ABSTRACT OF PhD THESIS

MICROALBUMINURIA AND OTHER RISK MARKERS IN HYPERTENSIVE PATIENT

SCIENTIFIC COORDINATOR

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- addendum
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**Note:** This summary selectively renders references and iconography in the text, with respect to numbering and table of contents from the thesis. A limited number of figures and tables with numbering from the thesis were selected.
# TABLE OF CONTENTS

Abbreviation list .................................................................................................................. 4

GENERALITIES ........................................................................................................................ 5

INTRODUCTION ..................................................................................................................... 5

CHAPTER I. Cardiovascular risk score .................................................................................. 5

CHAPTER II. New directions in the physiopathology of hypertension .................................... 7

CHAPTER III. Target organ damage in hypertension ............................................................... 10

 III.1 Mechanisms of target organ damage in hypertension ......................................................... 11
 III.2 Left ventricular hypertrophy .............................................................................................. 12
 III.3 Atherosclerosis ................................................................................................................ 14
 III.4 Renal damage .................................................................................................................. 17

CHAPTER IV. Markers of atherosclerosis in the hypertensive patient .................................... 19

 IV.1 Microalbuminuria ........................................................................................................... 19

 IV.1.1 Definition and evaluation .............................................................................................. 19
 IV.1.2 Microalbuminuria and the other forms of target organ damage .................................. 22
 IV.1.3 Microalbuminuria and the evaluation of cardiovascular risk ........................................ 25
 IV.1.4 Pathological mechanisms of microalbuminuria onset .................................................... 30
 IV.1.5 Microalbuminuria as therapeutical target ..................................................................... 34

 IV.2 Midkine ............................................................................................................................ 36

 IV.2.1 Midkine, a new heparin-binding factor – physiopathological aspects .............................. 36
 IV.2.2 Midkine in inflammation and renal pathology ................................................................. 37
 IV.2.3 Cardioprotector and neuroprotector effect of midkine .................................................... 39
 IV.2.4 Midkine, mediator in atherosclerosis ................................................................ .......... 41
 IV.2.5 Research on midkine in human pathology .................................................................... 43

PERSONAL CONTRIBUTIONS ................................................................................................. 48

CHAPTER V. Work hypothesis ............................................................................................... 48

CHAPTER VI. General methodology ....................................................................................... 49
CHAPTER VII. Clinical studies

VII.1. CLINICAL STUDY 1. Assessment of microalbuminuria in hypertensive patients with established coronary artery disease

VII.1.1. Introduction ..................................................................................50
VII.1.2. Methods ........................................................................................51
   Patients ..................................................................................................51
   Study design and procedures .................................................................51
   Statistical analysis .................................................................52
VII.1.3. Results .........................................................................................52
VII.1.4. Discussion ....................................................................................70
   Study limits .......................................................................................75
VII.1.5. Conclusions ................................................................................75

VII.2. CLINICAL STUDY 2. Microalbuminuria, left ventricular hypertrophy, and coronary artery disease in patients with essential hypertension

VII.2.1. Introduction ..................................................................................77
VII.2.2. Methods ........................................................................................77
   Patients ..................................................................................................77
   Study design and procedures .................................................................78
   Statistical analysis .................................................................78
VII.2.3. Results .........................................................................................78
VII.2.4. Discussion ....................................................................................98
VII.2.5. Conclusions ................................................................................103

VII.3. CLINICAL STUDY 3. Serum levels of midkine, a heparin-binding growth factor, inversely correlate with angiotensin and endothelin receptor autoantibody titers in patients with macroangiopathy

VII.3.1. Introduction ..................................................................................104
VII.3.2. Methods ........................................................................................105
   Patients and study design ..................................................................105
   Analytical methods (blood) .................................................................106
   ELISA .................................................................................................106
   Immunohistochemistry (endarterectomy piece) ..................................107
   Statistical analysis .............................................................................107
VII.3.3. Results .........................................................................................107
VII.3.4. Discussion ....................................................................................123
Study limits.............................................................................................................................................128
VII.3.5. Conclusions ..................................................................................................................................128
GENERAL CONCLUSIONS ..........................................................................................................................129

Originality and innovative contributions of the thesis............................................................................132
REFERENCES ...........................................................................................................................................133
ADDENDUM ............................................................................................................................................148
GENERALITIES

The motivation for the doctoral study

Despite the progress in diagnosis and treatment of the cardiovascular risk factors, cardiovascular and renal diseases are still the leading cause of mortality in the general population. In the last few years, hypertension has been recognised as a major contributor of the risk for cardiovascular diseases, also being the factor with the highest global prevalence. It has been demonstrated that, with all treatment, hypertensive patients still have a poorer survival and a higher cardiovascular mortality compared with age-matched non-hypertensive individuals. A delay of the therapy, an inadequate control and/or type of therapy or an incomplete covering of various components of the cardiovascular added risk could contribute to a worse prognosis in hypertensives.

The risk for developing complications and for cardiovascular outcomes does not depend only on the magnitude of blood pressure, but also on the presence of associated risk factors, comorbidities, and target organ damage. All these define the so-called global cardiovascular risk.

Even if prevention and treatment measures were taken against established risk factors – smoking, obesity, sedentariness, diet, hypertension, dyslipidaemia, and diabetes mellitus, the incidence of coronary artery disease is still rising. The interest for improving the assessment of the cardiovascular risk, derived from a better understanding of the pathogenesis of atherosclerosis and the identification of new targets for atherosclerosis therapy, has always stimulated the research of new risk factors. Up to 20% of the patients with coronary artery disease do not have any risk factor and 40% only have one. In the general population, individuals with low or intermediate risk based on traditional criteria are responsible for the highest prevalence of the cardiovascular risk. Thus, the identification of new risk markers for cardiovascular diseases have a meaningful potential of improving selection of individuals for prevention strategies.

Clinical trials have brought evidence in favour of the potential importance of some variables or physiopathological conditions, which can be considered prognosis intermediate endpoints, even if they can not be regarded as established risk factors for now. It is the case for a multitude of biomarkers and inflammatory markers of coronary artery disease, like lipoprotein (a), homocisteine, fibrinogen, plasminogen activator inhibitor, C-reactive protein, different cytokines, and microalbuminuria. Among all, microalbuminuria has earned recognition as a simple marker of an atherogenic milieu.

Even if Framingham study has established since 1984 that proteinuria is an important risk marker for cardiovascular mortality in the general population, it did not lead to adding albuminuria on the list of the most important factors or cardiovascular risk markers and it did not modify the cardiovascular risk calculators. 20 years have passed until this subject received a proper attention. Important studies appeared one by one, MONICA (Multinational Monitoring of Trends and Determinants in Cardiovascular Disease), PREVEND (Prevention of Renal and Vascular End-stage Disease), HUNT (The Nord-Trøndelag Health Study) and EPIC (European
Prospective Investigation into Cancer). All of them showed that microalbuminuria predicts cardiovascular events, and data suggest that pathological changes that cause microalbuminuria and disorders that determine early atherosclerosis are the same.

