inflammation (I) is

now considered to be fundamental in both the onset and growth of PAD. It is closely linked to endothelial injury and endothelial dysfunction. The lack of endothelium-derived nitric oxide release is the most likely symptom for the induction of endothelial vasodilator endothelial vascular dysfunction and is a well-recognized crucial step in atherosclerosis. Moreover, it contributes to arterial wall disarticulation, arterial distensibility, stiffness, vasomotion and vasomotility.

The new circulating markers could provide additional information on the risk of developing PAD and could add prognostic information beyond what is possible by measuring ABI, particularly in terms of predicting cardiovascular events and mortality.

MicroRNAs (miRNAs) were discovered in 1993 and have since then had a substantial impact on the study of epigenetics. Chapter 3 extensively discusses the details of miRNAs in peripheral arterial disease and sets the stage for new data to be uncovered in the thesis study.

Considerable evidence supports the association of novel circulating markers with several aspects of PAD, including: (1) the risk of developing symptomatic or asymptomatic PAD; (2) progression of PAD; (3) functional impairment; and (4) adverse cardiovascular events and mortality. However, significant knowledge gaps remain. Given the complexity of atherosclerotic vascular disease, a single marker is unlikely to provide significant predictive or prognostic information, and a multimarker approach is more likely to be useful.



**The special part, the personal contributions** are meant to answer the above-mentioned questions:

- An epidemiologic and risk factor study in critical ischemia
- A biomarker profile to predict amputation-free survival in patients with critical ischemia
- miRNA in the diagnosis and treatment of critical ischemia

**The first study focuses on angiographic imaging evidence and its correlation with specific risk factors for peripheral arterial disease.** This study was included in a project developed by the Academy of Medical Sciences, which generated the first peripheral arterial disease registry. Seven Romanian hospitals treating patients with this pathology were included in the study. A total of 2859 patients were included, which constituted a peripheral vascular disease registry. In total, 1745 patients have been included in the register so far. Preliminary analysis of the data revealed a duplication of records for some patients, so these were removed from the analysis. This resulted in 1528 patients. Most of the patients came from the County Clinical Emergency Hospital "Pius Brinzeu" in Timișoara, followed by the Emergency Institute for Cardiovascular Diseases and Transplantation in Târgu Mureș and the County Emergency Hospital "St. John the New" in Suceava. The Buzău County Emergency Hospital has a much smaller number of patients compared to the other centers included in the analysis. The age of patients ranges from 27 to 95 years, with an average of 65.91 years and a median of 66.00 years. The results show that the odds of a woman not developing insulin-balanced type 2 diabetes mellitus are 0.65 times lower than for a man. There are no significant differences between men and women in elementary hypertension (p-value = 0.135), insulin-dependent type 1 diabetes mellitus (p-value = 0.523), oral antidiabetic balanced type 2 diabetes mellitus (p-value = 0.254), obesity (p-value = 0.284), antecedent stroke (p-value = 0.538), heart failure (p-value = 0.479), coronary artery disease (p-value = 0.692) and chronic renal failure (p-value = 0.789).

Smokers are more prevalent among men. Men are also 1.55 times more likely to develop carotid disease than women.

Patients with elementary hypertension are older than those without, and the same result holds for three other risk factors, history of stroke, heart failure and chronic renal failure, which are predominantly present in older patients. However, smokers are

younger than non-smokers, with a mean age of 63.41 years, compared with a mean age of 67.74 years for non-smokers.

Next, a score of the risk factors (antecedents) present in a patient was constructed. This risk score takes values in the range 0 (no risk factors/ antecedents present) and 11 (all risk factors/ antecedents present). To carry out this procedure, only complete observations related to the risk factors analyzed were kept. Thus 880 patients were analyzed.

Analysis of the arterial tree of the lower limbs revealed the following:

- The proportion of males with occlusion exceeds the proportion of females.
- The majority of the analyzed patients have patent vessel of the analyzed arteries.
- The main risk factors leading to lower arterial lesions are smoking, obesity, history of chronic renal failure and coronary artery disease. Cramer's V Cramer correlation coefficient indicated a positive association between the above risk factors and the lesions identified. However, individual associations were not found to be strong, indicating a multifactorial element of risk factors contributing to lower limb arterial lesions

Evidence has supported traditional cardiovascular risk factors in PAD, such as diabetes, smoking, dyslipidemia and hypertension. A sedentary lifestyle also increases the risk in developing PAD. The Edinburgh study reported that the risk of PAD is inversely related to physical activity. Of these conventional risk factors, diabetes and smoking are particularly linked to the development of PAD.

**The second study** aims to create a profile of relevant biomarkers in critical ischemia.

Inclusion criteria were: patients over 18 years of age, who agreed to participate in the study, confirmed with critical ischemia according to current ESVS guidelines (ischemic rest pain is usually described as affecting the forefoot and usually aggravated by the supine position; for diagnosis, pain must have been present for more than 2 weeks and associated with one or more abnormal parameters: ankle-brachial index  $< 0.4$ , ankle pressure  $< 50$  mmHg in absolute value, ankle-ankle pressure  $< 30$  mmHg in absolute value, a transcutaneous oxygen pressure  $< 30$  mmHg and a flat or weakly pulsating waveform (equivalent to WIfI classification grade III ischemia).

Exclusion criteria were patients under 18 years of age, patients who did not give consent for participation, patients with previous revascularization (not more than 3

months) on the index leg, patients on steroid medication, pregnancy, chronic kidney disease, active neoplasia, Child Pugh B minimal chronic liver disease, thrombophilia, other autoimmune diseases, which could affect inflammatory samples.

In these patients, pre- and postoperative biological samples were collected and the following markers were quantified:

- ✚ Matrix metalloproteinase 2
- ✚ Matrix metalloproteinase 9 -
- ✚ Human endothelial nitric oxide synthase 3 eNOS/NOS3
- ✚ Tumor necrosis factor -
- ✚ Cytokine transforming growth factor  $\beta$ 1
- ✚ Interleukin 12
- ✚ Cystatin-C, Homocysteine, hsCRP, Lipoprotein A
- ✚ IL-6

The quantification of the markers of interest was carried out after performing the calibration curves (optical density vs. standard amount for Elisa, respectively cycle threshold **Ct** vs. standard amount for QPCR), according to the manufacturer's instructions.

The role of inflammatory biomarkers in the atherosclerotic process has been extensively analyzed in both experimental and clinical studies. Although inflammatory mediators such as IL-6, TNF- $\alpha$ , C-reactive protein (CRP) have been identified as predictors of major cardiovascular events

Statistically significant variations were also found between the preoperative and postoperative values of these markers, but especially that in the critically ischemic patient the values are much higher than their baseline values.

**The third direction of study** concerns new therapeutic possibilities that can be applied in the early stages of the atherosclerotic process. In numerous literature studies, miRNAs have been identified as important markers of atherosclerosis. The present study aims to identify the expression of 3 miRNAs - *miR-199a*, *miR-20a* and *miR-30c*- in patients with limb-threatening chronic ischemic stroke in the pre- and post-revascularization periods. The aim of the study is to identify whether these 3 markers play a role in critical ischemia and whether they have potential for future use in new treatments of this pathology.

We enrolled 21 patients diagnosed with critical limb ischemia (CLTI). We collected

data 24 hours before and after the revascularization procedure, either open or endovascular. Inclusion criteria were: patients over 18 years of age who agreed to participate in the study, confirmed with CLTI according to current ESVS guidelines (ischemic rest pain is usually described as affecting the forefoot and usually aggravated by the supine position; for diagnosis, pain must have been present for more than 2 weeks and associated with one or more abnormal parameters: ankle-brachial index  $< 0.4$ , ankle pressure  $< 50$  mmHg in absolute value, ankle pressure  $< 30$  mmHg in absolute value, a transcutaneous oxygen pressure  $< 30$  mmHg and a flat or weakly pulsatile waveform (equivalent to grade III ischemia in the WIfI classification). Exclusion criteria were patients under 18 years of age or who did not consent to participate, patients with previous revascularization (not more than 3 months) on the index leg, patients on steroid medication, pregnancy, chronic kidney disease, active neoplasia, Child Pugh B minimal chronic liver disease, thrombophilia, other autoimmune diseases, which could increase microRNA expression.

Blood samples were meticulously collected in standard vacutainer tubes, each containing EDTA, which acts as an effective anti-coagulant to prevent clotting. We investigated three miRNAs: miR-199a, miR-20a, miR-30c.

The present study demonstrates the importance of microRNAs in chronic limb-threatening ischemia and their potential as biomarkers. The dynamics of their expression in different pre- and post-revascularization situations supports the hypothesis that they may also have an important role in the treatment of this pathology by targeting their regulation.

The study of miRNA is a new concern in chronic limb-threatening ischemia. Therefore, different markers are still being identified and it is necessary to further demonstrate in larger population-based studies which are the most diagnostically relevant and which may have potential in treatment.

The results of this thesis would not have been possible without an interdisciplinary collaboration between the Discipline of Pharmacology, the Discipline of Molecular

Biology, the Vascular Surgery Clinic, the Anesthesia and Intensive Care Clinic and the Clinical Laboratory of the "Pius Brînzeu" County Emergency Hospital Timisoara.

We consider that further studies are needed to identify the targeted feasibility of using inflammatory markers in the diagnosis, prognosis and treatment of critical ischemia in practice.

Thus, studies would be needed to identify different risk classes according to the value of markers, the possibility of identifying the prognosis according to the evolution of these markers, the clinical staging of the disease in the post revascularization phase, both in terms of events related to the revascularized limb and in terms of major cardiac events related to these patients.

Particular attention should be paid to diabetic patients and those with chronic kidney disease.

Ultimately, new and novel medical protocols that address these markers must be developed to protect against the inflammatory process and endothelial dysfunction.