Even if numerous trials have suggested that microalbuminuria is associated with a number of cardiovascular risk factors, i.e. age, male gender, hypertension, diabetes, smoking, obesity, and dyslipidaemia, it is obvious that none of these could explain the relation between microalbuminuria and atherosclerotic events. This might imply an inadequate quantification of these factors or that other factors could determine microalbuminuria and the associated cardiovascular disease. Whether microalbuminuria is a sensitive pathological indicator resulted from other risk factors for coronary disease, conclusions from trials admit that microalbuminuria could be useful in identifying patients with high risk for ischemic heart disease that could benefit the most from treatment.

Microalbuminuria fulfils the conditions for a cardiovascular biomarker, having applications in at least 4 areas: screening, diagnosis, prognosis, and treatment monitoring. This functional marker of arterial vulnerability offers information related to different stages of atherogenesis including subclinical cardiovascular disease and its sequelae, and probably the technological progress in the future would make possible the use of a multimarker profiling for individualising the treatment of cardiovascular diseases.

Further, we will present a new factor with potential to become a biomarker in cardiovascular pathology, but whose studies are still in early phase.

The 13 kDa heparin-binding growth factor midkine (MK) was first identified as the product of the Midkine gene, whose expression increased at early stage of the retinoic acid-induced differentiation of murine teratocarcinoma stem cells and also in the mid-gestation period of mouse embryogenesis (130). Midkine plays a major role in development and cell survival, neural growth, inflammation, hypertension, and malignancy (132). Numerous studies have demonstrated an amazing increase of midkine expression in different pathologies, compared to healthy subjects, especially in ischemia, inflammation, autoimmunity, but mostly in cancer. Midkine is important for the pathogenesis of vascular restenosis, after balloon angioplasty and vein grafts and also after stenting, thus being able to become a therapeutical target.

An earlier study (158) showed an important role of midkine in cardiac remodelling after myocardial infarction due to activation of angiogenesis. Potent angiogenic and anti-apoptotic activities of midkine from the chronic stage of myocardial infarction could act synergistically to prevent left ventricular remodelling (156). Anti-apoptotic action of midkine could determine a reduction of secondary necrosis, which would further activate inflammation (158).

Ischemia/reperfusion injury followed by angiogenesis and cardiac remodelling, accompanied by vascular stenosis have a few mechanisms in common (infiltration of inflammatory cells, cytokines and growth factors expression). Therefore we consider that midkine's major functions in cardiovascular diseases depend on these events.
If at experimental level on different animal models studies results are promising and open new perspectives on possible therapeutical applications, research in human pathology is still poor.

Because midkine is expressed in cardiomyocytes during ischemia and overexpressed after myocardial infarction, midkine's increase after cardiac transplant could be due to ischemia/reperfusion and protection against remodelling of the transplanted heart.

By enhancing the inflammatory response, midkine might contribute to development and progression of cardiac dysfunction in heart failure, high levels being suggestive for cardiac damage and/or congestive heart failure. Midkine might also represent a surrogate marker for subclinical inflammation in this population.

A necessary condition for midkine to become a biomarker with clinical application is to elucidate its specific indications, to standardise analytical methods, to characterise analytical features, to evaluate performance characteristics, additional benefit of different markers for a given clinical indication and demonstration of cost-efficiency. In Clinical Study 3 in Personal Contributions of the thesis we will try to add a new clinical use of midkine to the list for human pathology.

**PERSONAL CONTRIBUTIONS**

V. Work hypothesis

The first 2 studies aim to assess the clinical relevance of microalbuminuria in patients with established cardiovascular disease and to underline the significance of this factor. The fact that microalbuminuria could be regarded as an early marker of vascular damage may partly explain why the relationship between cardiovascular comorbidities and microalbuminuria had been observed irrespective of target organ damage (e.g. estimated glomerular filtration rate (eGFR) or left ventricular hypertrophy (LVH)) or the presence of diabetes mellitus. An aggregation of multiple pathological conditions could be associated and aggravated by endothelial damage.

While several studies proving the implication of classical risk factors in vascular ischemic pathology exist, more and more evidence become available for the role of microalbuminuria, and this was the purpose of the introduction in Generalities. Further on we wish to define this role in clinical practice by analysing a significant group of hypertensive patients with coronary artery disease that underwent coronarography and to whom we have tested microalbuminuria. We want to determine the extent of this risk marker of the high-risk hypertensive patient and its relation to the other classical factors. The importance of microalbuminuria as a screening tool should be supported by a powerful connection with the burden of risk factors in high-risk patients.

It is also of interest to establish the link with other markers of target organ damage, and in this case we easily assessed left ventricular hypertrophy by ecocardiography. To define more
precisely the involvement of microalbuminuria in the coronary risk profile, the study group has been compared with a control group of hypertensives without coronary artery disease (Clinical Study 2).

The third study opens a new perspective on a multifunctional cytokine, midkine, a possible factor with pathological involvement in patients with peripheral artery disease that are submitted to endarterectomy. Data from literature show the participation of midkine in regulating renin-angiotensin system in hypertension, but also in atherosclerosis process, data being only experimental. In the present study we wanted to look for and to highlight midkine in atherosclerotic arteries and in sera from patients with ischemic disease.

The next step was represented by searching correlations of midkine with classical cardiovascular risk factors, but also with a few new factors, meaning autoantibodies of renin-angiotensin system and autoantibodies of endothelinic receptor. Next to the classical risk factors, there are important evidence in the literature for the involvement of autoimmune systemic factors in accelerated atherosclerosis. Renin-angiotensin system and endothelin system are participants at the atherosclerotic process, therefore the observation of any molecular interactions at this level may open new perspectives in the physiopathology of atherogenesis.

VI. General methodology

The first 2 studies have been conducted in the Cardiology Clinic of the Cardiovascular Diseases Institute "Prof. dr. George I.M. Georgescu" from January 2012 – December 2013. The study group was represented by hypertensive (controlled under medical treatment) patients in whom the angiography objectified significant coronary stenosis, and were diagnosed with different forms of angina pectoris. Patients with acute myocardial infarction were excluded.

Data included in the study were obtained from medical history, laboratory investigations, and echocardiography. Microalbuminuria was measured in all patients by a quantitative method – immuneturbidimetry. A statistical analysis was carried on the collected data – clinical and laboratory features – in relationship with microalbuminuria.

The control group included patients with hypertension under treatment, who had no clinical or angiographical evidence of ischemic heart disease.

All patients signed the informed consent for participating in the study. This study was conducted in accordance to the Declaration of Helsinki and was approved by the local ethical Committee.

The third study was conducted at the University of Medicine "Otto von Guericke", Magdeburg, Germany, in the Clinic of Nephrology, Hypertension, Diabetes, and Endocrinology and in the Department of Vascular Surgery, from July 2010 – March 2012. The study group included patients operated on for peripheral artery disease stages III-IV Fontaine. Data collected was represented by medical history and usual lab analysis, but also functional autoantibodies (for
angiotensin receptor and endothelin receptor), that had been analysed at CellTrend, Luckenwalde, Germany.

As biological materials, we have collected blood (prior to the operation) that had been centrifuged and the serum obtained was kept at -80°C until analysis, and an endarterectomy piece immediately after operation, that was methacarn fixed and parafined, and later processed in histological sections.

ELISA for quantification of midkine in serum and immunohistochemistry applied for histological sections to identify midkine in the artery, followed by computer analysis, took place in the Laboratory of the Clinic of Nephrology, Hypertension, Diabetes, and Endocrinology of the University Hospital, Magdeburg.

V. 1. CLINICAL STUDY 1
ASSESSMENT OF MICROALBUMINURIA IN HYPERTENSIVE PATIENTS WITH ESTABLISHED CORONARY ARTERY DISEASE

VII.1.1. Introduction
In hypertensive subjects, microalbuminuria has often been correlated with an excess of atherosclerotic disease and with a high level of atherosclerotic risk markers. The relationship between microalbuminuria and the development of vascular disease is strengthened by its presence in acute myocardial ischemia, congestive heart failure, stroke, peripheral artery disease, and carotid atherosclerosis. Therefore, microalbuminuria could serve as a "diagnosis window" for the whole vasculature, thus becoming an indicator of generalized endothelial damage.

Both the presence of microalbuminuria and its level offer important information regarding the determination of risk profile of hypertensive patients, and there are conclusive evidence from randomised trials that show that early detection of microalbuminuria and strict control of blood pressure can slow the progression and onset of cardiovascular disease (125). Cardiovascular risk and mortality of these patients are strongly influenced by comorbidities and risk factors.

In the present study we have investigated the clinical relevance of microalbuminuria in high-risk hypertensives with coronary disease and we have underlined the significant correlations with traditional risk factors. The primary objective was to establish the prevalence of microalbuminuria in hypertensives with ischemic disease and to identify the role in establishing the cardiovascular risk. The secondary objectives were to determine the correlations between microalbuminuria and classical risk factors, but also with left ventricular hypertrophy.
VII.1.2. Methods

Patients

94 patients with hypertension (on medication) and stable coronary disease (documented with angiography, with > 50% stenosis of one or more coronary arteries) admitted in the Cardiovascular Diseases Institute from January 2012 – July 2013 have been screened for microalbuminuria. Microalbuminuria was tested in all individuals excluding the ones with fever (> 38°C), renal disease (glomerular filtration rate <60 ml/min/1,73 m², Cockroft-Gault formula), urinary infection or who have performed strenuous physical activity during the last 24 hours, but also pregnant or menstruating women (high probability of false positive results).

VII.1.3. Results

Normality tests for microalbuminuria (MAU) distribution show that the values are not normally distributed. This can be observed from the histogram of MAU values distribution for the 94 patients, presented in fig.7.1.

In fig. 7.2 – 7.7 are represented the 2 groups from the study population (normoalbuminuric and microalbuminuric group), and respectively the proportion of traditional cardiovascular risk factors and left ventricular hypertrophy among them. Clinical and biological characteristics of the 2 groups are presented in table 7.II.

![Figure 7.1 Distribution of microalbuminuria values in the study population](image)

In the study population, 57,4% (54 subjects) were male and 42,6% (40 subjects) were female (fig. 7.2). Among them, 54,5% (24 subjects) of males and 45,5% (20 subjects) of females were in the normoalbuminuric group, and 60% (30 male subjects) and 40% (20 female subjects) represented the microalbuminuric group.
Figure 7.2 Gender distribution in the normoalbuminuric and the microalbuminuric group

Of all patients, 46 (48.9%) were non-smokers and 48 (51.1%) smokers (fig. 7.3). Of these, 25 smokers (52.1%) were in the normoalbuminuric group and 23 smokers (47.9%) in the microalbuminuric group.

Figure 7.3 Risk factor Smoking in the normoalbuminuric and the microalbuminuric group

Of all patients, 49 (52.1%) were non-diabetic and 45 (47.9%) diabetic (fig. 7.4). Of these, 23 (51.1%) diabetic subjects represented the normoalbuminuric group and (48.9%) the microalbuminuric group.
Of all patients, 20 (21.3%) were normolipidemic and 74 (78.7%) dyslipidemic (fig. 7.5). Of these, 34 (45.9%) dyslipidemic subjects represented the normoalbuminuric group and 40 (54.1%) the microalbuminuric group.

**Figure 7.4** Risk factor Diabetes mellitus in the normoalbuminuric and the microalbuminuric group

**Figure 7.5** Risk factor Dyslipidaemia in the normoalbuminuric and the microalbuminuric group
Of all patients, 55 (58.5%) had normal weight and 39 (41.5%) were obese (fig. 7.6). Of these, 16 (41%) obese subjects represented the normoalbuminuric group and 23 (59%) the microalbuminuric group.

**Figure 7.6** Risk factor Obesity in the normoalbuminuric and the microalbuminuric group

Of all patients, 39 (41.5%) did not have LVH and 55 (58.5%) had LVH (fig. 7.7). Of these, 22 (40%) subjects with LVH represented the normoalbuminuric group and 33 (60%) the microalbuminuric group.

**Figure 7.7.** Presence of left ventricular hypertrophy in the normoalbuminuric and the microalbuminuric group
Table 7.II. Clinical and biological characteristics of the study population

<table>
<thead>
<tr>
<th></th>
<th>normoalbuminuric group</th>
<th>microalbuminuric group</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 44</td>
<td>n=50</td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>60 (50-72)</td>
<td>62.5 (55-75)</td>
<td>p=0.29</td>
</tr>
<tr>
<td>Male, %</td>
<td>54.50%</td>
<td>60%</td>
<td>p=0.67</td>
</tr>
<tr>
<td>Smokers, %</td>
<td>56.80%</td>
<td>46%</td>
<td>p=0.31</td>
</tr>
<tr>
<td>Hypertension, y</td>
<td>5 (2-9)</td>
<td>6 (3-10.25)</td>
<td>p=0.26</td>
</tr>
<tr>
<td>Dyslipidaemia, %</td>
<td>77.30%</td>
<td>80%</td>
<td>p=0.8</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>52.30%</td>
<td>44%</td>
<td>p=0.53</td>
</tr>
<tr>
<td>Obesity, %</td>
<td>36.40%</td>
<td>46%</td>
<td>p=0.4</td>
</tr>
<tr>
<td>LVH, %</td>
<td>50%</td>
<td>66%</td>
<td>p=0.144</td>
</tr>
<tr>
<td>EF (%)</td>
<td>45 (35-50)</td>
<td>45 (40-50)</td>
<td>p=0.676</td>
</tr>
</tbody>
</table>

Laboratory data

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dl)</td>
<td>117 (105-180)</td>
<td>108 (100.25-173)</td>
<td>p=0.179</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>50 (32-59)</td>
<td>48 (38.75-54)</td>
<td>p=0.964</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>113 (95-164)</td>
<td>119 (93-139)</td>
<td>p=0.578</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>185 (159-221)</td>
<td>193 (163.5-215.25)</td>
<td>p=0.894</td>
</tr>
<tr>
<td>Triglycerides (mg/l)</td>
<td>154 (108-200)</td>
<td>118 (73-212.25)</td>
<td>p=0.1</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73m2)</td>
<td>89.6 (64.3-117.97)</td>
<td>84.65 (68.64-99.75)</td>
<td>p=0.74</td>
</tr>
<tr>
<td>Microalbuminuria (mg/l)</td>
<td>10.12 (6.21-15.77)</td>
<td>48.65 (30.62-87.06)</td>
<td></td>
</tr>
<tr>
<td>logmicroalbuminuria</td>
<td>1 (0.78-1.19)</td>
<td>1.68 (1.48-1.93)</td>
<td></td>
</tr>
</tbody>
</table>

LVH, left ventricular hypertrophy; LVEF, left ventricular ejection fraction; HDL, high density lipoprotein; LDL, low-density lipoprotein; eGFR, estimated glomerular filtration rate

Urine analysis showed that a substantial proportion (53.2%, 50 patients) of the study population represented by high-risk hypertensives had microalbuminuria, fig. 7.8.
Figure 7.8 Proportion of patients in the 2 groups, normoalbuminuric and microalbuminuric

Patients with microalbuminuria are older, mostly male, with a longer duration of hypertension and with a higher prevalence of left ventricular hypertrophy. A significant percentage are dyslipidemic (80%).

A correlation analysis was made in order to evaluate the relationship of microalbuminuria with traditional risk factors:
- male gender (p=0.244),
- age (p=0.011),
- smoking (p=0.089),
- diabetes mellitus (p=0.179),
- dyslipidemia (p=0.970),
- obesity (p=0.781).

The only factor with statistical significance was the age. An important correlation was established with the presence of left ventricular hypertrophy (p=0.004, fig. 7.17) and with the duration of hypertension (p=0.044).
Binary logistic regression shows that MAU influences significantly the presence or absence of LVH (Exp(B)=1.021, p=0.004). While MAU increases, the probability of LVH rises with 2%.

### Variables in the Equation

<table>
<thead>
<tr>
<th>Step 1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>B</th>
<th>S.E.</th>
<th>Wald</th>
<th>df</th>
<th>Sig.</th>
<th>Exp(B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAU</td>
<td>.020</td>
<td>.007</td>
<td>8.307</td>
<td>1</td>
<td>.004</td>
<td>1.021</td>
</tr>
<tr>
<td>Constant</td>
<td>-.387</td>
<td>.287</td>
<td>1.816</td>
<td>1</td>
<td>.178</td>
<td>.679</td>
</tr>
</tbody>
</table>

<sup>a</sup> Variable(s) entered on step 1: MAU.

After inserting more variables in multiple regression models, LVH and age are the only independent variables correlated with MAU.

### VII.1.4. Discussion

First of all, this study shows a high prevalence of microalbuminuria (51.4%) among patients with coronary artery disease, supporting the theory of generalised extent of inflammation and endothelial dysfunction, the albumin being able to pass through the damaged vascular endothelium.

Earlier studies reported a 10% - 15% and respectively 15% - 20% prevalence of microalbuminuria in hypertensive and diabetic patients, which is higher than the prevalence reported in the general population (6% - 8%) (184). Other studies report a prevalence as high as 40% in diabetic patients without known renal disease, a percentage similar to ours of 44%.
Losartan Intervention for Endpoint Reduction (LIFE) trial on hypertensive patients with electrocardiographical signs of LVH showed a 23% prevalence. From what we know so far there are no large trials concerning the incidence and prevalence of microalbuminuria in a specific cohort of hypertensives, that is why our results bring a novel point of view on hypertensives with coronary artery disease. The prevalence of microalbuminuria in the present study was close to the one reported in I-SEARCH which included high- and very high risk patients with hypertension.

In our study the patients presented an important aggregation of risk factors, taking into account the fact that 80% were dyslipidemic, and almost 50% were either smokers, obese, or diabetics. The strong link between microalbuminuria and the accumulation of risk factors emphasize the importance of microalbuminuria as screening element to identify patients at high risk. The fact that microalbuminuria could be seen as an early marker of subclinical vascular damage could partly explain why the relationship between cardiovascular comorbidities and microalbuminuria was observed irrespective of target organ damage or diabetes mellitus.

The Copenhagen City Heart study revealed a strong correlation between microalbuminuria and metabolic syndrome (186), showing that microalbuminuria is associated with a high risk of death and cardiovascular disease at a similar level as metabolic syndrome, irrespective of its concomitant presence. A recent analysis of the same study showed that microalbuminuria gives a risk of death similar to obesity, irrespective of its concomitant presence or other risk factors. The population in the present study had a high prevalence of the components of the metabolic syndrome. Like in the aforementioned study, our study neither could demonstrate a meaningful connection between obesity and coronary disease when the population was divided according to albumin excretion, the reason probably being the small number of subjects.

Our results are in agreement with data from I-SEARCH, where patients with LVH had the highest prevalence of microalbuminuria (68%) compared to the presence of other cardiovascular conditions (185).

Microalbuminuria is a biological indicator or a marker of structural and/or functional damage of small vessels. Taking into consideration the imaging difficulties for arterioles, microalbuminuria is probably the best available method as a measure for microangiopathy. By demonstrating its high prevalence in hypertensive patients with coronary disease we also support its important role as an indicator of macroangiopathy.

**VII.1.5. Conclusions**

By proving that in a given population of hypertensive patients with ischemia microalbuminuria is widespread and that it is in a direct relation to left ventricular hypertrophy, we have highlighted the importance of carefully examining patients with high levels of albuminuria in order to detect target organ damage and other cardiovascular comorbidities, to avoid underdiagnosis and insufficient therapy.
VII.2. CLINICAL STUDY 2
MICROALBUMINURIA, LEFT VENTRICULAR HYPERTROPHY, AND CORONARY ARTERY DISEASE IN PATIENTS WITH ESSENTIAL HYPERTENSION

VII.2.1. Introduction
It has been suggested that microalbuminuria is just a marker of generalised atherosclerosis and that this explains its association with clinical cardiovascular disease. Microalbuminuria and cardiovascular disease could be connected not only by a common risk factor, but even by a physiopathological process. Actually, it has been a long time since the idea that vascular endothelium dysfunction causes both microalbuminuria and cardiovascular disease has emerged. Generalised endothelial dysfunction is now considered a translator of atherogenic risk factors and is assumed to play an important role in the initiation and progression of atherosclerosis. Therefore, a coupling of microalbuminuria with generalised endothelial dysfunction, if it really exists, could explain the reason why microalbuminuria is an important predictor of cardiovascular disease.

It has been reported that patients with microalbuminuria are characterised by signs of diffuse vascular injury and target organ damage, like left ventricular hypertrophy, supporting the affirmation that microalbuminuria is an early marker of target organ damage and of cardiovascular disorders in hypertensive patients. We do not have yet enough data about the interconnection of the two conditions of target organ damage in hypertension, meaning microalbuminuria and left ventricular hypertrophy in ischemic patients.

The primary objective of this study was to establish the prevalence of microalbuminuria in hypertensive patients with and without coronary artery disease by introducing a control group to prove the role of microalbuminuria as a marker of coronary artery disease in hypertensive patients. The secondary objectives were to determine the correlations between microalbuminuria and classical risk factors in the 2 groups, study group and control group, as well as left ventricular hypertrophy, as target organ in hypertension. Even though the relationship between microalbuminuria and coronary atherosclerosis has been widely investigated, it is not the same case for the superposition and association of the two parameters of target organ damage.

VII.2.2. Methods
Patients
157 eligible patients with hypertension under treatment admitted in the Cardiovascular Diseases Institute between January 2012 – December 2013 have been screened for microalbuminuria. Besides what was mentioned in the Clinical Study 1, the patients were divided in 2 groups based on the medical history, the clinical and biochemical evaluation and diagnosis procedures (coronarography): with coronary artery disease (CAD, n=109) and without coronary artery disease (n=48). Patients without CAD represent the control group.
VII.2.3. Results

General characteristics of the study population with and without coronary artery disease are presented in the table 7.XIV. The average age of the 157 subjects was 65.9 years (58-75 y, percentile 25-75), 44.6% were male, and the average duration of hypertension was 7 years.

51.6% of the population had microalbuminuria, without significant differences of prevalence between the group with coronary disease and the one without.

Regarding the aggregation of the other cardiovascular risk factors one may observe an almost equitable distribution, representing approximately one third of the patients, of smoking, diabetes, and obesity. Almost ¾ of the subjects are dyslipidemic.

Half of the studied patients presented LVH and half of them had prior treatment with angiotensin converting enzyme inhibitors (ACEI) or angiotensin receptor blockers (ARB).

Table 7.XIV. Basic characteristics of the study population

<table>
<thead>
<tr>
<th></th>
<th>All (n = 157)</th>
<th>With CAD (n = 109)</th>
<th>Without CAD (n = 48)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>65.93±10.91</td>
<td>63.2±11.01</td>
<td>72.1±7.78</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Male, %</td>
<td>44.6</td>
<td>49.5</td>
<td>33.3</td>
<td>0.061</td>
</tr>
<tr>
<td>Hypertension, y</td>
<td>7 (3-10)</td>
<td>5 (3-9)</td>
<td>9 (7-13)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smoker, %</td>
<td>37.6</td>
<td>46.8</td>
<td>16.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes, %</td>
<td>34.4</td>
<td>46.8</td>
<td>6.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Dyslipidemic, %</td>
<td>74.5</td>
<td>84.4</td>
<td>52.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Obese, %</td>
<td>31.8</td>
<td>39.4</td>
<td>14.6</td>
<td>0.002</td>
</tr>
<tr>
<td>LVH, %</td>
<td>48.4</td>
<td>56.9</td>
<td>29.2</td>
<td>0.001</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>50 (45-55)</td>
<td>50 (40-50)</td>
<td>55 (50-60)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ACEI/ARB, %</td>
<td>52.2</td>
<td>53.2</td>
<td>50</td>
<td>0.713</td>
</tr>
<tr>
<td>Glycemia (mg/dl)</td>
<td>134.8±67.03</td>
<td>149.7±75.25</td>
<td>100.98±14.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>189.66±39.07</td>
<td>192.36±39.91</td>
<td>183.5±36.76</td>
<td>0.148</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl)</td>
<td>49.01±10.62</td>
<td>47.06±11</td>
<td>53.4±8.21</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dl)</td>
<td>116.75±34.43</td>
<td>121.5±35</td>
<td>105.9±30.71</td>
<td>0.006</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>152.46±74.53</td>
<td>161±82.7</td>
<td>132.8±46.4</td>
<td>0.105</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73 m²)</td>
<td>78(62.4-97.1)</td>
<td>82.1(68.47-103.3)</td>
<td>62.85 (60.5 – 76.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Microalbuminuria (%)</td>
<td>51.6</td>
<td>51.4</td>
<td>47.9</td>
<td>0.542</td>
</tr>
<tr>
<td>Microalbuminuria (mg/l)</td>
<td>19.76</td>
<td>20.79</td>
<td>18.76</td>
<td>0.047</td>
</tr>
</tbody>
</table>

LVH, left ventricular hypertrophy; LVEF, left ventricular ejection fraction; ACEI, angiotensin converting enzyme inhibitors; ARB, angiotensin receptor blockers; eGFR, glomerular filtration rate
The patients with coronary disease were younger, with a shorter duration of hypertension, and mostly male. The probability to be smokers, diabetics, dyslipidemias, and obese was higher than for the control group.

Compared to the group without CAD, patients with CAD had a higher prevalence of LVH (56.9% vs. 29.2%, p=0.001, fig. 7.26) and higher values of albuminuria (median 20.79 mg/l vs. 18.76 mg/l, p=0.047, fig.7.27). The percentage of normo- and microalbuminuric patients was not different in the 2 groups.

Figura 7.26 Distribution of normo- and microalbuminuric patients and with left ventricular hypertrophy (HVS) respectively in the 2 groups of the study population (with and without coronary artery disease (BCI))

Figura 7.27 Microalbuminuria in patients with and without coronary artery disease (BCI) (A) and in those with and without left ventricular hypertrophy (HVS) (B)
A correlation analysis established the statistical significant relationship between MAU and age (p=0.048), diabetes mellitus (p=0.016), duration of hypertension (p=0.038), and prior treatment with ACEI/ARB (p=0.002), table 7.XV.

**Table 7.XV** Significant correlations with microalbuminuria

<table>
<thead>
<tr>
<th></th>
<th>MAU</th>
<th>Vârsta</th>
<th>DZ</th>
<th>Durata HTA</th>
<th>IECA_BRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAU</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pearson Correlation</td>
<td>1</td>
<td>.158*</td>
<td>.193*</td>
<td>.165*</td>
<td>-.251**</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td></td>
<td>.048</td>
<td>.016</td>
<td>.038</td>
<td>.002</td>
</tr>
<tr>
<td>N</td>
<td>157</td>
<td>157</td>
<td>157</td>
<td>157</td>
<td>157</td>
</tr>
</tbody>
</table>

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

The other cardiovascular risk factors (male gender, obesity, dyslipidemia, smoking) included in the analysis were not correlated with microalbuminuria (table 7.XVI).

**Table 7.XVI** Insignificant correlations with microalbuminuria

<table>
<thead>
<tr>
<th></th>
<th>MAU</th>
<th>Sex</th>
<th>Obezitate</th>
<th>HLP</th>
<th>Fumat</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAU</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pearson Correlation</td>
<td>1</td>
<td>-.103</td>
<td>.063</td>
<td>.091</td>
<td>-.006</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td></td>
<td>.198</td>
<td>.435</td>
<td>.255</td>
<td>.942</td>
</tr>
<tr>
<td>N</td>
<td>157</td>
<td>157</td>
<td>157</td>
<td>157</td>
<td>157</td>
</tr>
</tbody>
</table>

A statistically significant correlation is seen between MAU, HVS and BCI (table 7.XVII).

**Table 7.XVII** Significant correlations with microalbuminuria

<table>
<thead>
<tr>
<th></th>
<th>MAU</th>
<th>HVS</th>
<th>BCI</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAU</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pearson Correlation</td>
<td>1</td>
<td>.311**</td>
<td>.194*</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td></td>
<td>.000</td>
<td>.015</td>
</tr>
<tr>
<td>N</td>
<td>157</td>
<td>157</td>
<td>157</td>
</tr>
<tr>
<td>Pearson Correlation</td>
<td></td>
<td>.311**</td>
<td>1</td>
</tr>
<tr>
<td>HVS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td></td>
<td>.000</td>
<td>.001</td>
</tr>
<tr>
<td>N</td>
<td>157</td>
<td>157</td>
<td>157</td>
</tr>
<tr>
<td>Pearson Correlation</td>
<td></td>
<td>.194*</td>
<td>.255**</td>
</tr>
<tr>
<td>BCI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td></td>
<td>.015</td>
<td>.001</td>
</tr>
<tr>
<td>N</td>
<td>157</td>
<td>157</td>
<td>157</td>
</tr>
</tbody>
</table>

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

**. Correlation is significant at the 0.01 level (2-tailed).**
In a univariable linear regression, microalbuminuria positively correlated with the presence of coronary disease ($\beta = 0.194 \pm 0.001$, $p=0.015$) and LVH ($\beta = 0.311 \pm 0.001$, $p<0.001$), table XVIII.

**Table 7.XVIII. Univariable linear regression**

<table>
<thead>
<tr>
<th>Model</th>
<th>Unstandardized Coefficients</th>
<th>Standardized Coefficients</th>
<th>t</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Constant)</td>
<td>.608</td>
<td>.050</td>
<td>12.042</td>
<td>.000</td>
</tr>
<tr>
<td>MAU</td>
<td>.003</td>
<td>.001</td>
<td>.194</td>
<td>2.467</td>
</tr>
</tbody>
</table>

a. Dependent Variable: BCI

<table>
<thead>
<tr>
<th>Model</th>
<th>Unstandardized Coefficients</th>
<th>Standardized Coefficients</th>
<th>t</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Constant)</td>
<td>.334</td>
<td>.053</td>
<td>6.298</td>
<td>.000</td>
</tr>
<tr>
<td>MAU</td>
<td>.005</td>
<td>.001</td>
<td>.311</td>
<td>4.070</td>
</tr>
</tbody>
</table>

a. Dependent Variable: HVS

60.5% of the subjects with microalbuminuria also had LVH. Compared to the patients without LVH (n=81 patients, 51.6%), those with LVH (n=76 patients, 48.4%) had higher albuminuria values (fig. 7.27 B): median 26.85 mg/l (16.21 – 54.72 mg/l) vs. median 16.7 mg/l (8.84 – 25.36 mg/l), $p<0.001$.

Further on, binary logistic regression showed that high albuminuria rates influence the presence or absence of LVH (Exp(B)=1.025, $p=0.001$) and the presence or absence of coronary disease (Exp(B)=1.019, $p=0.023$).

**VII.2.4. Discussion**

We have observed a high prevalence of microalbuminuria in the study population, more than 50% of the subjects having albuminuria values of 20-200 mg/l. This could be explained by the characteristics of the study group, where besides a longstanding hypertension (median of 7 years), 34.4% of patients also had diabetes. The accumulation of all risk factors with important proportions defines a high-risk population, and this explains why 109 out of 157 patients already have ischemic heart disease. Even if there are no significant differences between normo- and
microalbuminuric subjects in the groups with and without coronary artery disease (for the same reasons already mentioned), there is a tendency towards higher microalbuminuria values in the ischemic group, pointing out the importance of this factor in the orchestra of cardiovascular risk factors. We also support with these results the need to redefine microalbuminuria cut-off values, because we have observed lower values than initially defined in our coronary patients.

We cannot overlook the fact that almost half of the study population had LVH, with a considerable difference between the two groups with or without CAD (56.9% vs. 29.2%), and having an inverse relationship with the duration of hypertension. Apparently patients with a longer duration of hypertension and without coronary disease were better treated than those who developed ischemic disease (also taking into consideration the low prevalence of the other risk factors), or it could suggest that LVH itself is an important condition of the coronary artery disease.

Our results show an independent association between microalbuminuria and LVH, strengthening the idea that multiple target organ damage contributes to high risk and to occurrence of ischemic disease in hypertensives. Microalbuminuria and LVH are independent predictive factors for coronary disease.

It would be of interest the follow-up of the 2 groups to assess the consequences of changes in albuminuria for the cardiovascular outcome.

VII.2.5. Conclusions

Microalbuminuria is very common in high-risk hypertensive patients, but it is still poorly diagnosed, and the awareness of its importance as a cardiovascular risk marker is very low. Given its quality of early, strong and independent marker of cardiovascular morbidity and mortality, our study emphasize the importance of evaluating the presence of microalbuminuria followed by that of target organ damage – focusing on left ventricular hypertrophy – and not in the last place, the necessity of a more aggressive medical therapy in hypertensive patients beyond strictly treating high blood pressure.
VII.3. CLINICAL STUDY 3.
SERUM LEVELS OF MIDKINE, A HEPARIN-BINDING GROWTH FACTOR, INVERSELY CORRELATE WITH ANGIOTENSIN AND ENDOTHELIN RECEPTOR AUTOANTIBODY TITERS IN PATIENTS WITH MACROANGIOPATHY

VII.3.1. Introduction
In the following study we have selected a cohort of patients with clinically apparent peripheral arterial occlusive disease undergoing revascularisation through bypass operations. These patients are at high risk for cardiovascular morbidity and mortality. They have not developed sufficient arterial collateralisation. Our hypothesis is that midkine plays an important role in the atherosclerotic pathology and that this cytokine together with humoral immune response represented by autoantibodies against angiotensin 1 receptor (AT1R) and endothelin A receptor (ETAR) depict a functional link in the atherosclerotic process.

VII.3.2. Methods

Patients and study design
This study initially included 200 patients that underwent an endarterectomy intervention for severe peripheral artery disease stages III-IV Fontaine between July 2010 – March 2012. Out of the 200 patients, 118 individuals with hypertension and normal renal function have been selected for the final study.

19 endarterectomy pieces have been collected from the operated patients, and they were later analysed by immunohistochemistry.

Analytical methods (blood)
Serum midkine levels were quantified by immunoenzymatic assays with human midkine enzyme-linked immunosorbent assay (ELISA) provided by PeproTech (Hamburg, Germany), according to manufacturer’s instructions by means of a spectrophotometer (TECAN Infinite 200; Tecan Group US, Inc., Durham, NC). Antibodies against AT1R and ETAR were analysed by CellTrend, Luckenwalde, Germany (European patent number 1393076, assay validated according to the Guidance for Industry: Bioanalytical Method Validation, US FDA May 2001). Complete blood count, creatinine, serum lipids, and fasting glucose were analysed by standard laboratory methodology at the Clinical Chemistry Institute of the Otto-von-Guericke University, Magdeburg, Germany.
100 blood samples from healthy volunteers have been analysed for serum levels of midkine and they served as controls. The volunteers were asymptomatic for any form of arterial disease (coronary or peripheral) and they did not present clinical signs of inflammatory status at the time of blood collection.

**VII.3.3. Results**

By immunohistochemistry and microscopic analysis, in comparison with a positive control, midkine was found in the thickened intima of fatty streaks and of advanced atherosclerotic lesions (fig. 7.32).

![Figura 7.32. Midkine expression in an advanced atherosclerotic plaque; objective's power 40x](image)

After qualitatively objectifying the cytokine by means of immunohistochemistry in atherosclerotic plaques of femoralis artery and branches, we have quantitatively determined midkine in serum from patients in the study group serum. Midkine titers obtained by ELISA (fig. 7.35) have been significantly higher among patients with macroangiopathy (mean midkine value 1364 (657-2214) pg/ml, logmidkine = 3.14 ± 0.53) than in the control group (mean midkine value 239 (141-961) pg/ml, log midkine control = 2.5 ± 0.47, p < 0.001). Correlation analysis have been carried out to determine if changes in serum level of midkine are associated with clinical or laboratory data.
**Figura 7.35** ELISA plate after adding the ABTS substrate, before the spectrophotometric analysis

Midkine values distribution graph show that the variable is not normally distributed (fig. 7.36).

**Figura 7.36** Histogram of midkine distribution

Clinical and biological characteristics of the study patients are summarised in the table 7.XXXI. The majority of the study population is represented by male smokers. Traditional cardiovascular risk factors were present at most of the patients. 39% had diabetes mellitus.
We have compared mean midkine values in different atherosclerotic risk subgroups: male gender (p=0.34), age >60 years (p=0.34), obesity (p=0.27), diabetes mellitus (p=0.23), and dyslipidaemia (p=0.33). None of them showed a statistically significant correlation. The assessment of midkine in relationship with glycemia did not reveal any connection (r = 0.41, p=0.66).
Midkine was not influenced by systolic or diastolic blood pressure (r = 0.128 and 0.104 respectively), neither by glomerular filtration rate (r = 0.08) or CRP (p=0.052). Prior treatment with ACEI/ARB did not reach statistical significance in relation with midkine (p=0.927).

Besides inhibiting renin-angiotensin system (RAS) medication, there have been described autoantibodies against angiotensin receptors (AT1R) and endothelin receptors (ETAR). These autoantibodies have been quantified in serum samples by means of an ELISA technique based on a recombinant protein. Correlative analysis demonstrated a strong inverse relationship between serum levels of midkine and AT1R (r=−0.215, p=0.019) and ETAR (r=−0.265, p=0.004) titers, fig. 7.44.

Figura 7.44 Relationship between serum levels of (A) midkine and anti-AT1R (units) and (B) midkine and anti-ETAR (units) titers.

VII.3.4. Discussion

The primary objective of this study was the investigation of the cytokine midkine in relationship with atherosclerotic risk factors. Therefore, we have collected hystopathological specimens and blood samples from a cohort of patients with macroangiopathy that have undergone a revascularisation procedure. After qualitative reveal of midkine in atherosclerotic plaques of different stages (fatty streaks and advanced plaques), we have continued the research by quantitatively evaluating this cytokine in our patients. The first results showed high serum levels in these patients when compared to control group.

In the few studies on patients from literature high midkine levels have also been found in different chronic inflammatory pathologies in comparison with healthy volunteers. Ulcerative colitis (179), septic shock, and sepsis (182) are just a few examples. Experimental studies on
animals are more numerous and support these results, as we have presented in the Generalities part of the thesis.

Contrary to our belief, we have not found any positive correlation between midkine and CRP, nor with clinical or laboratory parameters routinely collected for the surgical preparation (blood pressure, leukocytes count, etc.). This is not unusual, given the great acceptance of the theory that atherosclerosis is not completely explained by the prevalence of ordinary cardiovascular risk factors. The lack of correlation with CRP orients us towards another substrate of action other than the inflammatory one in elucidating the implication of midkine in the studied pathology. Similarly, we could not detect any difference between midkine levels in patients with RAS interfering medication. In order to prove the new role of midkine in peripheral artery disease we have further looked for other relevant factors for the physiopathology of this phenomenon.

In the present study we have hypothesized that midkine serum values are influenced by RAS, probably by ACEI or ARB medication. Consequently we have evaluated whether the presence of functional autoantibodies directed against angiotensin and endothelin receptors are correlated with midkine. Our data support the connection between midkine and RAS, but at the autoimmunity level.

This study brings into question AT1R and ETAR autoantibodies, which can also be identified in significant proportion in the investigated atherosclerotic group, thus being proposed as factors related to atherosclerosis pathogenesis (232). It has been earlier demonstrated that their titers are independent of classical risk factors, a strong positive correlation being found between anti-AT1R and anti-ETAR titers.

AT1R and ETAR are expressed in advanced atherosclerotic lesions in patients with severe peripheral artery disease. Both receptors mediate unfavourable vasoconstriction and have proinflammatory, proproliferative, and profibrotic actions which are relevant for the physiopathology of atherosclerosis. There are only few studies in the literature about the involvement of AT1R and ETAR autoantibodies in human diseases.

Our data suggest that midkine is an important factor in patients with advanced atherosclerosis and could be one of the missing links in the pathomechanism of this disease. Just as midkine is expressed in cardiomyocytes in ischemic conditions and overexpressed after myocardial infarction, probably the same mechanism takes place in peripheral ischemia, with higher midkine values for acute ischemia.

Of particular interest is the inverse association with the signalling induced by autoantibodies against AT1R and ETAR. Midkine, as a RAS regulator, induces the expression of ACE in microvascular endothelial cells. Significantly lower serum titers of midkine have been found in cardiac transplantation patients treated with ACEI or ARB. The same mechanism could be valid also for AT1R and ETAR autoantibodies, which are in inverse correlation with midkine.

Pooled data bring together renin-angiotensin system and endothelin system as important effectors in atherosclerosis, and an angiogenesis mediator (fig. 7.46). A possible explanation for this phenomenon would be "the inner balance" of the atherosclerotic process as multifactorial
disease. The more important the general state of inflammation, angiogenesis, and proliferation in the vascular wall, represented by high midkine levels, the lower the activation of the immune system involved in atheroprogession, in this case represented by functional autoantibodies against well-known effectors of the atherogenic process.

**Figure 7.46** Model on pathophysiological link between midkine and autoantibody formation against angiotensin1 and endothelin-A receptors. With limb ischemia, a counterregulatory neoangiogenesis is initiated, amongst others by midkine expression. An inadequate neoangiogenetic response is observed in those patients with autoantibody formation against AT1R and ETAR, that exhibit lower midkine levels.

### VII.3.5. Conclusions

Although the concept of atherosclerosis as a chronic inflammatory disease as well as an autoimmune one is well known, the way in which proinflammatory cytokines, autoantigens, and autoantibodies contribute to atherogenesis in humans stays mostly un unknown territory. This study opens a new perspective on the interference of a multifunctional cytokine with the autoantibodies from the renin-angiotensin system formation, all of these seeming critical in regulating atherosclerotic lesions, neoangiogenesis, and peripheral ischemia.

Midkine is involved in a variety of physiological and physiopathological processes, and we have added a new role in the present study. In the future there will certainly be new researches, and new roles in pathological processes will be discovered. What matters is to discover the way to apply this information in the clinic and to use midkine as therapeutical target.
• Urine analysis showed that a substantial proportion (53.2%, 50 patients) of the study population represented by high-risk hypertensives had microalbuminuria (MAU).
  • Patients with MAU are older, mostly male, with a longer duration of hypertension and with a higher prevalence of left ventricular hypertrophy.
  • A significant percentage are dyslipidemic (80%).
  • There are no significant differences between the normoalbuminuric and the microalbuminuric group regarding ACEI/ARB treatment.
  • The only cardiovascular risk factor with statistical significance with MAU was the age; male gender, smoking, diabetes mellitus, dyslipidaemia, and obesity are not in direct relationship with MAU in the study group.
  • MAU values in coronary hypertensives are not influenced by glycemia, lipid serum levels, GFR or LVEF.
  • An important correlation was established with the presence of left ventricular hypertrophy (p=0.004) and with the duration of hypertension (p=0.044).
  • Binary logistic regression shows that MAU influences significantly the presence or absence of LVH (Exp(B)=1.021, p=0.004). While MAU increases, the probability of LVH rises with 2%.
  • LVH and age are the only independent variables correlated with MAU.
  • After introducing a control group with hypertensives without coronary artery disease (CAD) in the study, we have demonstrated that the patients with coronary disease were younger, with a shorter duration of hypertension, and mostly male. The probability to be smokers, diabetics, dyslipidemias, and obese was higher than for the control group.
  • Compared to the group without CAD, patients with CAD had a higher prevalence of LVH and higher values of albuminuria. The percentage of normo- and microalbuminuric patients was not different in the 2 groups.
  • A correlation analysis established the statistical significant relationship between MAU and age, diabetes mellitus, duration of hypertension, and prior treatment with ACEI/ARB.
  • The other cardiovascular risk factors (male gender, obesity, dyslipidaemia, smoking) included in the analysis were not correlated with microalbuminuria.
  • A statistically significant correlation is seen between MAU, HVS and BCI.
  • 60.5% of the subjects with microalbuminuria also had LVH. Compared to the patients without LVH, those with LVH had higher albuminuria values.
  • The best predictive factor for the presence of MAU is LVH.
• Cytokine midkine is present in atherosclerotic plaques of femoralis artery and its branches (objectified by immunohistochemistry and microscopic analysis) in patients with peripheral artery disease stages III-IV Fontaine.

• Serum midkine titers obtained by ELISA were significantly higher in patients with macroangiopathy than in the control group.

• There are no statistically significant correlations between midkine and the following factors: male gender, age > 60 years, obesity, diabetes mellitus, dyslipidaemia, glycemia, GFR, CRP, or prior treatment with ACEI/ARB.

• Correlative analysis proved a strong inverse relationship between serum levels of midkine and AT1R and ETAR titers.

The originality and the innovative contributions of the thesis

By demonstrating the high prevalence of microalbuminuria in coronary disease patients we have emphasized the necessity of routinely investigating this parameter in our daily practice, anytime during the antihypertensive treatment. A tighter blood pressure control is requested when we detect microalbuminuria and even more so when it is associated with left ventricular hypertrophy and a longer hypertension duration. By inversely approaching the role of microalbuminuria in patients with already established coronary artery disease and by showing the extent of this marker in association with left ventricular hypertrophy, we support the insertion of microalbuminuria on the list of cardiovascular risk factors in hypertensive patients, also playing an important role in calculating the risk of hypertensive patients. In the same time we affirm the possibility of using microalbuminuria as surrogate endpoint, our results being suggestive even on a small number of patients.

Another idea arising from the 2 studies would be that the hypertensive patient with microalbuminuria could be considered, until proven otherwise, a possible coronary patient. If left ventricular is added to microalbuminuria, the patient is old and has a longer duration of hypertension, then the chances to have ischemic heart disease are higher, irrespective of the other risk factors.

The pathology of atherosclerosis is not currently fully understood, new mechanism and new incriminating factors in its progression appearing constantly. In the same time, the factors and the known courses of action can not entirely explain the phenomenon. Therefore, it is exciting to look for new molecules and interactions to clarify it.

Another novel and original element is represented by the third study, where we bring to the fore a new molecule, less clinically researched. Midkine has been intensively studied in oncology, but its role in inflammatory processes has drawn attention to experimental studies regarding hypertensive and atherosclerotic pathology. From what we know so far, this is the first study that investigates and highlights midkine’s presence in atherosclerotic plaques in humans.
and establishes correlations with midkine serum levels in preoperation patients. Introducing the
analysis of functional autoantibodies in the same study group is another interesting point, these
autoantibodies being mainly objectified in autoimmune diseases. Because atherosclerosis also
has an autoimmune component, we considered of interest their analysis in patients with ischemic
disease.

The use of more molecular biology methods, immunohistochemistry, and ELISA, besides
the usual analysis methods, represents an originality element of the thesis.

The correlations established in this thesis open a new perspective on the complex
physiopathological mechanisms between known risk factors, with an emphasize on hypertension,
and emergent factors that could explain many unknown links so far in ischemic pathology.

The use of autoantibodies and of biomarkers of endothelial cells activation could contribute
to our attempt to assign an adequate cardiovascular risk stratification for our patients, therefore
the results of these studies could be useful in the management of patients as well as in
researching new therapeutic target or developing new drugs for delaying the onset or
progression of atherosclerotic disease.

Selected references

125. Ibsen H, Olsen MH, Wachtell K, et al. Reduction in albuminuria translates to reduction in
cardiovascular events in hypertensive patients: losartan intervention for endpoint reduction in
130. Kadomatsu K, Tomomura M, Muramatsu T. cDNA cloning and sequencing of a new gene
intensely expressed in early differentiation stages of embryonal carcinoma cells and in mid-
132. Muramatsu T. Midkine, a heparin-binding cytokine with multiple roles in development, repair
156. Takenaka H, Horiba M, Ishiguro H, et al. Midkine prevents ventricular remodeling and improves
296(2):H462-9
ischemia/reperfusion injury in Swine hearts: a novel therapeutic approach for acute coronary
syndrome. Front Physiol. 2011; 2:27
179. Krzystek-Korpacka M, Neubauer K, Matusiewicz M. Clinical relevance of circulating midkine in
184. Garg AX, Kiberd BA, Clark WF, et al.. Albuminuria and renal insufficiency prevalence guides